To the Graduate Council:

I am submitting herewith a thesis written by Holiday Agnes Durham entitled "Insulin Sensitivity and the Onset of Hyperphagia in Fatty Rats." I have examined the final electronic copy of this thesis for form and content and recommend that it be accepted in fulfillment of the requirements for the degree of Master of Science, with a major in Nutrition.

Gary E. Truett
Major Professor

We have read this thesis
and recommend its acceptance:

Betsy Haughton

Naima Moustaid-Moussa

Accepted for the Council:

Ann Mayhew
Vice Chancellor and Dean of
Graduate Studies

(Original signatures are on file with official student records.)

INSULIN SENSITIVITY AND THE ONSET OF HYPERPHAGIA IN FATTY RATS

A Thesis
Presented for the
Master of Science Degree
The University of Tennessee, Knoxville

Holiday Agnes Durham
December, 2004

DEDICATION

I dedicate this thesis to my parents, Dwight and Gay Durham, my brother and sister, Mack and Carolyn Durham, and to my grandparents. All of their love, guidance, encouragement, and humor allowed me to "keep on truckin" and pursue my goals. I love each of you and appreciate all you do for me always!

ACKNOWLEDGEMENTS

I would like to thank all those who supported me through my graduate studies at the University of Tennessee. I am especially grateful to my advisor, Dr. Gary Truett, for his patience, constructive advice, guidance, and support through this process. Thank you for providing me with this invaluable experience; it has definitely been a "good" one. Also, I would like to thank my committee members, Dr. Betsy Haughton and Dr. Naima Moustaid-Moussa, for their instrumental support and time. I am indebted to Dr. Ruth Harris at the University of Georgia for her technical expertise and advice.

I am appreciative of my lab mates, Mary Brown and Taryn Stewart, fellow graduate students and staff in the Department of Nutrition, for their assistance in countless ways. Also, a special thank you goes to Alycia and Gracie Truett, who, behind the scenes, provided support, memorable stories, and an array of pictures to fill the office walls with happiness.

Finally, I would like to thank my family for their love and support during this chapter in my life. Thank you!

ABSTRACT

Hyperphagia emerges suddenly after 22 days of age in Zucker fatty (*fa/fa)* rats, and fatties become visibly obese within days. This rapid increase in growth rate is likely to be associated with substantial changes in glucose metabolism. The objective of these experiments was to determine the relationship between insulin resistance and the onset of hyperphagia. Insulin tolerance tests showed that fatties were remarkably insulin resistant prior to the onset of hyperphagia, hyperinsulinemia, and obesity, but become as insulin sensitive as their lean littermates after the onset of hyperphagia. Furthermore, fatties became increasingly insulin resistant prior to the onset of hyperphagia, and then became as insulin resistant as their lean littermates after the onset of hyperphagia. To determine whether these changes in insulin resistance are associated with changes in expression of the nuclear transcription receptor peroxisome proliferator-activated receptor gamma (PPARγ), which improves insulin resistance by increasing lipogenesis in adipose tissue, we measured PPARγ and fatty acid synthase (FAS) mRNA and protein at several points in development. PPARγ and FAS mRNA expression was induced in fatties after the onset of hyperphagia. PPARγ and FAS protein expression increased gradually after the onset of hyperphagia, similar to the changes in insulin sensitivity. These observations suggest that changes in PPARγ signaling may play a critical role in promoting the increase in insulin sensitivity after the onset of hyperphagia and perhaps in the promotion of hyperphagia itself.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

ACC	Acetyl-CoA carboxylase
ACTH	Adrenocorticotropin
AgRP	Agouti gene-related protein
ASP	Agouti signaling protein
bp	Base pair
BMI	Body mass index
C/EBP	CCAAT/enhancer-binding protein
db	*diabetes*
fa	*fatty*
FAS	Fatty acid synthase
IRE	Insulin response element
LPL	Lipoprotein lipase
MCR	Melanocortin receptor
α-MSH	α-melanocyte stimulating hormone
NADPH	Nicotinamide adenine dinucleotide phosphate
NHANES	National Health and Nutrition Examination Survey
ob	*obese*
PC1	Prohormone convertase-1
PCR	Polymerase chain reaction
POMC	Pro-opiomelanocortin

PPAR	Peroxisome proliferator-activated receptor
RT-PCR	Real time-polymerase chain reaction
RXR	Retinoid X receptor
SREBP	Sterol regulatory element-binding proteins
TAG	Triacylglycerols
TULP1	Tubby
TZD	Thiazolidinedione
VLDL	Very low density lipoproteins
WHO	World Health Organization

PART 1. INTRODUCTION

The obesity epidemic in the United States has been accompanied by an alarming increase in the prevalence of overweight children (1). Data from the National Health and Nutrition Examination Survey (NHANES) indicate that the number of overweight children has increased among all age, race, and sex groups. Overweight children are at high risk for becoming overweight adults and developing hypertension and type II diabetes (2). These observations underscore the importance of understanding the early development of energy balance regulatory systems, particularly during critical periods of rapid growth.

The Zucker fatty (*fa/fa)* rat is a unique model for the early development of obesity. Fatties grow at the same rate as their lean littermates for the first 22 days of age (3). At 23 days of age, fatties develop robust hyperphagia, which dramatically increases growth rate and expansion of fat mass (3). Such a rapid increase in growth is likely to be accompanied by a fundamental change in regulation of glucose metabolism.

There are gradual changes in glucose metabolism during development of obesity. As juvenile fatties accumulate excess adipose mass, they become increasingly insulin resistant. When insulin resistant adult fatties are treated with the thiazolidinedione (TZD) rosiglitazone (4; 5), they become more insulin sensitive. Rosiglitazone binds to peroxisome proliferator-activated receptor gamma (PPARγ), a transcription factor that belongs to the nuclear hormone receptor family responsible for control of adipocyte gene expression and differentiation, and glucose homeostasis. PPARγ is expressed predominately in adipose tissue (6). This transcription factor improves insulin sensitivity by upregulating genes for glucose uptake and lipogenesis in adipose tissue. As a result,

TZDs promote elevated food consumption and increase lipogenesis in the Zucker fatty rats (4; 5) and in humans with diabetes (7) .

PPARγ mRNA expression in adipose tissue increases during the suckling period, reaches a plateau after weaning, and does not change thereafter (8). The same increase in PPARγ occurs whether pups are weaned onto high fat or high carbohydrate diets. In contrast, fatty acid synthase (FAS) mRNA is low during suckling and increases only when pups are weaned onto a high carbohydrate diet. This rise in FAS mRNA is driven by a sharp increase in insulin. When animals are weaned onto a high fat diet, insulin levels remain low, and FAS mRNA is undetectable. These results indicate that PPARγ expression is developmentally regulated and that FAS is regulated by diet composition (8; 9). These observations suggest that an increase in PPARγ-mediated insulin sensitivity could also play a role in the emergence of hyperphagia in fatties during the weaning transition.

The purpose of these studies was to characterize the relationship between insulin resistance and the emergence of hyperphagia in fatty rats. An initial series of experiments characterized changes in insulin sensitivity under several developmental conditions. Secondly, we evaluated the activation of the PPARγ and FAS expression at various ages, using real time-polymerase chain reaction (RT-PCR) assay and Western blot analysis, relative to the onset of hyperphagia in the fatty rats.

PART 2. LITERATURE REVIEW

Adult and Childhood Obesity

Epidemiology and characterization

Body fat ordinarily accounts for 25% of weight in women and 18% in men. Adults with an excess amount of body fat are classified as either overweight or obese, depending on the amount of excess body fat (10). According to the National Center for Health Statistics, approximately 64% of Americans are either overweight or obese (11). This problem is not exclusive to the United States; the World Health Organization (WHO) has reported that obesity is a health concern in other developed countries as well (12). As a result, the WHO has characterized obesity as a global epidemic.

Medical hazards and chronic diseases associated with obesity have been increasingly well documented in recent years, and obesity has been conceptualized as a chronic disorder (13). In adults, increased adiposity is associated with an increased risk for cardiovascular disease, type 2 diabetes, and several cancers (14). It was recently estimated that obesity contributes to 300,000 deaths per year in the United States and cost our nation over $100 billion a year in direct and indirect cost (15-17).

Body mass index (BMI) is used as a screening tool for the classification of individuals as normal weight, overweight, or obese. It is widely used primarily because it is easy to calculate, noninvasive, and inexpensive; BMI is body weight in kilograms divided by height in meters squared. Current guidelines consider a BMI of 25 to be overweight and a BMI more than 30 to be obese (15). While BMI is a convenient screening tool, it has several limitations. It can overestimate body fat in individuals who

are muscular and underestimate body fat in individuals who have lost muscle mass, such as the elderly. The relationship between adipose mass and BMI can also vary among ethnic groups. For these reasons, it may be necessary to estimate body fat by more direct measures, such as skin-fold thickness, underwater weighing, or dual energy X-ray absorptiometry, to determine whether someone actually has excess body fat.

Childhood prevalence and significance

The obesity epidemic has been accompanied by an alarming increase in the prevalence of overweight children (1; 2). Currently, statistical percentiles are used to classify children as at risk for overweight. These guidelines define children at or above the 85th percentile BMI to be at risk for overweight and those at or above the 95th percentile to be overweight (18). Because BMI changes as a function of age, BMI growth curves for children published by the Centers for Disease Control and Prevention (CDC) are used to chart an individual child's BMI relative to the population (19). According to data from the National Health and Nutrition Examination Survey (NHANES), the number of obese children in United States has doubled in the last two decades (1; 20). Currently, 15.3% of 6 to 11 year olds and 15.5% of 12 to 19 year olds are at or above the 95th percentile for BMI on the CDC growth charts.

It is rare that mortality of children relates to body fat, but the contribution of excess body fat to morbidity in children is increasing. The obese child can suffer from serious morbidity, including hyperlipidemia, type 2 diabetes, orthopedic, neurological, pulmonary, gastroenterological, and endocrine complications (21; 22). Overweight also

contributes to early pubertal development in girls and delayed pubertal development in boys (23-25). Aside from the physiological constraints, children often suffer from the psychological repercussions associated with overweight and obesity, such as teasing and discrimination (22; 26). With advancing age, obesity also promotes physical, economic, and emotional hardship (27). In most circumstances, the complications from childhood obesity do not become a major health burden until decades later, although the medical consequences may still exist early in life (13; 28). Numerous studies have demonstrated a correlation between childhood morbidity and adulthood morbidity and mortality (29). Approximately 25 - 50% of obese adolescents will become obese adults (22). Moreover, the pathological processes and long term risk factors beginning in childhood may be more detrimental for adults who became obese as children than the 30% of obese adults who were not obese during childhood (18). Early in life, obesity plays a critical role in promoting adult onset insulin resistance syndrome, which includes hyperinsulinemia, hypertension, hyperlipidemia, type 2 diabetes mellitus, and heightens the risk for cardiovascular disease (30). The implication that childhood obesity is a critical starting point to the manifestation of obesity in adults creates an intriguing area of research. This period of rapid growth needs to be better understood and used as a strategy to control associated health problems.

Etiology

Obesity can be described as an imbalance between caloric intake and energy expenditure (13). Although the mechanisms that promote this imbalance are not

completely known, genetics and environment play important roles in the development of obesity. The rise of the obesity epidemic can be partially attributed to unlimited access to the convenience of energy dense, highly palatable foods, together with an inactive lifestyle (2; 31). However, evidence for genetic contribution to this epidemic is well established from family and twin studies which indicate fat mass is also heritable (32). A number of studies reveal a close relationship between the weight of adopted children and their biological parents rather than their adoptive parents, indicating environment is only a portion of the equation (33).

Realistically, obesity has many causes, each of which have variable genetic components. Genes may attribute to 25%-40% of the variability in BMI and contribute to differences among individual resting metabolic rate, weight gain in response to over consumption, and physical regions of fat storage (34). Obesity can also be explained by rare occurrences, such as single-gene mutations or by various diseases in subjects for whom obesity otherwise would not occur (34). Therefore, some individuals are more likely to be predisposed to obesity through their genetic makeup, while others respond to a lifestyle which promotes obesity. Bray has summarized the etiology of obesity: "Genes load the guns, the environment pulls the trigger" (34).

Critical periods and the development of obesity

Critical periods in growth and development are periods when environmental influences alter growth and development with possible lifetime consequences. Three critical periods in development have been identified as influencing early onset childhood

obesity and risk for experiencing adulthood obesity (35; 36). These crucial periods are the prenatal and postnatal period, the adiposity rebound period, and adolescence (37).

Prenatal and postnatal period.

Gestation is sensitive to environmental effects that can influence the long term health, behavior, and function of an individual. During the first and second trimester of pregnancy the hypothalamus begins to develop; availability of metabolic fuels may alter development of the hypothalamus and sympathetic nervous system to permanently influence the individual's response to nutrients (38).

Maximal adipocyte replication occurs during the third trimester. The availability of metabolic fuels and the hormonal environment may influence the number of adipose cells that develop during this period and may also influence leptin, insulin, lipoprotein lipase, and other key regulatory mechanisms (37).

Postnatally, infants undergo another critical period, the transition from nursing to weaning. The weaning period is a time of much nutritional significance; the infant shifts from dependence on the mother for nutrition to independent control of food intake. Moreover, evidence from several species indicates that the mechanisms that activate overeating are dormant in early development and then become active around the weaning transition, eventually causing increased growth and adiposity (3; 39).

Animal studies were instrumental in revealing how early nutrition may impact growth and development later in life. One of the first to initiate work of this kind was McCance in 1962, when he manipulated litter size such that pups in large litters received

less milk than pups in smaller litters (40). After suckling for a 21 day period, each group was weaned and provided unlimited stock diet. Pups from large litters remained smaller than those from the small litters. Furthermore, the smaller pups from the large litters continued to diverge in body size from the larger animals in spite of unlimited food. This early nutrition intervention created a lifelong programming of growth (40). McCance proved that it was the early weeks in postnatal development that promoted this growth trajectory, when he conducted the same nutritional manipulation several weeks later for three weeks. Under these circumstances, the animals underfed were smaller, but they displayed catch-up growth when re-fed, indicating the period for growth programming had passed (41). Other studies indicated that briefly overfeeding pups from small litters during the suckling period induced permanently elevated plasma insulin and cholesterol levels in adulthood (42).

Metabolic programming was demonstrated more recently by feeding neonate pups a high carbohydrate milk formula, rather than mother's milk. This intervention caused substantial changes in pancreatic islet function increasing insulin biosynthesis as well as producing alterations in metabolic responses of peripheral tissues, each persisting into adulthood. Pups fed the high carbohydrate diet initially grew at the same rate as pups raised by their dams, but during adulthood, they developed obesity even though they were fed the same diet as those raised by their dams (43). Finally, in primates, such as female baboons, overfeeding in infancy resulted in the onset of obesity only later in life (44). Evidence from these animal models indicates early events in energy balance regulatory systems control metabolic occurrences later in life.

There is also evidence for early developmental imprinting in humans. Near the end of World War II, Germany began to restrict food to Northern Holland for over a 6 month period beginning in October, 1944. The Dutch famine was a "natural experiment," as individual caloric intake declined from ~1500 calories to ~1000 calories per day over a six-month period and was further minimized to ~500 calories per day for 1 month until the famine ended in May 1945. Ravelli *et al* showed that those exposed to famine during the last trimester had a reduced prevalence of obesity at 18 years old (45). These data suggest leanness later in life may be a result of reduced fat deposition late in gestation. In fact, it is during the last trimester that adipocyte replication and increases in body fat become evident (37). In contrast, individuals exposed to famine during the first and second trimester of gestation had a higher incidence of obesity by 18 years of age. During the first 2 trimesters, the hypothalamus begins to develop, possibly setting caloric cues with the sympathetic nervous system (45).

Several factors alter interpretation of this experiment, such as lack of data pertaining to pregnancy weight gain and birth weight measurements. It is also reasonable to consider those women who carried a fetus to term during famine may have differed from those women who experienced miscarriages or failed to become pregnant. Regardless, these data provide evidence that occurrences during the gestational period may be associated with the prevalence of adiposity later in life (37; 38). The Dutch Famine study revealed early evidence that maternal energy intake even during pregnancy can impact the risk of offspring becoming obese later in life (45).

Infants of diabetic mothers also show how birth weight can affect adiposity later in life. Gestational diabetes leads to an increase in delivery of glucose to the fetus. In response, the fetus increases insulin production, which increases growth rate and development of adipose tissue. Pima Indian infants exposed to diabetes during gestation were more likely to suffer from obesity at birth and later in life than those infants born to mothers who were prediabetic or never affected by diabetes (46). This is not only true for the Pima Indians, but also for infants born to diabetic mothers in other populations (46). These infants tend to be larger at birth and are at higher risk for growing up to be overweight or obese adults (37; 38; 47). Animal studies indicate fetal hyperinsulinemia can effect and even alter neurotransmitters, eventually leading to the hyperphagia and increased body weight in adult offspring (47; 48).

A number of studies indicate low birth weight is directly linked to the prevalence of obesity and subsequently Metabolic Syndrome X later in life (37; 49). It is important to consider that low weight births only account for a small percentage of the general population, therefore, low birth weight alone can only contribute minimally to adult obesity (50).

Adiposity rebound.

Adiposity rebound is a period in a child's development that may predict increased incidence of obesity later in life. First described by Roland, this period captures BMI increasing during infancy, then declining to nadir levels between 6 to 8 years of age, and then beginning to increase with age thereafter (36). Children who undergo an earlier

rebound period may, in fact, be at greater risk for being overweight or obese later in life (18; 51; 52). For example, the Fels Longitudinal Study recorded numerous measurements of 338 individuals until 25 years of age; 159 of the subjects were studied from 35 to 45 years of age. The data indicated that those with earlier adiposity rebound showed maximum BMI at an earlier age and a higher BMI when maximum BMI was achieved (18; 53). Therefore, this period appears to partially predict adult weight status.

Other mechanisms are likely to contribute to this early effect. During this period, early childhood behaviors associated with food intake and activity begin to be recognized. Maternal regulation of food plays a central role in the capability of the child to regulate food later in life. Those children living in strictly regulated environments are susceptible to impaired self-regulation of food and increased risk for adiposity (54). Early adiposity rebound may also be related to infant exposure to gestational diabetes (37), while other research indicates early maturation can be correlated to increased levels of adiposity (23; 37; 55).

The age of adiposity rebound may reflect a period of altered genetic programming, inherited susceptibility to obesity, and/or modifiable environment influences (38). It is important to recognize that adiposity rebound is based on a number of population studies defining BMI as the primary marker for adiposity. Because BMI is a measure of weight divided by height squared, without other anthropometric measures, it is not completely clear whether BMI reflects increased weight (50). Nevertheless, if BMI does reflect increased weight, other measures are needed to determine if such weight is a result of body fat rather than increased bone or muscle mass. The time of

adiposity rebound does appear to be a critical time development, yet biological and behavior determinants as well as secondary anthropometric measurements should be evaluated more closely to determine if adiposity rebound is a modifiable risk factor for adult obesity which could be prevented and treated (52).

Adolescence.

Adolescence is a third critical period in childhood. Evidence shows the older a child is when becoming at risk of overweight or overweight, the greater the risk that the overweight will persist into adulthood (18; 22). It is predicted that 25-50% of overweight adolescents grow up as obese adults. In fact, obese adolescents are 2 to 11 times more likely to become obese adults than non-obese adolescents (22).

Overall, the studies conducted in this area indicate childhood obesity is often likely to persist into adulthood. However, the criteria for obesity can vary between studies as well as the statistical interpretation and the time interval studied from childhood to adulthood. Guo *et al* showed in a long-term prospective study that 18 year old adolescents at $\geq 95^{th}$ percentile BMI were at significantly higher risk for overweight by 35 years of age (35). The probability for adult overweight was 78% for male and 66% for female adolescents. Similar findings from a 1958 British birth cohort suggest the persistence of obesity into adulthood is similar in males and females. Data indicates at 33 years of age 17% of males and 18% of females were $>95^{th}$ percentile BMI at 7 years of age (22). However, other findings suggest that gender may affect the persistence of obesity into adulthood. A follow-up of the National Survey of Health and Development

examined males and females who were obese by 36 years of age, using an internal criteria for overweight as >130% of BMI. This study found 11% of men and 24% of women were obese at 20 years of age, yet at 14 years of age, 14% of males and 32% of females were considered obese (56). One of the most extensive studies was conducted over a 55 year span, tracking 181 subjects in the Harvard Growth Study from adolescence to old age (57). Individuals in the study were considered overweight as adolescents with >75th percentile BMI for at least two years. Results indicated male participants who were overweight during adolescence suffered from increased risk of mortality from all causes and disease-specific mortality, yet women did not. However, risk of morbidity from coronary heart disease and atherosclerosis was increased in both sexes who had been overweight as children. Data suggested that being an overweight adolescent is a more accurate predictor of these risks later in life than being overweight in adulthood (57).

Genetic and Biological Determinants of Obesity

In the last several years, 7 human genes and more than 20 mouse genes have been associated with fat mass accumulation (2). Rodent research provides valuable insights into energy balance that are often relevant to humans. Table 1 provides a summary of some of the genes that influence fat mass in humans and mice. Although rare, several human disorders of energy balance can be attributed to genetic mutations that produce morbid obesity in childhood. Such genetic diseases include Prader-Willi Syndrome, Cohen's Syndrome and Bardet-Biedl Syndrome.

Table 1. Genetic mutations in humans and mice

Gene Name	Will the genetic mutation cause human obesity?	Will the genetic mutation cause increased fat mass in mice?
Leptin	Yes, rare	Yes
Leptin receptor (central and peripheral)	Yes, very rare	Yes
Melanocortin receptor-4 (MC4R)	Yes, 3%-5% of morbidly obese	Yes
α-melanocyte stimulating hormone (α-MSH)	Yes, very rare, red hair, and obesity	Yes
Prohormone convertase-1 (PC1)	Yes, one known case	Yes
Bardet-Biedl	Yes, very rare	Unknown
Dunnigan partial lipodystrophy	Yes, rare	Unknown
Agouti gene-related protein (Agrp)	Unknown	Yes
Tubby (TULP1)	Unknown	Yes

Prader-Willi Syndrome

Prader-Willi syndrome (PWS) is caused by genetic mutations in a region of chromosome 15 that is subject to genomic imprinting (58). Genomic imprinting during gametogenesis suppresses expression of maternal alleles of genes in this region. This means that only paternally inherited alleles are expressed in normal development.

Many Prader-Willi children inherit chromosomal deletions from their fathers that eliminate the paternally derived alleles, leaving only the silenced maternal alleles in the genome (59). Others develop Prader-Willi Syndrome because of maternal uniparental disomies, in which both copies of chromosome 15 are inherited maternally and none are

inherited paternally. In both cases, it is the loss of paternal alleles that determines the phenotype.

The Prader-Willi Syndrome phenotype severely affects appetite and feeding in infancy and childhood (60). Prader-Willi Syndrome infants have minimal appetite and often have such difficulty suckling as infants that they require nutritional support. A marked change occurs during the first year or two of life. Appetite and feeding ability improve so much that these infants develop hyperphagia. The onset of hyperphagia initially produces an improvement in growth, but because hyperphagia never resolves, it rapidly leads to the development of obesity. This developmental pattern suggests that the pathways that mediate hyperphagia are not fully developed in infants, but become activated during the first two years of life.

These symptoms have been attributed to hypothalamic dysfunction and decreased energy utilization (61-63). These dysfunctions have lead some researchers to hypothesize this syndrome produces a perception of starvation in hypothalamic feeding centers (62). Individuals with Prader-Willi syndrome, produce normal increased levels of cholecystokinin and leptin after food intake (62; 64). They also have reduced levels of orexigenic agents, such as neuropeptide Y and agouti-related protein in the hypothalamus, similar to other obese individuals (61; 62). Under normal conditions, such levels of cholecystokinin, leptin, neuropeptide Y, and agouti-related protein are associated with satiety, but in Prader-Willi Syndrome hyperphagia exists despite the metabolic and physiological consequences of such peripheral factors controlled by the hypothalamus (62). Therefore, it appears there is an alteration in the pathways controlled

by the hypothalamus that allows the body to incorrectly act as if in a state of starvation. Not only is appetite enhanced, but metabolic rate is low, and gonadotropin release is impaired as in other states of starvation.

Proopiomelanocortin C (POMC)

Proopiomelanocortin (POMC) is a single gene product that is spliced into several different peptides, including α-melanocyte–stimulating hormone (α-MSH). POMC is produced in the arcuate nucleus in the hypothalamus and acts to integrate hormonal signals from adipose cells. The activation of POMC increases α-MSH, which binds to melanocortin-3 and melanocortin-4 receptors (MC3R and MC4R).

A mutation in POMC identified in humans affects hair color, skin color, food intake, and energy expenditure due to its inactivation in the hypothalamus. The first known case was documented in 1998 by Krude *et al,* revealing a red haired, fair skinned girl who had a normal birth weight, and developed cholestasis at 3 weeks of age (65). Because her older brother had died at 7 months of age and was diagnosed postmortem with adrenal insufficiency which caused hepatic failure and severe cholestasis, this girl was diagnosed with adrenocorticotropin (ACTH) deficiency at 23 days of age. She was successfully treated and the cholestasis resolved. She grew normally during the first 3 months of life, but by 4 months of age, the child's parents noticed a substantial increase in appetite, which produced early-onset obesity. The large weight gain during this short period prevented the patient from walking until 2 years of age, even though mental development appeared to be normal (65).

Approximately seven other such cases have been reported worldwide. Although rare, these cases emphasize the importance of POMC in energy homeostasis indicating variants in POMC may be a contributing factor to the common form of early-onset obesity (65; 66). Like children with Prader-Willi Syndrome, the pathways for hyperphagia appear to have been inactive during infancy in the girl with a POMC mutation, and emerged later in early development.

Rodent Models

Parabiosis studies

Early parabiosis studies, which involve the surgical union of two animals to produce a cross-circulation of blood exchange, have assisted in revealing the metabolic role of hormones. Using rats, Hervey showed when one parabiotic partner was given lesions to the ventromedial hypothalamus that caused the rat to overeat and become obese, the non-lesioned parabiotic partner reduced its food intake and its adipose mass (67). Hervey proposed that a circulating satiety factor produced by the expanding fat mass of the lesioned rat was responsible for inhibition of food intake in the parabiotic partner. He proposed that the hypothalamic lesions prevented the rat from responding to the elevated circulating satiety factor. In fact, he hypothesized the elevated satiety factor in the obese rat produced effects to decrease food intake in the lean parabiotic partner without lesions, causing death by starvation.

Others extended these parabiosis studies to genetic models of obesity: *obese (ob), diabetes (db),* and *fatty (fa)* (68-70). These mutations are characterized by hyperphagia,

decreased energy expenditure and early-onset obesity. Coleman demonstrated when obese *db/db* mice are parabiosed to wildtype mice, the *db/db* is unaffected by the union, while the wildtype lost significant weight, experienced hypoglycemia, and eventually died (68). When *ob/ob* mice were parabiosed to their lean littermates, the obese animals did not experience rapid weight gain and in fact consumed less food but did not die (69). When the *db/db* and *ob/ob* were parabiosed, the *ob/ob* partner experienced weight loss, became hypoglycemic, and also died of starvation. Coleman suggested that the *ob* locus served as a humoral satiety factor, whereas the *db* locus functioned to produce a molecule or receptor to respond to this satiety factor (69).

Harris *et al* also demonstrated similar findings to those of Coleman's in the *db/db* mice, when performing parabiosis between fatty (*fa/fa*) and lean Zucker rats (70). The lean parabiotic partner of the fatty rats reduced its food intake and its adipose mass. Thus, fatty rats appeared to produce the humoral factor but failed to respond to the circulatory satiety factor.

Leptin and leptin receptors

The conclusions of Coleman's parabiosis studies were confirmed later with the cloning of *ob* and *db*. The *ob/ob* mouse inherits an autosomal recessive allele that increases energy efficiency and food intake, leading to early onset obesity, with the *ob/ob* mouse eventually tripling the weight of a normal mouse. The development of obesity in *ob/ob* mice is accompanied by fasting hyperglycemia, hyperinsulinemia, infertility, thyroid dysfunction, and stunted linear growth. The cloning of *ob* mutation by Zhang et

al in 1994 began a series of important discoveries in the molecular basis of energy balance (71). The *ob* gene was shown to encode a secreted protein, subsequently named leptin. Leptin is produced predominately by adipose tissue, released in the bloodstream, and detected by leptin receptors in the hypothalamus, as well as many other tissues. Exogenous leptin injected intracerebroventricularly suppresses food intake and increases energy expenditure in *ob/ob* mice, restoring body weight to normal levels (72). Most obese individuals are characterized by elevated circulating leptin, which correlates with adipose tissue mass. Although there are a few documented cases of individuals with congenital leptin deficiency who can be successfully treated with recombinant leptin, most of the obese population are resistant to the weight reducing effects of recombinant leptin (73; 74).

The *db/db* mouse is strikingly similar to the *ob/ob* mouse. It also inherits fasting hyperglycemia, hyperinsulinemia, infertility, thyroid dysfunction, and stunted linear growth. But the abnormalities found in the *db/db* mouse model are caused by a mutation in a leptin receptor splice variant predominantly expressed in the hypothalamus (75; 76). This splice variant causes the intracellular signaling domain of the leptin receptor splice variant to be defective, so that leptin signaling cannot be activated despite high circulating leptin levels (75; 77). Obesity in the *db/db* mouse cannot be resolved with administration of recombinant leptin (72). Thus, leptin and its receptors serve in a feedback loop to regulate fat mass.

There are several documented cases of children who fail to produce leptin because of genetic mutations in the human homologue of the *ob* gene. These children have

normal birth weight, but develop severe early-onset obesity. Like *ob/ob* mice, these individuals can be treated using recombinant leptin; decreasing hyperphagia and fat mass (2). Even with treatment of recombinant leptin, these children are above the 95th percentile for normal weight and age. Furthermore, they experience recurring episodes of increased hyperphagia during leptin therapy as the dose of leptin becomes ineffective. As a result, leptin dosages must be elevated when such episodes occur and these symptoms will then resolve temporarily (31; 78). In these children, leptin primarily affects food intake, and has no detectable effect on energy expenditure (31; 78). This suggests that leptin plays less of a role in regulating energy expenditure in humans than it does in mice.

Although leptin decreases fat mass in leptin deficient mice and humans, it is not as effective in humans and animals that have normal leptin expression. An increase in adipose mass normally leads to an increase in leptin levels. Although obese individuals tend to produce high levels of leptin, it appears they are resistant to its actions.

There is also evidence that responses to leptin change developmentally. Even though fat mass is relatively low during the first few days of life, there is a developmental surge in leptin levels (39). During the neonatal period, the physiological growth requirements, such as maximal food intake and maintenance of high metabolic rate for survival, appear to contradict the known role of leptin. Mistry *et al* observed the developmental progression of leptin's ability to alter food intake and energy expenditure early in life; leptin accelerates metabolic rate in lean and *ob/ob* mice early in life, but does not affect food intake until the fourth week of life (39). This provides evidence that

the leptin signaling pathways regulating hyperphagia are inactive during the neonatal period but are activated at a specific developmental age.

Lipodystrophy

Lipodystrophy syndromes are also characterized by low leptin levels (79). Lipodystrophy refers to a heterogeneous group of conditions classified as acquired or genetic, describing both partial and complete loss of subcutaneous adipose tissue at birth or later in life (80). The extent of fat loss in these patients defines the severity of their metabolic complications. Despite variations in fat loss and different etiologies pertaining to each type of lipodystrophy, these patients often share common metabolic abnormalities in varying degrees, such as insulin resistance, glucose intolerance or hyperglycemia, and hypertriglyceridemia (79). As a result of minimal adipose mass, there is a deficiency in leptin signaling, thought to contribute to many of the metabolic abnormalities characterized by lipodystrophy (81-83).

Patients diagnosed with localized lipodystrophy suffer from cosmetic abnormalities where fat loss is selective to various parts of the body, creating depressions and indentations subcutaneously. In contrast, patients with congenital generalized lipodystrophy suffer from nearly complete absence of adipose mass and adipocyte-derived hormones, such as leptin, causing severe hyperphagia early in life (84). Unger RH *et al* suggest that leptin functions regulate intracellular homeostasis of fatty acids and triglycerides in nonadipocytes, such as the muscle, liver, and pancreas, but prevent fatty acid overload (85). When leptin production is minimal, lipotoxicity occurs in

nonadipocytes due to ectoptic lipid accumulation (86; 87). These physiological occurrences lead to severe metabolic complications, such as hypertriglyceridemia, severe insulin resistance, diabetes, hepatic steatosis, and cardiac complications (88-90).

Over the last decade, advances have been made to understand the mechanism underlying this disease and why adipose tissue is vital in the prevention of metabolic abnormalities. One hypothesis is that leptin is a critical determinant in the prevention of insulin resistance and hypertriglyceridemia seen in lipodystrophy. The administration of recombinant leptin to lipodystrophic humans and rodents dramatically improves glucose and lipid abnormalities associated with this condition including insulin resistance, hypertriglyceridemia, and hepatic steatosis (82; 91; 92). Others suggest that the metabolic abnormalities are a result of problems in the differentiation of adipocytes (79). During adipogenesis, precursor cells undergo transient expression of transcription factors, thus becoming preadipocytes. Preadipocytes develop the ability to store triglycerides through the activation of multiple genes controlled by key transcription factors such as peroxisome proliferator-activated receptor gamma (PPARγ). Transcription factors, such as sterol regulatory element-binding proteins (SREBP-1)/adipocyte determination- and differentiation-dependent factor 1 (ADD-1), are also involved in activating key factors or controlling synthesis of their ligands. Defects during vital differentiation periods may lead to adipocyte deficiency. Evidence indicates that thiazolidinediones (TZDs), oral hypoglycemic agents, classified as agonists for PPARγ, stimulate adipocyte differentiation in vitro and increase insulin sensitivity in vivo (6; 93). Therefore, thiazolidinedione compounds appear to be a favorable treatment in alleviating the

metabolic abnormalities found in lipodystrophy syndromes. When troglitazone, a member of this class of drugs, was given to rodents with lipodystrophy syndromes, the effects were positive, improving metabolic condition and increasing adipose mass (94). Human treatments also provide similar results. Lipodystrophic patients treated with troglitazone for 6 months showed improvement in metabolic control, with decreased hemoglobin A1c, triglyceride levels, and free fatty acids levels. Furthermore, patients experienced a 3% increase in subcutaneous adipose tissue over the treatment period (95). These findings suggest TZDs stimulate adipocyte differentiation and possibly promote normal metabolic function, alleviating glycosuria and sparing of fat stores in this population. Nevertheless, more human trials are still needed over a longer period of time, using not only troglitazone but more recent classes of TZDs, such as rosiglitazone and pioglitazone. Such studies would be helpful in further evaluating the benefits as well as the potential risks involved.

Many of the metabolic complications characteristic of lipodystrophy are similar to a cluster of metabolic abnormalities faced by obese individuals collectively known as "metabolic syndrome X" or "insulin resistance syndrome," characterized by glucose intolerance, noninsulin-dependent diabetes mellitus (NIDDM), dyslipidemia, and hypertension (96; 97). As a result of these similarities, genetic lipodystrophy defines an extreme example of monogenic insulin resistance, providing a research model to study the underlying mechanism of insulin resistance associated with the obese phenotype.

Melanocortin pathway

Leptin is produced by the peripheral adipose tissue and acts on the arcuate nucleus in the hypothalamus to activate proopiomelanocortin C (POMC) (66). POMC is a single gene product that is spliced into several different peptides, including α-melanocyte–stimulating hormone (α-MSH). The activation of POMC increases α-melanocyte–stimulating hormone (α-MSH), which binds to melanocortin-3 and melanocortin-4 receptors (MC3R and MC4R).

The discovery of the melanocortin pathway, as it relates to food intake and energy expenditure, began in a mouse strain known as *agouti yellow mice*. These mice inherit a dominant mutation that causes obesity and yellow coat color. The mutation causes ectopic expression of a protein called *agouti* or *agouti signaling protein* (ASIP), which is normally expressed only in the skin (2; 98). When agouti protein is expressed in the hypothalamus, it blocks binding of α-MSH to MC3R and MC4R. Each of these receptors is expressed in the centers of the hypothalamus that regulate weight. Although only the MC4R mutations cause increased food intake and decreased energy expenditure in humans, reports indicate MC3R may also be associated with obesity but more research is needed. In fact, mutations in MC4R accounts for nearly 4% of all common cases of human obesity (2; 99).

The Zucker fatty (*fa/fa*) Rat

The Zucker fatty (*fa/fa*) rat inherits an autosomal recessive mutation that causes early onset obesity (100). The fatty phenotype includes increased energy efficiency,

hyperphagia and infertility. As fatties become obese they also develop hyperlipidemia, insulin resistance, renal disease, and other pathologies. Female fatties are essentially sterile, displaying a small, underdeveloped uterus, while males are classified are infertile because they are occasionally able to breed. As a result, heterozygote Zucker rats are the most appropriate breeders to maintain a colony (100).

The *fa* mutation was mapped to rat chromosome 5, in a region that shares synteny homology with the region of mouse chromosome 4 that contains the *db* mutation (101). This suggested that *fa* and *db* affect rat and mouse homologs of the same gene. This was confirmed after the *db* mutation was cloned and shown to be a leptin receptor (102; 103).

The fatty rat inherits an amino acid substitution at position 269 of the leptin receptor protein sequence, changing glutamine to proline within the extracellular domain of the leptin receptor, causing a disruption in leptin signaling (104). There are six splice variants of the leptin receptor in the rat, and the *fa* is incorporated into all the splice variants (105). This differs from the *db* mutation, which affects the intracellular signaling domain of only one leptin receptor splice variant, the B form (102). As a result, the fatty rat leptin receptor has a reduced ability to respond to leptin, although large doses of leptin can stimulate some signaling through the mutant receptor (104).

In the initial description of the fatty mutation, it was noted that fatties became visibly obese after weaning, and also developed elevated amounts of a "white, milky substance" in serum samples at this age (100). This substance was found to be circulating lipids (106). This observation provided the first clue that a developmental program was involved in the onset of obesity in fatties.

Thermogenesis

Preobese suckling fatties accumulate excess adipose mass by maintaining a lower body temperature than their lean littermates, even when food intake is similar (107). They remain visibly indistinguishable from their lean littermates until the fourth week of life (107; 108). When fatties and lean pups are reared in a neutral thermal environment, both genotypes accumulate the same amount of adipose mass (109). This demonstrates that energy expenditure produces the difference in adipose mass between the fatty and lean pups with similar energy intakes. These data suggest that a portion of the leptin signal transduction pathway affecting energy expenditure functions normally during the neonatal period in normal rats, but is defective in fatties (107; 108; 110).

During the early developmental period, a reduction in sympathetic activity controlling thermogenesis in brown adipose tissue appears in the fatty rat, which partly contributes to the etiology of genetic obesity in fatties (108). Brown adipose tissue develops early in embryonic development, functioning to primarily dissipate energy in the form of heat during adaptive non-shivering thermogenesis through activation of the mitochondrial β-oxidative pathway by uncoupling proteins (111; 112). When this system is suppressed or ablation of brown adipose tissue in rodent models occurs, there is an increase metabolic efficiency, resulting in obesity (113).

Weaning practices

Many of the metabolic abnormalities associated with altered energy balance in the Zucker fatty rat emerge during or immediately following the weaning period. During

this period that fatty rats becomes distinguishable from their lean littermates becoming visibly obese and displaying defects in glucose homeostasis (114). In this context, weaning is defined as a common husbandry practice of separating the pups from their dam at 3 weeks of age. This unnatural procedure promotes abrupt alterations in diet and thermal environment (115; 116). When pups remain with the dam, weaning is gradual and nursing can continue for up to 5 weeks of age (116).

It should be noted that early separation of pups from the dam promotes stress responses that alter the timing of developmental events (115; 116). When maternal separation precedes such events, it is difficult to differentiate between those that are stress-induced and those that are a result of spontaneous developmental occurrences (115). Several observations indicate that, when animals are weaned suddenly, weight gain is stimulated in the fatty rat by maternal separation rather than natural developmental occurrences (3; 115). In fact, enzymatic activity appears to be impacted by weaning practices, whether this is a minute delay in maturation or an effect that is not detected until later in development weaning appears to be a contributing factor. Such enzymes include jejunal sucrase, hepatic glucokinase, pancreatic amylase, lipase, and chymotrypsin (115).

Weaning research provides evidence that developmental occurrences are affected by alterations in diet although diet is not the only initiator of the ontogenic changes. Such effects are demonstrated when pups that remain unweaned are compared to those that are weaned according to traditional practices. Initially, both groups display similar levels of jejunal sucrase activity, verifying these levels are not initiated by dietary

practices. With age, both groups diverge, indicating this early cue produces changes that eventually occur later in life (115).

Physical activity levels are also altered by the timing of weaning which may contribute to the early onset obesity seen in fatties. Moreover, the fatty does not appear to be born less active than lean littermates; rather inactivity appears to coincide with separation from the dam at early weaning. Although activity levels decline in fatties when unnatural weaning does not occur, this time point is much later in development and is also associated with a period in which the dam spontaneously weans pups (117).

It is evident that separating these developmental occurrences from stress responses is difficult especially when weaning precedes a defining developmental occurrence (3; 117). Studies by Truett et al revealed when maternal separation was no longer a limiting factor and pups were left with their dams up to 4 weeks of age, a reduction in the variation of growth with age occurred. Under these natural conditions, fatties suddenly developed robust hyperphagia after 22 days of age and rapidly developed overt obesity soon after. Because pups were not weaned this developmental physiological event appears to be independent of environmental factors induced by maternal separation (3).

Hyperphagia

The onset of hyperphagia in the Zucker fatty rat is an area that has been explored by several researchers using a variety of experimental techniques. Although several hypotheses have been proposed, the precise timing of this developmental occurrence

remains unknown due to inconsistent findings between investigators and different methodologies.

The portion of the leptin signal transduction pathway that suppresses energy intake in adulthood appears to be insignificant in neonate pups when maximal food intake is needed to support growth during this critical period (118). Exogenous leptin during the neonatal period in mice does not promote the anorectic effects seen later (39). In fact, the neonatal period may not be a time when animals are insensitive to leptin, but rather leptin appears to produce a developmental signal that assists in the formation hypothalamic circuitry pathways that maintain leptin signals to the brain later in life to mediate food consumption (119).

Several studies have demonstrated fatty and lean littermates consume approximately the same amount of milk during the suckling period (120; 121). Others have found that even after injection of leptin into wildtypes, energy intake remains unaffected during this critical period of growth and development (122).

Kowalski *et al* demonstrated a technique to determine the onset of hyperphagia by capturing ingestion practices of preweaned fatties independent of the dam. To stimulate feeding, pups were removed from the dam and placed in an incubator for 3.5 hours with only littermates. After fasting the animals and eliminating urine and fecal matter, pups were provided ad libitum access to half and half (milk and cream) for 20 minutes. Afterwards, animals were weighed to estimate food intake at 4 ages. Using this environment, fatty pups ate significantly more than lean pups at 12, 15, and 18 days of age (123).

Others have also concluded fatties experience hyperphagia early in development. McLaughlin and Baile observed food intake by training pups to release food pellets by pressing a bar, at which time several parameters were recorded for analyzed (124). Data indicated there was an elevation in food intake at 3 weeks of age due to increased meal frequency, which continued to be elevated by 4 weeks of age due to meal duration. Researchers did not specify the weaning or rearing practices used, but it is unlikely that at 3 weeks fatties were visibly obese; therefore, it is possible these animals were older (124).

Evidence also exists that fatty pups consume similar amounts of food as their lean littermates in early development, and the onset of hyperphagia does not become apparent until later in life (121). Buchberger and Schmidt demonstrated that 5 and 15 day old fatty and lean littermates raised in a thermoneutral environment and exposed to an abundant supply of milk for 1 hour, in fact, consumed similar amounts of milk based on body weight (121).

Together these observations offer evidence to suggest a developmental occurrence appears in early life that may alter the onset of hyperphagia, yet it is difficult assess realistic consumption levels of fatties. Each of the studies described creates an extremely unnatural environment that may induce a stress response, altering developmental timing. Truett et al demonstrated that changes in daily weight gains, stomach contents, liver weight, and plasma insulin are significantly increased at 22 days, when pups are reared in a natural, unaltered environment (3). Therefore, these findings provide evidence that the onset of hyperphagia occurs during the fourth week of life (3).

Diet composition

Transitioning from total dependence on the dam to independent control of food begins to take place during the third week of life. During this time, pups begin consuming chow, introducing carbohydrates as the primary macronutrient source rather than fat found in milk. Several have observed how this dietary modification alters hyperphagia. Vaselli and Maggio reviewed the course of hyperphagia when placing juvenile fatty rats on a high fat diet and found hyperphagia increased and there was an accelerated accumulation of adipose mass (125).

In contrast, others have compared the effect of weaning 16 day old fatties onto a diet similar in macronutrient content to the dam's milk versus a diet high in carbohydrates similar to chow. This study found those animals weaned onto the high fat diet were less obese than those fed the high carbohydrate diet (126). These studies provide contradicting results, failing to define the onset of hyperphagia as it relates to diet composition and inducing unnatural environmental practices when performing early weaning.

Lipid and Glucose Metabolism

Physiological Role of Adipose Tissue

Adipose tissue is a specialized tissue in the human body forming in areas with dense connective tissues, including the subcutaneous layers between the muscle and the

dermis, in addition to areas encompassing the heart, kidneys, and other internal organs. Fat cell development usually occurs in clusters and disperses in many sites of the body.

Adipose tissue is found in two forms within the body - white adipose tissue and brown adipose tissue (111). White adipose tissue serves to provide heat insulation, mechanical cushion, and most importantly is a source of energy. These unilocular cells appear as a single large lipid droplet, which pushes the cell nucleus against the plasma membrane.

Brown adipose tissue acts to dissipate heat rather than store it, essentially defending against cold and protecting against obesity. Therefore, heat production is used for non-shivering thermogenesis and for utilization of excess caloric intake via diet-induced-thermogenesis. The mechanism of heat generation occurs in the mitochondria of brown adipose tissue which has a specific carrier called uncoupling protein that transfers protons from outside to inside without further production of ATP tissue. Brown adipose tissue has a multilocular phenotype, containing many smaller lipid droplets. The brown color of this tissue is a result of cells' rich vascularization and densely packed mitochondria (111; 127).

Adipose tissue is comprised of triglycerides stored in the cytoplasm as lipid droplets constructing the adipocyte. Triglycerides are synthesized through circulating lipoproteins or *de novo* lipogenesis. In fact, triglycerides are constantly being synthesized and broken down in the adipose tissue due their short half-life. *De novo* lipogenesis is the metabolic process in which glucose is converted to triacylglycerols (TAG) when energy demands have been met; whereas, when energy levels are low, TAG

undergo a metabolic process called lipolysis to release fatty acids for energy demands (111; 127).

Many modern molecular advances have further defined adipose tissue not only as a storage unit for energy but also as an important component in the secretion of gene products or acting as a signaling molecule. These research efforts clearly define adipose tissue as a primary link to metabolic programming (111; 112).

De novo lipogenesis

De novo lipogenesis is an important metabolic process that allows the body to store calories efficiently in the form of fat. This process is essential during periods of dietary restriction, allowing for survival. The metabolic process is summarized as follows (128). Lipogenesis begins when glucose is ingested and converted through glycolysis to pyruvate. Pyruvate is oxidized by pyruvate dehydrogenase to produce acetyl-CoA which is then converted to citrate by citrate synthase. At this point, citrate can be oxidized by the TCA cycle to produce energy, or it can be exported from mitochondria to the cytosol, where it is converted back to acetyl-CoA by citrate lyase. Acetyl-CoA is converted malonyl-CoA by acetyl-CoA carboxylase (ACC). ACC is a rate- limiting step in fatty acid synthesis. Palmitate is synthesized from acetyl-CoA and malonyl CoA using the catalytic properties of fatty acid synthase (FAS) and nicotinamide adenine dinucleotide phosphate (NADPH) as the reducing equivalent. The NADPH required for the activity of FAS is derived from converting malate to pyruvate by malic enzyme or by the enzymes involved in the pentose phosphate shunt. De novo lipogenesis

is a functioning pathway as a result of ACC and FAS. ACC functions as the short-term rate limiting step in the regulation of fatty acids and FAS is the long-term rate limiting step in this process (129).

Fatty acids are ingested in the diet primarily as triglycerides or synthesized by the liver. Triglycerides are comprised of three long chain fatty acids attached to a glycerol backbone. They are carried from the intestines in chylomicrons or from the liver in very low density lipoproteins (VLDL). Lipoprotein lipase (LPL) in peripheral tissues hydrolyzes triglycerides to glycerol and free fatty acids. Lipoprotein lipase is synthesized in adipocytes and secreted into adjacent endothelial cells. Chylomicrons and lipoproteins (very low density lipoproteins) contain an apoprotein, which activates LPL allowing free fatty acids to be taken up by adipocytes. When inside the adipocyte, fatty acids become part of a pool that is constantly being replaced. Before the fatty acids can be stored in the adipose tissue, they must first combine with coenzyme A to form a thioester and then they are re-esterified to glycerol to form triglycerides. Glucose provides the majority of glycerol for this re-esterification process (127; 129).

Insulin also has multiple roles in the lipogenic process. Insulin stimulates LPL formation, allowing circulating triglycerides to be hydrolyzed and free fatty acids to enter the adipocyte. Insulin is also required for the transport of glucose, which is a primary component of the re-esterification of the triglycerides once inside the adipocyte. Lastly, insulin activates several enzymes that promote the conversion of glucose to fatty acids (127; 129).

Fatty acid synthase (FAS)

As previously explained, FAS is an essential component of *de novo* lipogenesis. Biosynthesis of fatty acids in simple life forms such as bacteria perform these steps by catalyzing separate peptides, whereas higher life forms such as mammals have consolidated these multiple steps into a single protein produced by a single gene. FAS is a homodimer with each identical peptide subunit being approximately 260 kDa in size (130). Each subunit contains 8 functional domains, 6 of which are required for synthesis and elongation of the fatty acid chain (129; 130).

Nutritional and hormonal regulation

Alterations in diet composition promote fluctuations in FAS expression. Carbohydrates are a key regulating factor of FAS expression in the animal model (131). In Zucker rats, *FAS* mRNA concentrations are low during the suckling period and dramatically increase when weaned onto a high carbohydrate diet, with fatties expressing the highest levels. Yet, when pups are weaned onto a high-fat diet these changes preclude this increase (8; 132). Others have shown there is also a fasting and refeeding response. When animals are fasted for a period of time and then refed a carbohydrate diet, hepatic FAS protein levels will increase (131). Feeding animals a diet consisting of polyunsaturated fatty acids will also decrease FAS expression at the level of gene transcription. This inhibitory effect of FAS by dietary fatty acids has not been observed with saturated or monounsaturated fatty acids (133).

Sterols also have properties that regulate gene expression of FAS. Sterols interact with sterol regulatory element-binding proteins (SREBP-1 and SREBP-2). FAS contains 2 SREBP-1 binding sites activating transcription of this enzyme (134). Like FAS, SREBP-1 is also decreased by fasting and induced when refeeding a high carbohydrate diet, indicating SREBP-1 may mediate insulin's stimulatory effects of FAS during these periods (135). Hormones also play a crucial role in FAS expression. It is during fasting and refeeding of high carbohydrate diets that insulin levels will increase and glucagon will decrease causing FAS mRNA induction to be stimulated. When animals are insulin deficient, such as diabetic mice, FAS mRNA levels will fall, yet this effect can be reversed when injecting these animals with insulin (136). It appears activation of FAS by insulin mediation is a result of a insulin response element (IRE) found on the FAS promoter (137; 138). Yet when glucagon or dibutyryl cAMP is administered, FAS expression is suppressed (136). Thus, it appears glucagon acts through cAMP-dependent mechanisms to decrease synthesis of FAS (136).

Thyroid hormone (T3) also increases FAS expression in adipocytes through gene transcription (137). Hyperthyroid rats show FAS mRNA levels are increased 9- fold in the liver and 3- fold in the white adipose tissue in comparison to hypothyroid rats (139).

Transcriptional regulation of adipose differentiation

Peroxisome proliferator-activated receptors (PPARs)

Peroxisome proliferator-activated receptors (PPARs) are members of a subfamily of nuclear hormone receptors which contain type II zinc finger DNA binding motif (140).

Such ligand-activated nuclear receptors are pivotal components of gene transcription and intracellular function in a diverse number of biological functions, including lipid and glucose homeostasis, cellular proliferation, and differentiation (141). As shown in Figure 1, the PPAR subfamily form heterodimers with another nuclear hormone receptor, retinoid X receptor (RXR), bind peroxisome proliferator response elements on target genes, and become transcriptionally active (142). Protein-protein interactions are formed between different nuclear proteins called coactivators and corepressors, interacting with the PPAR-RXR heterodimer to upregulate or down regulate gene expression (143). Three subtypes of the PPAR family exist

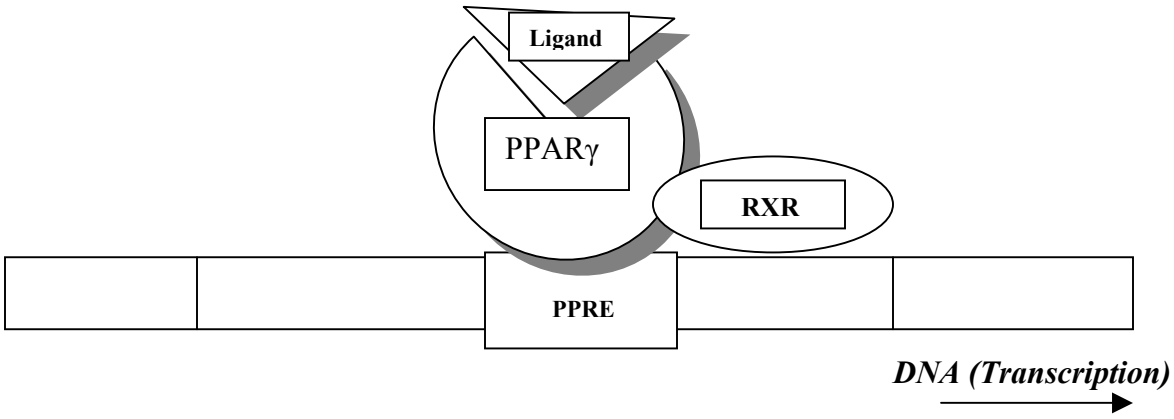

Figure 1. Peroxisome proliferator-activated receptors (PPARs) regulation of gene expression.

(PPARγ, PPARα, PPARδ), each processing unique biological characteristics and tissue specifies.

PPARα is primarily expressed in the muscle, heart, liver, and kidney where it is involved in the regulation of fatty acid catabolism (144; 145). In fact, gene targets of PPARα function to conserve energy during fasting and feeding. PPARδ is located ubiquitously with less defined function. Research does indicate its role in keratinocyte differentiation, wound healing, and mediation of VLDL signaling (146; 147). PPARγ is expressed in the adipocyte and macrophage where it functions in adipocyte differentiation, lipid storage, and glucose homeostasis.

Physiological role of PPARγ and ligand activation

PPARγ is the most adipose tissue specific of the 3 isoforms, and has been exhaustively researched as it pertains to lipid metabolism as well as other basic and clinical functions. PPARγ exist in 2 isoforms: PPARγ1 and PPARγ2. PPARγ2 is 30 amino acids longer than PPARγ1 at the N terminus and the dominant isoform selective for adipose tissue (148; 149). PPARγ is regulated at the level of gene expression by pharmacological and endogenous ligand availability and through the phosphorylation status of PPARγ activity. PPARγ appears to be an important component in the regulation of developmental genes. When the PPARγ gene is deleted in mice, the embryo is aborted at approximately 10 days of gestation due to placental insufficiency in the adipose, placenta, and cardiac tissues (150). In one instance, the PPARγ-null homozygous mutant developed to term, yet the animal lacked adipose tissue and died by postnatal day 5,

further suggesting PPARγ is critical in the development of adipose tissue *in vivo* (150; 151). *In vitro* studies indicate similar findings. The differentiation of adipocytes from PPARγ null embryonic stem cells were dependent on PPARγ gene dosage, while other organ tissues developed normally regardless of dose (152). These data show PPARγ has a significant role in the formation of adipose tissue in vivo and adipocyte differentiation in vitro.

PPARγ is activated by synthetic and natural ligands binding to the carboxyl-terminal ligand-biding domain. There are several naturally occurring ligands that bind to PPARγ with a low affinity; it has yet to be revealed a naturally occurring high affinity PPARγ ligand. Several types of polyunsaturated fatty acids, such as linolenic acid, linoleic acid, and eicosapentaenoic acid, activate PPARγ at a micromolar concentration. Although some nuclear receptors bind to ligands in nanomolar concentrations, free fatty acids circulate within the human serum in a micromolar concentration; therefore, this is consistent (153; 154). Diets containing polyunsaturated fatty acids have been shown to promote the same physiological effects as the synthetic PPARγ ligands, such as increasing insulin sensitivity and decreasing triglyceride levels (155). Certain prostaglandins, such as 15-deoxy-$\Delta^{12,14}$ –PGJ_2, also function to activate PPARγ demonstrating an anti-inflammatory response (156). Although it remains unclear whether this prostaglandin is present in adipose tissue, in vitro studies indicate it acts to stimulate adipocyte differentiation (93; 157). Although a high affinity natural ligand has not been discovered, it appears PPARγ binds to a multitude of polyunsaturated fatty acids serving to regulate levels and mediate genes involved in glucose and lipid homeostasis.

Multiple synthetic PPARγ activated ligands have been identified, of which the most well known are the thiazolidinediones (TZD) or glitazones. This class of drugs was first described for their ability to reduce insulin resistance and glucose levels without overexerting the pancreas to secrete insulin in type 2 diabetic rodent models (158). In addition, these compounds were also associated with minimizing triglyceride and free fatty acid levels. It was not until 1995 that TZDs were recognized as agonist for PPARγ (159). Several classes of glitazones exist to treat type 2 diabetes in humans, including rosiglitazone (Avandia) and pioglitazone (Actos). One medical issue surrounding the use of TZDs is their ability to promote increased food consumption and lipogenesis in rodents and humans. When adult fatty rats are treated with insulin-sensitizing rosiglitazone, the effects are strikingly similar to those changes that take place with the onset of hyperphagia (4; 5). Non-insulin dependent diabetic individuals clinically taking this medication often gain additional weight, when usually already overweight. It is uncertain whether this effect is primarily a result of increased insulin sensitivity, increased fat cell differentiation, or both. Some research suggest the activation of PPARγ by TZD may increase fat cell number while decreasing fat cell size, possibly explaining this receptors role in increased insulin sensitivity and adipogenesis (160). The mechanism underlying the activation of PPARγ by TZDs to produce insulin sensitivity remains unclear. Obesity and insulin resistance are linked, yet this receptor is primarily found in the adipose tissue and is known to produce adipogenesis; therefore, this receptor is a novel physiological control. Nevertheless, it is important to understand obesity and adipose cell differentiation are not identical. Obesity is a disorder in which energy

balance is not achieved, whereas increased fat cell number does not always lead to obesity and insulin resistance unless total energy stores are increased. Although TZDs are synthetic ligands, they have provided extensive insight into the biological function of PPARγ, providing explanations for such effects.

PPARγ is predominantly found in adipose tissue but is also expressed in other tissues such as the liver and muscle (161). High affinity ligands such as TZDs may stimulate all these tissue sites to produce alterations in gene expression that eventually induce insulin sensitivity. Cell culture studies indicate ligand activation of the PPARγ is part of a positive feedback loop. When treated with the TZD, troglitazone, mice genetically altered to lack adipose tissue showed improved insulin sensitivity (94). This suggests that the regulation of PPARγ can take place in the absence or near absence of adipose tissue, indicating the action of PPARγ receptors partially occurs in adipocyte-independent pathways. PPARγ ligands produce a coordinate activation of this receptor both directly and indirectly to increase expression in the skeletal muscle and liver to promote insulin sensitivity.

PPARγ agonists may produce insulin sensitivity indirectly by lowering fatty acid levels in the muscle and liver thus altering glucose metabolism in these tissues (162). In muscle, PPARγ agonists provoke the Randle cycle, which occurs when glucose and fatty acids compete for use as energy substrate (163). This influx of free fatty acid levels prevents glucose disposal in the muscle. In the liver, this influx of fatty acids promotes increased gluconeogenesis. These physiological occurrences in the muscle and liver

induced by PPARγ agonists increase uptake and storage of fatty acids in the adipocyte, causing decreased triglyceride and free fatty acids in circulation (163).

TZDs activation of PPARγ also increases adipose cell differentiation. When energy intake does not exceed energy expenditure, the body produces more fat cells that are smaller in size yet more sensitive to insulin. These occurrences may invoke insulin sensitivity (94; 160; 164).

As discussed above, TZDs promote glucose utilization in skeletal muscle and decrease de novo glucose synthesis in the liver of type 2 diabetics (7; 165). PPARγ found in adipocytes may regulate glucose metabolism in the liver and muscle by the secretion of endocrine signaling molecules. Two possible signaling molecules produced by adipose cells are the cytokines, TNF-α and leptin, both of which are repressed by PPARγ agonists (166; 167). TNF-α is known as an inhibitor of adipocyte differentiation, mediated by the down regulation of PPARγ. Research shows elevated levels of TNF-α in vivo and in vitro may cause insulin resistance. In fact, mice knockouts lacking TNF-α or its receptor develop insulin resistance (168; 169). Hofmann et al showed treatment with TZDs for a 2 week period reduced adipose TNF-α mRNA expression by 50% (166). The role of leptin on insulin sensitivity remains unclear, but in vitro studies suggest leptin can interfere with insulin signaling in various cell types (7). Qian et al showed rodents treated with leptin for 5 days increased PPARγ expression by 70-80% at both 3 and 8 months, suggesting PPARγ may be associated with leptin-induced adipocyte apoptosis signal pathway (170). PPARγ agonists may induce insulin sensitivity by decreasing TNF-α and increasing adipose tissue for secretion of leptin.

Transcriptional Cascade and Adipogenesis

Adipogenesis is the differentiation of fat cells from preadipocytes into adipocytes. These mature adipocytes are lipid filled expressing and secreting several hormones and cytokines. To support cell turnover and excess energy storage, adipogenesis is a normal developmental occurrence, taking place during the prenatal and postnatal periods. Several transcription factor families have emerged that are pivotal components in the early phases of cell growth into the differentiated adipocyte. These are PPARγ, CCAAT/enhancer-binding protein (C/EBP), and sterol regulatory element-binding protein 1 (SREBP-1) (112; 171).

Several studies first described expression and activation of PPARγ driving fibroblast and myoblasts to efficiently differentiate into mature adipocytes (6; 172). Other studies further demonstrated insulin resistant rodents treated with PPARγ agonists instead showed minimal large adipocyte formation, while producing more small adipocytes in the white adipose tissue (160; 164). These detailed studies provide strong evidence to support PPARγ's pivotal role in adipocyte differentiation and the foundation to research linking PPARγ to other transcription factors. PPARγ interacts with other transcription factors such as C/EBPs and ADD1/SREBP1. Together, these factors regulate adipogenesis through a sequential cascade of events regulated at the level of transcription, essentially explaining activation of C/EBP and PPARγ. C/EBPβ and C/EBPδ induce expression of PPARγ through transcriptional control (173). PPARγ is stimulated by ligands and thus begins adipogenesis. C/EBPα is induced later in

differentiation, yet it is capable of synergizing with PPARγ to maintain elevated PPARγ levels. The mechanism responsive for their interaction remains unclear. C/EBPα is expressed in several tissue types, even inducing adipogenesis when expressed at high levels (174). However, when expressed in adipose tissue, C/EBPα functions with PPARγ through binding sites in the PPARγ promoter to create an adipogenic response.

ADD1/SREBP1, a second transcription factor, was first recognized for its effects in cholesterol regulatory genes (175) also acting as a key component in adipogenesis and fatty acid metabolism (176). In addition, this transcription factor has also been shown to function with PPARγ in adipocyte determination and differentiation. Coexpression increases PPARγ expression without ligand activation. It appears this activation is possible because ADD/SREBP1 has a unique ability to induce the expression of several major genes in fatty acid metabolism, such as fatty acid synthase and lipoprotein lipase, providing high levels of endogenous ligands for PPARγ, causing transcriptional activity (135).

The implication that childhood obesity is a critical starting point to the manifestation of obesity in adults creates an intriguing area of research. Moreover, this period of rapid growth needs to be better understood and used as a strategy to control associated health problems. Therefore, these research projects described in the following sections were completed to better understand the coordinated changes in genes and pathways that mediate hyperphagia during a brief developmental period in the fatty rat. This model system provides a foundation to understand cellular and molecular mechanisms that may

influence the early onset of obesity in children. We propose the activation of pathways regulated by PPARγ promote insulin sensitivity and the onset of hyperphagia.

PART 3. INSULIN SENSITIVITY AND DEVELOPMENT OF HYPERPHAGIA IN ZUCKER FATTY RATS

Abstract

The onset of hyperphagia in the Zucker fatty (*fa/fa*) rat occurs on a single day in postnatal development and could be driven by increased insulin sensitivity. To test this hypothesis, we performed insulin tolerance tests at several points in development. In rapidly growing juvenile rats, fatties are as insulin sensitive as leans at 4 weeks of age, and became increasingly insulin resistant as they become obese. During the suckling to weaning transition, fatties are insulin resistant at 2 weeks of age, when they are exclusively suckling; they are also insulin resistant at 3 weeks of age, when they are suckling and consuming solid food, but not hyperphagic. By 4 weeks of age, when fatties are hyperphagic, fatties are as insulin sensitive as their lean littermates. These data indicate that fatties experience two phases of insulin resistance, punctuated by a brief period of insulin sensitivity that follows the onset of hyperphagia. To determine whether the increase in insulin sensitivity could be driving the onset of hyperphagia, insulin tolerance tests were performed from 21 to 27 days of age. Fatties and leans became increasingly insulin resistant from 21 days to 23 days of age, then became as insulin sensitive as leans by 25 days of age. These data show that increased insulin resistance precedes the onset of hyperphagia and increased insulin sensitivity follows the onset of hyperphagia. This pattern suggests that developmental perturbations in insulin signaling are likely to be involved in the onset of hyperphagia.

Introduction

Zucker fatty (*fa*/*fa*) rats inherit a leptin receptor mutation that causes early onset obesity (104). Fatties grow at the same rate as their lean littermates for the first 22 days-of-age, then suddenly begin to grow at a much higher rate (3). This dramatic increase in growth rate corresponds with the emergence of hyperphagia in fatties, and is accompanied by the onset of hyperinsulinemia. Fatties become visibly distinguishable from their lean littermates at this age by the expansion of their bellies with food, and within a few days they also become visibly obese (3).

This increase in growth is easily detectable when fatties remain with their dams through the fourth week of life, but is obscured when fatties are separated from their dams at three weeks-of-age, which is a common husbandry practice. The fact that the onset of hyperphagia occurs at such a precise age without the introduction of environmental changes suggests that a developmentally regulated mechanism determines the timing of the onset of hyperphagia.

The weaning transition is associated with many physiological changes, including changes in insulin sensitivity in adipose tissue in normal rats. Suckling rats consume a high fat, low carbohydrate diet and are somewhat insulin resistant (177-179). Furthermore, the expression of PPARγ, a transcription factor that controls the expression of insulin sensitizing pathways, is very low during the first two weeks of life and increases to adult levels during the weaning transition (8). An increase in insulin

sensitivity during this period could contribute to the onset of hyperphagia by stimulating pathways for glucose disposal.

An analogous situation occurs when adult fatties are treated with PPARγ receptor agonists. Fatties become increasingly insulin resistant as they become obese (180), and their hyperphagia also lessens as they become obese and insulin resistant (125). However, treatment of insulin resistant obese adult fatties with thiazolidinediones increases insulin sensitivity and stimulates hyperphagia (4). These observations suggest that an increase in insulin sensitivity could also play a role in the emergence of hyperphagia in fatties during the weaning transition. To determine the relationship between insulin resistance and the emergence of hyperphagia in fatty rats, we measured insulin tolerance under several developmental conditions.

Materials and Methods

Animals

Animals were produced in the principal investigator's colony of partially inbred (F15-F17) ZUCKER-*fa* rats. Animals are housed in plastic breeder boxes with ad libitum access to Teklad 8640 Rodent Diet and tap water. The light cycle is maintained at 12 hours of dark, followed by 12 hours of light. The room temperature is maintained at 68 to 74°F and 55 to 60% humidity. All animal studies were reviewed and approved by the University of Tennessee Institutional Animal Care and Use Committee.

The population is maintained with brother by sister matings to minimize extraneous genetic variation and with selection for large litter size to minimize inbreeding

depression. Sires are removed when the dams appear pregnant. The day of birth is considered 0 days of age. Neonates are genotyped for *fa* during the first week of life, as described in detail below. Litters are reduced to 8 pups during the first ten days of life by decapitating excess pups. This is done to reduce variation in growth due to litter size. Pups are weighed daily from 14 days of age to the end of each experiment to acclimate them to handling and to generate growth curves. Pups remain with their dams until 28 days of age.

Genotype detection

Pups are marked by toe-clipping, and a single toe from each pup is saved to prepare DNA by the HotSHOT method (3; 181). Briefly, toes are collected into 200 µl thermal cycler tubes. Seventy-five microliters of 25 mM NaOH/ 0.2 mM EDTA/ pH 12 are added to the tubes. Samples are heated to 95 degrees C for 30 minutes, and then cooled to room temperature. Seventy-five microliters of 40 mM Tris HCl/ pH 5 are added to each tube to adjust the pH to 8. The DNA is then ready for PCR amplification.

The *fa* mutation creates a restriction site for *Msp* I that can be used to detect *fa* genotype (104). However, *Msp* I occasionally fails to cut DNA to completion in our hands, which can cause genotype misclassification. To overcome this problem, we engineered an alternative restriction site by a strategy that has been described previously (182). PCR primers were designed to amplify 101 base pair (bp) of DNA flanking the *fa* mutation. The reverse primer, RLepr, was selected to anneal adjacent to the site of the mutation, and a single base substitution (C→G) was made in the second base from the 3'

end of the primer to create a *Pvu* II restriction site in the wildtype allele and no restriction site in the mutant allele. The primer sequences are fLepr, 5'-CGTATGGAAGTCACAGA-3', and RLepr, 5'GAATTCTCTAAATATTTCAGC-3'; the base substitution in RLepr is underlined. Primers are mixed at 500 nM each in 50mM KCl, 10mM Tris-HCl (pH 9 at 25°C), 0.1% Triton X-100, 1.5 mM $MgCl_2$, 0.2 mM each dATP, dCTP, dGTP and dTTP, 0.25 units *Taq* DNA polymerase and 2 µl of HotSHOT DNA in a 22 µl volume. PCR amplification is carried out in a Robocycler thermal controller (Stratagene, La Jolla, CA) at 95°C for two min, followed by 40 cycles of 95°C for 30 sec, 45°C for 45 sec and 72°C for 30 sec. Twenty µl of PCR product are digested with 1 unit *Pvu* II (New England Biolabs, Beverly MA) in a 30 µl volume for 1 h at 37°C. Thirty µl of *Pvu* II treated PCR product are loaded onto a 3% 3:1 high-resolution blend agarose (Amresco) gel and electrophoresed 30 minutes at 5 volts/cm. Gels are stained with ethidium bromide and exposed to UV light. The wildtype genotype produces an 80 bp band, and the mutant genotype produces a 101 bp band; both bands are visible in heterozygotes.

Insulin Tolerance Tests

Preliminary insulin tolerance tests, in which blood glucose was measured at multiple time points after insulin injection, determined that blood glucose levels reach a nadir 30 minutes after injection of 0.5 units/kg of insulin (Humulin R, Eli Lilly Pharmaceuticals). Therefore, we used a simplified insulin tolerance test to assess the development of insulin resistance in fatty rats. Rats were fasted two hours, then the tip of

the tail was nicked with a scalpel blade, a drop of blood was expressed onto a blood glucose test strip, and glucose values were measured with a glucose meter (Prestige Smart System, Walgreens). After measuring fasting glucose, rats were injected subcutaneously with 0.5 units/kg of insulin. A second glucose measurement was made 30 minutes after insulin injection, and the rats were returned to their home cage with *ad libitum* access to food and water. This simplified insulin tolerance test reduces the stress to the animals. It also limits the number of incisions that must be made in the tail and enables the same subjects to be tested repeatedly across development.

Experimental design

Development of insulin resistance in juvenile fatties

An initial experiment was performed to establish the course of development of insulin resistance in fatty rats under our experimental conditions. Sex-matched fatty and wildtype littermates (N=24) were separated from their dams at 24 days of age and housed in pairs. Insulin tolerance tests were performed at 28, 35, 42, 49, and 56 days of age on the same set of rats.

Development of insulin sensitivity during the weaning transition.

The transition from suckling to weaning includes substantial changes in diet composition and food intake in fatties and their lean littermates. At 14 days of age, pups obtain all their nutrients from the dam. At 21 days of age, pups are suckling and consuming solid food and fatties are not yet hyperphagic, hyperinsulinemic or visibly

obese. By 28 days of age, pups are obtaining most of their nutrients from solid food and fatties are hyperphagic, hyperinsulinemic and visibly obese. To test the hypothesis that changes in insulin sensitivity occur across this interval, insulin tolerance tests were performed at 14, 21 and 28 days of age on the same set of rats. Rats were housed with their dams throughout this period, except during fasting and insulin tolerance tests to minimize investigator-induced changes in experimental conditions.

Development of insulin sensitivity and hyperphagia in fatty rats.

The results of the previous experiment suggested that changes in insulin sensitivity could be driving the onset of hyperphagia. To test this hypothesis, rats were housed with their dams throughout this period, except during fasting and insulin tolerance tests. Insulin tolerance tests were performed at 21, 22, 23, 24, 25, 26, and 27 days of age, using different litters for each age group. It was necessary to use different sets of rats for each age because it takes a couple of days to recover from insulin tolerance tests.

Statistical analyses

Data were analyzed under mixed linear models using Proc Mixed of SAS Version 8.2. Genotype and sex were treated as fixed effects factors. Age and litter were treated as random effects factors.

Results

Development of insulin resistance in juvenile fatty rats

Fatties begin to develop overt obesity during the fourth week of life and rapidly diverge from their lean littermates in subsequent weeks. To establish the development of insulin resistance in fatty rats during this period of rapid growth and expansion of adipose mass, we performed insulin tolerance tests weekly from 28 to 56 days of age. Fatties were already visibly obese by 28 days of age and slightly heavier than their wildtype littermates (wildtypes 64.3 g vs fatties 67.2 g, Fig. 2). They continued to diverge thereafter, with fatties gaining 46 grams per week compared to 29 grams per week for wildtypes. During this period of rapid growth, fasting glucose levels were similar among all genotype and age groups (Fig. 3). In contrast, the response of blood glucose to insulin injection showed marked age and genotype differences. At 28 days of age, blood glucose after insulin injection was similar in wildtypes and fatties. By 42 days of age, blood glucose values after insulin injection were higher in fatties, and by 56 days of age, fatties did not lower their blood glucose in response to this dose of insulin (Fig. 4). These data demonstrate that fatties are insulin sensitive at 4 weeks of age and become increasingly insulin resistance in parallel with the increase in body weight during this period of rapid growth.

Insulin resistance in suckling fatties

From the second week of life to the fourth week of life, rat pups change from reliance on their dam for all their nutrients to feeding independently on solid food. To

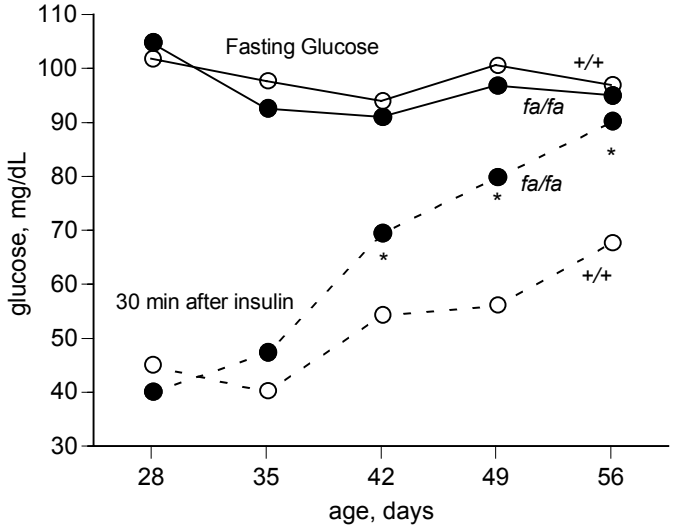

Figure 2. Daily body weight during juvenile development. Fatties are visibly obese by 28 days of age, and their body weights are significantly heavier. Fatties gain 67.2 g per week over the next 4 weeks, while wildtypes gain 64.3 grams per week.

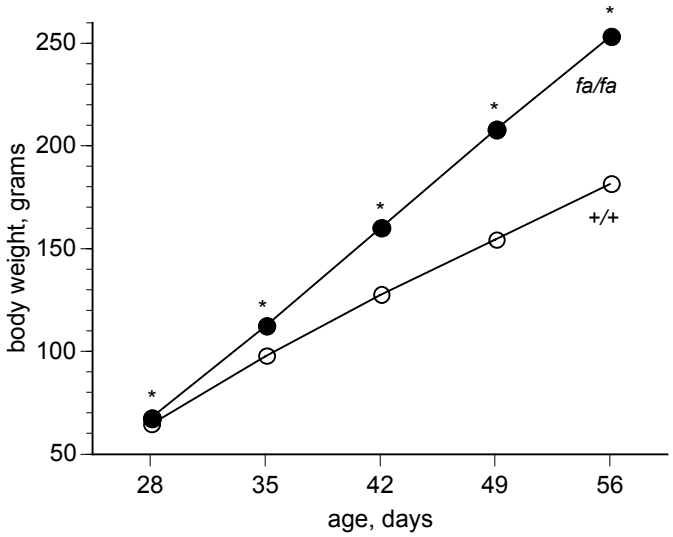

Figure 3. Insulin tolerance during juvenile development. Fasting glucose levels are not affected by genotype from 4 to 8 weeks of age. Fatties are as sensitive to insulin as wildtypes at 28 days of age. Fatties become insulin resistant by 42 days of age. By 56 days of age, fatties are unresponsive to 0.5U/kg insulin; genotype by age *P < .0001.

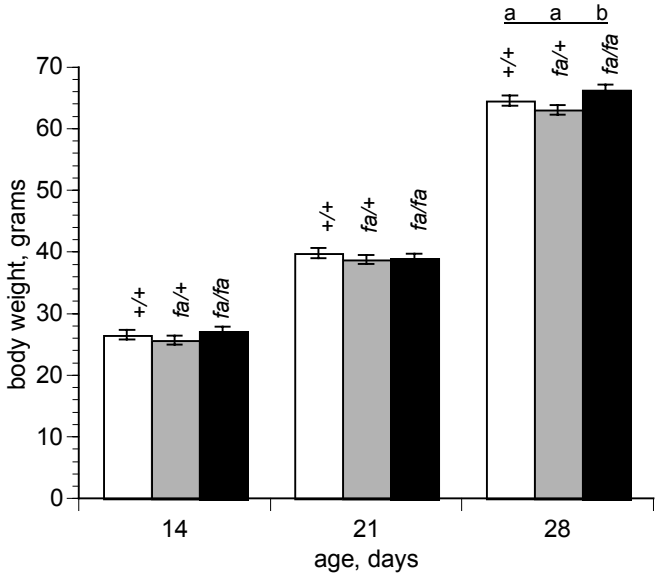

Figure 4. Body weights during the suckling to weaning transition. Body weights of fatties and wildtypes were similar at 14 and 21 days of age. By 28 days, fatties were significantly heavier than heterozygotes ($P < 0.0001$) and wildtypes ($P = 0.0449$). Letters above the bars are similar for groups with similar means.

establish the response to insulin across this transition, we performed insulin tolerance tests at 14, 21 and 28 days of age. At 14 and 21 days of age all genotypes had similar body weights (Fig. 4). However, by 28 days of age, fatties were slightly heavier than lean rats and were visibly obese. In all genotypes, fasting glucose values at 14 and 21 days of age were higher than levels at 28 days of age (Fig. 5). These observations indicate glucose metabolism is altered in both lean and fatty rats between 21 and 28 days of age. Thirty minutes after a 0.5 U/kg insulin injection, fatty rats maintained higher glucose levels than lean rats at 14 and 21 days of age (Fig. 5). By 28 days of age, fatty and lean rats converged at similar low levels of blood glucose following the same dosage of insulin. Thus, fatty rats become insulin sensitive between 21 and 28 days of age.

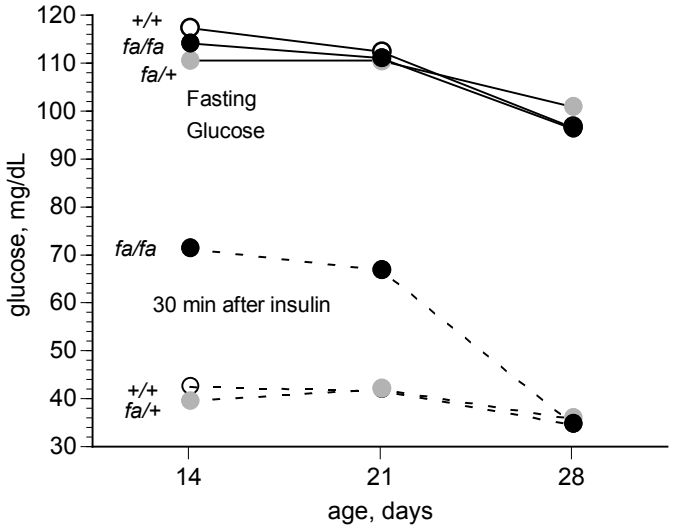

Figure 5. Insulin tolerance during the suckling to weaning transition. Fasting glucose levels decrease between 21 and 28 days of age in all genotypes groups; age, $P < 0.0001$. Fatties are insulin resistant at 14 and 21 days of age and, by 28 days of age, fatties become as sensitive to insulin as lean rats ; age X genotype, $P < 0.0001$.

These data indicate that fatty rats are insulin resistant prior to the onset of hyperphagia, hyperinsulinemia, and overt obesity, but are insulin resistant after the onset of hyperphagia.

Response to insulin during the fourth week of life

We have seen that fatties change from being insulin resistant to insulin sensitive from 21 to 28 days. This interval includes the age when fatties begin to gain weight at a higher rate than lean rats as a consequence of the emergence of hyperphagia (3). In many experiments, we have found that fatty rats develop robust hyperphagia quite suddenly after 22 days of age. This increase in food intake can be detected as an increase in daily weight gain at 23 days of age. To determine when this change in insulin sensitivity occurs relative to the onset of hyperphagia, we performed insulin tolerance tests daily from 21 to 27 days of age.

In this experiment, daily weight gain of fatty rats exceeded that of lean rats at 23 days of age, precisely the same age as in many previous experiments (Fig. 6). At 21 days of age blood glucose levels 30 minutes after insulin injection were higher in fatties compared to their lean littermates (Fig. 7). Although this pattern was maintained at 22 and 23 days of age, blood glucose 30 minutes after insulin injection shifted upwards in both lean and fatty rats at these ages, suggesting that both groups became slightly more insulin resistant at these ages. After 23 days of age, the peak of insulin resistance decreased. By 25 days of age, fatties and their lean littermates had similar levels of blood

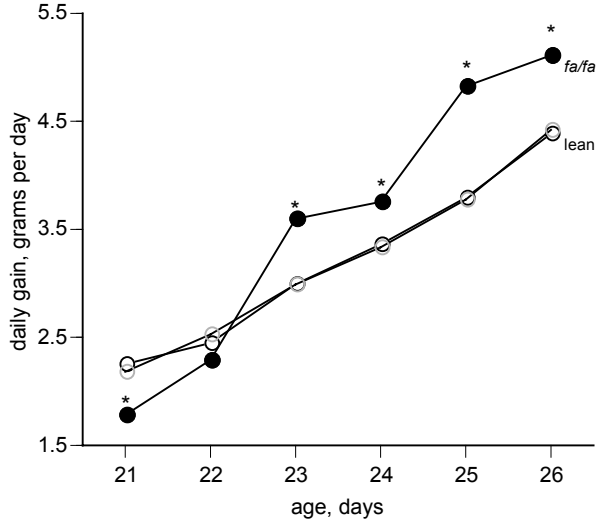

Figure 6. Daily weight gain during the fourth week of life. Fatties gain less weight than wildtypes at 21 days of age. At 22 days of age, daily gains of fatties and wildtypes are similar. At 23-26 days of age, daily gains are significantly higher in fatties than wildtypes; genotype by age *P < 0.0001. These data replicate a pattern we have described previously – daily gain increases in fatty rats after 22 days of age. This corresponds with the onset of hyperphagia.

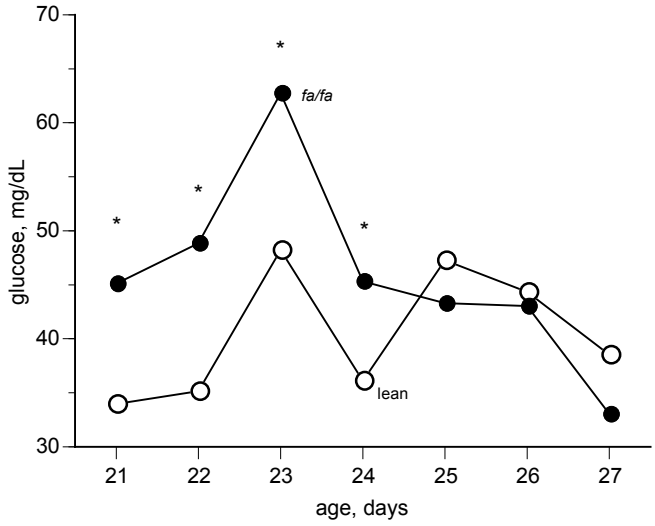

Figure 7. Insulin tolerance during the fourth week of life. For clarity, only glucose values 30 minutes after insulin injection are shown here. Fatties are insulin resistant from 21 to 24 days of age. By 25 days of age, fatties have become insulin sensitive; genotype by age *P < 0.0001. These data demonstrate that whole body insulin sensitivity increases in fatty rats after the onset of hyperphagia.

glucose thirty minutes after insulin injection (Fig. 7). These data indicate that a transient increase in insulin sensitivity begins prior to the onset of hyperphagia, and that an increase in insulin sensitivity evolves a few days after the onset of hyperphagia.

Discussion

The Zucker fatty rat (*fa/fa*) experiences insulin resistance during two phases of development. The early phase of insulin resistance occurs during the suckling period before the onset of hyperphagia, hyperinsulinemia and overt obesity. Hyperphagia emerges in fatties at 23 days of age and is accompanied by elevated plasma insulin and rapid development of overt obesity (3). After the onset of hyperphagia, fatties rapidly become as insulin sensitive as their lean littermates. This insulin sensitive phase is fairly brief; as they become more obese over the next few weeks, fatties gradually become insulin resistant again.

The early phase of insulin resistance probably reflects a lack of leptin signaling uncomplicated by hyperinsulinemia and obesity. While fatties have high leptin levels, their leptin signaling is low because of a defect in the leptin binding domain of the leptin receptors (104). In this respect, they are similar to mice that are leptin deficient because they lack adipose tissue. These mice produce very little leptin and are also insulin resistant. Treatment with exogenous leptin improves insulin resistance in this model, and in humans with lipodystrophies (82; 91). Because leptin signaling cannot be restored in fatty mutants after the onset of hyperphagia, the development of insulin sensitivity in this period must be mediated by mechanisms that are independent of leptin signaling.

The transition from the early phase of insulin resistance to the insulin sensitive phase is more complex than a simple increase in responsiveness to insulin. Instead, the response of blood glucose to insulin injection resembles a roller coaster ride – there is a gradual increase in insulin resistance prior the onset of hyperphagia, a peak at the onset of hyperphagia and a drop in insulin resistance following the onset of hyperphagia. This pattern has two implications. First, it suggests that increased insulin sensitivity does not drive the onset of hyperphagia as we initially proposed. Instead, increased insulin sensitivity seems to follow the onset of hyperphagia. Second, the fact that an increase in insulin resistance precedes the onset of hyperphagia and peaks at the onset of hyperphagia suggests that the mechanism that activates hyperphagia is related to increased insulin resistance.

An increase in lipogenesis in adipose tissue is one factor that probably contributes to the development of insulin sensitivity after the onset of hyperphagia. The increase in insulin secretion that accompanies a higher level of food intake contributes to the increase in lipogenesis at this age. However, the onset of hyperphagia and hyperinsulinemia emerges with striking amplitude at 23 days of age. We have shown elsewhere that the weight of the stomach contents increases in fatties by approximately 80% at 23 days of age and insulin levels increase by 140 % at this age (3). In contrast, the transition from an insulin resistant state to an insulin sensitive state requires a period of adaptation of a couple of days. This is likely to involve changes in other signaling molecules that improve the ability to respond to the increased insulin levels, such as PPARγ.

PPARγ is a transcription factor that improves insulin sensitivity by stimulating the expression of genes for glucose disposal and lipogenesis in adipose tissue. PPARγ mRNA levels are low at 15 days of age and reach a peak at 23 days of age in wildtype rats (8). This corresponds with the ages when rats in our studies become increasingly insulin resistant, which suggests that the changes in insulin resistance and PPARγ mRNA expression could be linked. Furthermore, when insulin resistant adult fatty rats or human diabetics are treated with thiazolidinediones, a class of PPAR agonists, their insulin sensitivity improves and their appetite is also stimulated (4; 7). These observations suggest that developmental changes in PPARγ expression could be an important part of the changes in insulin sensitivity in response to hyperphagia, or perhaps a part of the mechanisms that triggers the onset of hyperphagia.

Although we have shown that the onset of hyperphagia occurs at 23 days of age in fatties raised under standard husbandry conditions, it should be noted that others have demonstrated that fatties will consume greater amounts of food earlier than this age when individual food intakes are measured under other experimental conditions. When rats are separated from their dams for four hours and housed with their siblings, then transferred to individual test chambers with access to half and half (milk and cream), fatties consumed more half and half at 12, 15 and 18 days of age (123). This experiment suggests that the pathways that stimulate hyperphagia can influence food intake earlier than 23 days of age. It is possible that these pathways are stimulated by maternal deprivation or some property of the diet. The fact that fatties are not hyperphagic under standard husbandry conditions suggests that the onset of hyperphagia is also dependent

on other factors. These factors could be related to diet composition, the thermal environment, maternal interactions, or developmentally programmed changes in metabolism. On this note, the increase in PPARγ expression during the weaning transition does not differ between rats weaned onto high carbohydrate versus high fat diets, but occurs independent of diet composition. Thus, PPARγ expression is regulated by developmental factors rather that diet during the weaning transition (8).

These data show a dynamic relationship between insulin sensitivity and the fatty phenotype that is closely related to hyperphagia. Not only are there long term changes in insulin resistance from the age when fatties are not obese or hyperinsulinemic to the age when they are massively obese, but there are also short term changes in insulin sensitivity around the onset of hyperphagia that suggest that some developmental perturbation of insulin signaling is related to the onset of hyperphagia. The increase in expression of PPARγ at this age, and the fact that PPARγ agonists not only improve insulin signaling, but also stimulate lipogenesis and hyperphagia in adult fatties (4), suggest that this transcription factor could be a critical component of the activation of hyperphagia.

PART 4. PPARγ EXPRESSION RELATIVE TO THE ONSET OF HYPERPHAGIA IN ZUCKER FATTY RATS

Abstract

Hyperphagia emerges suddenly after 22 days of age in Zucker fatty (*fa/fa)* rats, and fatties become visibly obese within days. This rapid increase in growth rate was associated with increased insulin sensitivity. The objective of these experiments was to determine whether changes in expression of PPARγ, a transcription factor that enhances insulin sensitivity and lipogenesis, were related to the onset of hyperphagia in fatties. Quantitative RT-PCR for *PPARγ* and *FAS* mRNA showed that neither of these were differentially expressed before the onset of hyperphagia, but both were induced in fatties after the onset of hyperphagia. Western blots showed PPARγ and FAS proteins were low in adipose tissue of lean and preobese rats at 21 days of age, before the onset of hyperphagia. A large induction of PPARγ and FAS occurs after the onset of hyperphagia, as fatties become insulin sensitive. By 28 days of age, PPARγ and FAS expression were dramatically induced in fatties, but remain low in their lean littermates. These data show that PPARγ expression increased in fatty rats in parallel with the increased insulin sensitivity that follows the onset of hyperphagia, suggesting that PPARγ signaling may contribute to the timing of the onset of hyperphagia.

Introduction

The obesity epidemic in the United States has been accompanied by an alarming increase in the prevalence of overweight children (1). Data from the National Health and Nutrition Examination Survey (NHANES) indicate that the number of overweight children has increased among all age, race, and sex groups. Overweight children are at high risk for becoming overweight adults and developing hypertension and type II diabetes (2). These observations underscore the importance of understanding the early development of energy balance regulatory systems, particularly during critical periods of rapid growth.

The Zucker fatty (*fa/fa)* rat provides a unique model for understanding the early development of obesity. Fatties grow at approximately the same rate as their lean littermates for the first 22 days of age (3). At 23 days of age, fatties develop robust hyperphagia, which dramatically increases growth rate and expansion of fat mass (3). Fatties become visibly obese within a few days of the onset of hyperphagia. This developmental pattern suggests that a fundamental change in the regulation of energy balance occurs at this age.

The onset of hyperphagia is accompanied by a marked change in insulin sensitivity. It is well known that juvenile fatty rats are insulin sensitive and become increasingly insulin resistant as their adipose mass expands. We have shown that fatties are also insulin resistant prior to the onset of hyperphagia, hyperinsulinemia and overt obesity, and then become insulin sensitive with the emergence of hyperphagia. Thus,

fatties experience two phases of insulin resistance separated by a brief phase of insulin sensitivity that follows the onset of hyperphagia (Thesis Chapter 3).

It is likely that the early phase of insulin resistance that exists prior to the onset of hyperphagia primarily reflects the absence of leptin signaling. The best evidence for a role of leptin in countering insulin resistance is derived from animals that lack adipose tissue. These models produce very little leptin and are insulin resistant. Small amounts of adipose tissue or exogenous leptin injections improve their insulin sensitivity (91). Exogenous leptin injections also improve insulin resistance in people with congenital generalized lipodystrophies (80; 82). These observations support the hypothesis that insulin resistance develops early in life in fatty rats, before the onset of hyperinsulinemia and obesity, because of the reduction in leptin signaling.

Leptin promotes insulin sensitivity by enhancing the expression of peroxisome proliferator-activated receptors (PPARs) (170). Peroxisome proliferator-activated receptor gamma (PPARγ) is a transcription factor that stimulates adipocyte differentiation and expression of genes for glucose uptake and lipogenesis (6; 183). Thiazolidinediones (TZD) improve insulin sensitivity by activating PPARs, which stimulates glucose uptake and lipogenesis by adipose tissue in diabetics (7; 159). When insulin resistant adult fatties are treated with thiazolidinediones, they not only improve their insulin sensitivity, but also increase their food intake and growth (4). These responses are remarkably similar to those that occur developmentally in fatties at the onset of hyperphagia, suggesting that activation of PPARs could also be involved in the development of hyperphagia.

There are developmental changes in *PPARγ* mRNA levels during the same interval as the onset of hyperphagia in fatties. *PPARγ* mRNA expression in adipose tissue increases during the suckling period in rats; levels are low at 15 days of age and reach a maximum by 23 days of age (8). This developmental pattern is independent of changes in the fat content of the diet. It is possible that the increase in *PPARγ* expression contributes to the onset of hyperphagia at this age by inducing genes for glucose utilization and lipogenesis in adipose tissue, just as the activation of PPARγ does in adult fatties.

The fact that a rise in *PPARγ* mRNA expression occurs at the same age as an increase in insulin sensitivity, food intake and growth in fatties suggests that these two events are linked. The purpose of this study was to compare the expression of PPARγ in lean and fatty rats at key points in development to determine the relationship between PPARγ expression and the development of hyperphagia and obesity. We also evaluated the expression of fatty acid synthase (FAS) as a marker for lipogenesis at these ages.

Materials and Methods

Animals

Animals were produced in the principal investigator's colony of partially inbred (F15-F17) ZUCKER-*fa* rats. Animals are housed in plastic breeder boxes with *ad libitum* access to Teklad 8640 Rodent Diet and tap water. The light cycle is maintained at 12 hours of dark, followed by 12 hours of light. Room temperature is maintained at 68 to

74°F and 55 to 60% humidity. All animal studies were reviewed and approved by the University of Tennessee Institutional Animal Care and Use Committee.

The population is maintained with brother by sister matings to minimize extraneous genetic variation and with selection for large litter size to minimize inbreeding depression. Sires are removed when the dams appear pregnant. The day of birth is considered 0 days of age. Neonates are genotyped for *fa* during the first week of life, as described in detail below. Litters are reduced to 8 pups during the first ten days of life by decapitating excess pups.

Pups are weighed daily from 14 days of age to the end of each experiment to acclimate them to handling. Daily gain is calculated as the difference in weights on consecutive days. Daily gain provides a marker for the onset of hyperphagia.

Genotype detection

Pups are marked by toe-clipping, and a single toe from each pup is saved to prepare DNA by the HotSHOT method (3; 181). Briefly, toes are collected into 200 µl thermal cycler tubes. Seventy-five microliters of 25 mM NaOH/ 0.2 mM EDTA/ pH 12 are added to the tubes. Samples are heated to 95 degrees C for 30 minutes, and then cooled to room temperature. Seventy-five microliters of 40 mM Tris HCl/ pH 5 are added to each tube to adjust the pH to 8. The DNA is then ready for PCR amplification.

The *fa* mutation creates a restriction site for *Msp* I that can be used to detect *fa* genotype (104). However, *Msp* I occasionally fails to cut DNA to completion in our hands, which can cause genotype misclassification. To overcome this problem, we

engineered an alternative restriction site by a strategy that has been described previously (182). PCR primers were designed to amplify 101 base pair (bp) of DNA flanking the *fa* mutation. The reverse primer, RLepr, was selected to anneal adjacent to the site of the mutation, and a single base substitution (C→G) was made in the second base from the 3' end of the primer to create a *Pvu* II restriction site in the wildtype allele and no restriction site in the mutant allele. The primer sequences are fLepr, 5'-CGTATGGAAGTCACAGA-3', and RLepr, 5'GAATTCTCTAAATATTTCAGC-3'; the base substitution in RLepr is underlined. Primers are mixed at 500 nM each in 50mM KCl, 10mM Tris-HCl (pH 9 at 25°C), 0.1% Triton X-100, 1.5 mM $MgCl_2$, 0.2 mM each dATP, dCTP, dGTP and dTTP, 0.25 units *Taq* DNA polymerase and 2 μl of HotSHOT DNA in a 22 μl volume. PCR amplification is carried out in a Robocycler thermal controller (Stratagene, La Jolla, CA) at 95°C for two min, followed by 40 cycles of 95°C for 30 sec, 45°C for 45 sec and 72°C for 30 sec. Twenty μl of PCR product are digested with 1 unit *Pvu* II (New England Biolabs, Beverly MA) in a 30 μl volume for 1 h at 37°C. Thirty μl of *Pvu* II treated PCR product are loaded onto a 3% 3:1 high-resolution blend agarose (Amresco) gel and electrophoresed 30 minutes at 5 volts/cm. Gels are stained with ethidium bromide and exposed to UV light. The wildtype genotype produces an 80 bp band, and the mutant genotype produces a 101 bp band; both bands are visible in heterozygotes.

Experimental Design

Expression of PPARγ and FAS mRNA before and after the onset of hyperphagia.

This experiment was performed to determine whether *PPARγ* and *FAS* mRNA expression were affected by the onset of hyperphagia. Rats were weighed daily from 14 days of age until they were killed. Daily weight gain was calculated as a marker for the onset of hyperphagia. Sex-matched wildtype and fatty littermates were killed by decapitation at 20 or 24 days of age. Stomachs and liver were dissected and weighed at the time of decapitation. Stomach contents were removed and the empty stomachs were reweighed. The difference between full stomach weight and empty stomach weight was calculated as a measure of stomach contents. Inguinal adipose pads were dissected, weighed and frozen in liquid nitrogen for preparation of RNA and quantification of gene expression by real time-polymerase chain reaction assays (RT-PCR), as described below.

Developmental changes in FAS and PPARγ protein expression.

Because PPARγ expression is regulated at the level of translation, mRNA levels may be a poor indicator of PPARγ protein levels. To establish the pattern of expression of PPARγ and FAS at key points in development, we measured the expression of PPARγ and FAS protein at 21, 28 and 60 days of age. These ages represent different metabolic conditions. At 21 days of age, fatties are insulin resistant compared to their littermates, but they have normal insulin levels, and they are not hyperphagic or visibly obese. At 28 days of age, fatties are insulin sensitive, hyperphagic, hyperinsulinemic, and mildly

obese. At 60 days of age, fatties are insulin resistant, hyperphagic, hyperinsulinemic and massively obese.

Rats were killed by CO_2 asphyxiation for collection of inguinal adipose pads. Western blots were prepared with inguinal adipose tissue of lean and fatty sex matched pairs at 21, 28, and 60 days of age to determine PPARγ and FAS expression levels. Seven replicate western blots were prepared for PPARγ and 5 replicate blots were used for FAS expression analysis.

Expression of FAS and PPARγ relative to the onset of hyperphagia.

We have previously demonstrated that the onset of hyperphagia in fatties is accompanied by an increase in insulin sensitivity. To determine the relationship between PPARγ and FAS expression, rats were killed by decapitation from 21 to 28 days of age. Inguinal adipose tissue was dissected and frozen for analysis of PPARγ and FAS expression by western blots. There were 7 western blots prepared for PPARγ, and 5 blots were used for FAS expression analysis. Each western blot includes samples from lean and obese pairs killed at each age.

Tissue Processing

RNA for quantitative real time-polymerase chain reaction assays (RT-PCR) was extracted using a Totally RNA kit. The Totally RNA kit procedure for RNA extraction was modified to include two additional chloroform extractions immediately after homogenization of the tissues to remove lipids. After isolation, RNA was dissolved in

DEPC-treated water, precipitated with LiCl and washed with 70% ethanol. RNA was quantified spectrophotometrically in 1 mM Na_2HPO_4, pH 8.5 (184).

Fifty ng of total RNA were mixed with 5 mM $MgCl_2$, 300 μM each dNTPs, 62.5 units Moloney murine Leukaemia Virus (MuLV) Reverse Transcriptase, 10 units RNase inhibitor (GeneAmp RNA PCR Core Kit; Applied Biosystems), 0.625 units AmpliTaq Gold DNA Polymerase, 1X Taqman buffer A (TaqMan 1000 RXN Gold with Buffer A Pack; Applied Biosystems) in a 25 μl volume. The composition and concentrations of the primers and probes are listed in Table 2. Amplification was performed in a Cepheid SmartCycler dilutions of RNA pooled from multiple samples. *PPARγ* and *FAS* expression levels were expressed as a ratio of 18S RNA expression levels.

Table 2. RT-PCR primer and probe concentrations and compositions.

Oligo	Concentration	Sequence
FAS for	200 nM	CCCAGAGGCTTGTGCTGACT
FAS rev	400 nM	CGAATGTGCTTGGCTTGGT
FAS probe	100 nM	TET d(CCGATCTGGAATCCGCACCGG) TAMRA
PPAR for	200 nM	TGCGAGTGGTCTTCCATCAC
PPAR rev	400 nM	GCCTTGAGCACTTCACAAGAAATT
PPAR probe	100 nM	CAL RED d(TTTGCCAAGCTGCTCCAGAAAATGA) BHQ-2
18S for	200 nM	AGTCCCTGCCCTTTGTACACA
18S rev	400 nM	GATCCGAGGGCCTCACTAAAC
18S probe	100 nM	6-FAM d(CGCCCGTCGCTACTACCGATTGG) BHQ-1

Protein extracts were prepared by homogenization of inguinal adipose tissue in a buffer containing 100 mM Tris HCl, 510 mM sucrose, and 2 mM EDTA, pH 7.4. Protease inhibitors (2 μM phenylmethylsulfonyl fluoride, 1 μM pepstatin, 80 trypsin inhibitory milliunits aprotinin per ml, 10 μM leupeptin, 2 mM sodium orthovanadate, all from Sigma, St. Louis, MO) were added immediately before processing. Adipose tissue was homogenized in 1 ml of ice-cold homogenization buffer per gram of tissue. Homogenates were centrifuged at 5000g for 2 minutes at 4ºC. The supernatant was transferred to a fresh tube and centrifuged twice at 13000g for 15 minutes at 4ºC, and the supernatant was saved as the final sample protein extract. Protein concentration of each sample extract was quantified by the Bradford method (Amresco, Inc., Solon, OH).

SDS-PAGE and Western Immunoblotting

Eighteen μg of protein from each extract were loaded on 10% SDS-PAGE gel for PPARγ and 8% SDS-PAGE gel for FAS and electrophoresed at 50 volts for 4 to 5 hours. The SDS-PAGE gel was electroblotted onto a polyvinyldifluoride membrane overnight at 30 volts in 4 degrees C.

Blots were blocked in 25 ml of blocking buffer (1X TBS, 0.1% Tween-20, and 5% w/v nonfat dry milk) at room temperature for 1 hour. Blots were incubated overnight at 4ºC with a polyclonal PPARγ (Cell Signaling Technology, Beverly, MA) or polyclonal FAS (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) primary antibody diluted 1:500 in 10 ml of primary antibody dilution buffer (1X TBS, 0.1% Tween-20, and 5% w/v nonfat dry milk). After the first incubation, blots were washed with a 1X Tris-buffered

saline/0.1% Tween-20 solution, and then incubated at room temperature for 1 hour with the appropriate secondary antibody diluted 1:2000 in 20 ml of blocking buffer. Blots were washed with a 1X Tris-buffered saline/0.1% Tween-20 solution and bands corresponding to PPARγ and FAS were detected by chemiluminescence (Luminol, Santa Cruz Biotechnology, Inc., Santa Cruz, CA). Blots were stripped and immunoblotted as described above, using a primary antibody for β-actin (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) diluted 1:500 in 10 ml of primary antibody dilution buffer and an appropriate secondary antibody diluted 1:2000 in 20 ml of blocking buffer. Expression levels were quantified densitometrically.

Statistical analyses

Daily weight gain, body weights, tissue weights and stomach contents were analyzed using a mixed linear model, accounting for sex, genotype, and age as fixed effects, and litter as a random effect. RT-PCR data were analyzed under mixed linear models using sex, genotype and age as fixed effects and litter as a random effect. Western blot data were analyzed under mixed linear models using genotype and age as fixed effects and blot number as a random effect. These analyses were performed using Proc Mixed in SAS version 9.0 (The SAS Institute, Cary, NC).

Results

Expression of FAS and PPARγ mRNA before and after the onset of hyperphagia.

Many investigators routinely separate rat pups from their dams at 3 weeks of age. This type of investigator-initiated weaning typically disrupts growth of the pups. In these experiments, rat pups remained with their dams until they were killed or reached 28 days of age. Under these conditions, the daily gains of fatties exceed the daily gains of lean rats at 23 days of age (Fig. 8), replicating a pattern we have seen in many experiments. This increase in daily gain rapidly alters body weight; body weights are similar between wildtypes and fatties at 20 days of age, but fatties become heavier than wildtypes by 24 days of age (Table 3).

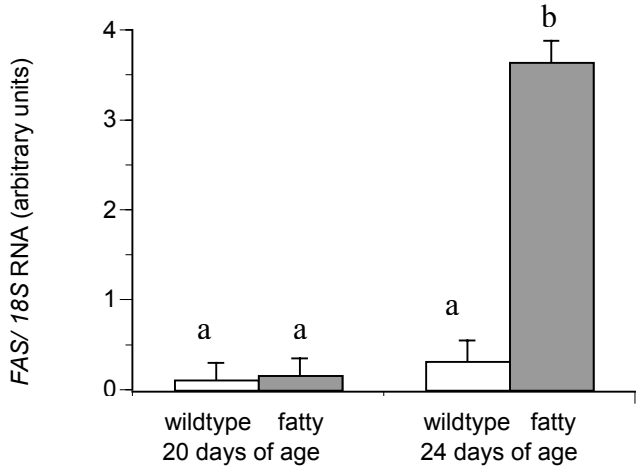

Figure 8. Expression of fatty acid synthase measured by quantitative RT-PCR in wildtype and fatty Zucker rats before and after the onset of hyperphagia. FAS expression and 18S RNA were quantified by RT-PCR and the data were expressed as a ratio of FAS

Table 3. Effects of *fa/fa* genotype on body weight and tissue weights at 20 and 24 days of age.

	DAY 20		DAY 24	
	+/+	*fa/fa*	+/+	*fa/fa*
Body weight, grams	35.0 ± 0.8	34.7 ± 0.9	49.7 ± 0.9	52.3 ± 1.1*
Stomach weight, grams	1.18 ± 0.09	0.99 ± 0.10	1.41 ± 0.16	2.22 ± 0.16*
Empty stomach weight, grams	0.28 ± 0.02	0.25 ± 0.02	0.41 ± 0.02	0.40 ± .02
Stomach contents weight, grams	0.90 ± 0.09	0.74 ± 0.10	1.01 ± 0.16	1.82 ± 0.16*
Liver weight, grams	1.14 ± 0.60	1.17 ± 0.06	1.90 ± 0.06	2.32 ± 0.06*
Inguinal adipose pads weight, grams	0.31 ± 0.03	0.75 ± 0.03*	0.62 ± 0.06	1.34 ± 0.06*

Values are means ± standard errors. Asterisks indicate where the mean of the fatties differ from the mean of age-matched wildtypes.

to 18S RNA in arbitrary expression units. FAS was not affected by genotype at 20 days of age, but became higher in fatties by 24 days of age, after the onset of hyperphagia. Letters above the bars are similar for groups with similar means.

This sudden increase in daily weight gain strongly suggests that fatties begin consuming more food at this age. While food intake is easily measured in adult rats, it is much more difficult to measure in suckling pups consuming both solid food and milk. However, the weight of the stomach contents is highly correlated with 24-hour food intake (185), making this measure a useful indicator of the level of food intake under the conditions of this experiment. In this experiment, full stomach weights of wildtypes and fatties were similar at 20 days of age, but by 24 days of age fatties experienced a 57% increase in full stomach weight over the wildtypes (Table 3). The weights of the

stomach tissues themselves were not affected by genotype at either age. However, the weight of the stomach contents were similar between genotypes at 20 days of age, but at 24 days of age the weight of the stomach contents was 80% higher in fatties than in wildtypes. Furthermore, liver weight was unaffected by genotype at 20 days of age, but became 22% greater in fatties compared to wildtypes at 24 days of age. These data confirm that the increased growth rate that emerges at 23 days of age is associated with the onset of robust hyperphagia.

Note that inguinal adipose mass is already elevated prior to the onset of hyperphagia (Table 3). The excess adipose mass that accumulates during the first three weeks of age is a consequence of reduced energy expenditure in fatties.

At 20 days of age, before the onset of hyperphagia, fatties and wildtypes have similar low levels of *FAS* mRNA (Fig. 8). From 20 to 24 days of age, *FAS* expression increases 22 fold in fatties. *PPARγ* mRNA expression was also similar between wildtypes and fatties at 20 days of age, and increased in fatties 2- fold from 20 to 24 days of age (Fig.9). These data demonstrate that the onset of hyperphagia is associated with a large induction of *FAS* mRNA in fatties, as expected, and a smaller induction of *PPARγ* mRNA.

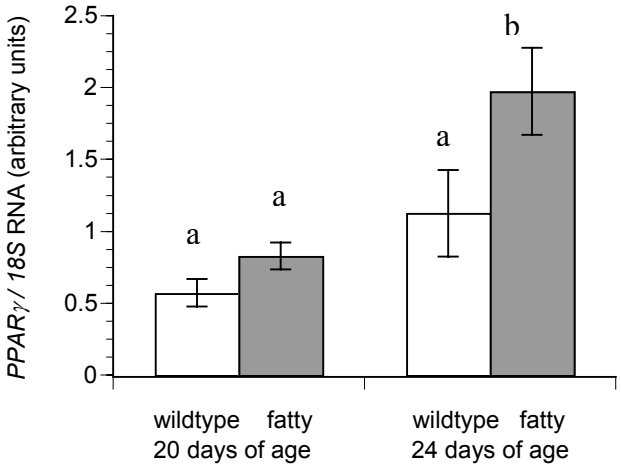

Figure 9. Expression of *PPARγ* measured by quantitative RT-PCR in wildtype and fatty Zucker rats at 20 and 24 day. *PPARγ* expression and *18S* RNA were quantified by RT-PCR and the data were expressed as a ratio of *PPARγ* to 18S RNA in arbitrary expression units. PPARγ was not affected by genotype at 20 days of age, but became higher in fatties by 24 days of age, after the onset of hyperphagia. Letters above the bars are similar for groups with similar means.

While FAS is regulated primarily at the level of transcription in response to insulin, expression of PPARγ expression is more dependent of translation, and analysis of expression of the protein could be more informative than the mRNA.

Developmental changes in FAS and PPARγ protein expression.

The purpose of this experiment was to determine the influence of the fatty genotype on PPARγ protein expression at three points in development that have different metabolic patterns.

At 21 days of age fatties are insulin resistant, but not hyperinsulinemic, hyperphagic or obese. FAS protein expression is low at this age in both lean and fatty rats (Fig. 10), which is consistent with the low levels of *FAS* mRNA in the previous experiment. These low levels of *FAS* protein reflect the fact that lipogenesis is very low at this age. PPARγ protein expression is also low in leans and fatties at 21 days of age (Fig. 10).

By 28 days of age fatties are insulin sensitive, hyperinsulinemic, hyperphagic and moderately obese. At this age, FAS protein expression is upregulated in lean rats and further upregulated in fatties (Fig. 10). PPARγ protein expression is also upregulated in fatties by 28 days of age (Fig. 11). These results are also consistent with the pattern of *FAS* and *PPARγ* mRNA expression after the onset of hyperphagia in the previous experiment.

By 60 days of age, fatties are again insulin resistant, hyperinsulinemic, hyperphagic and massively obese. At this age FAS expression in leans has returned to

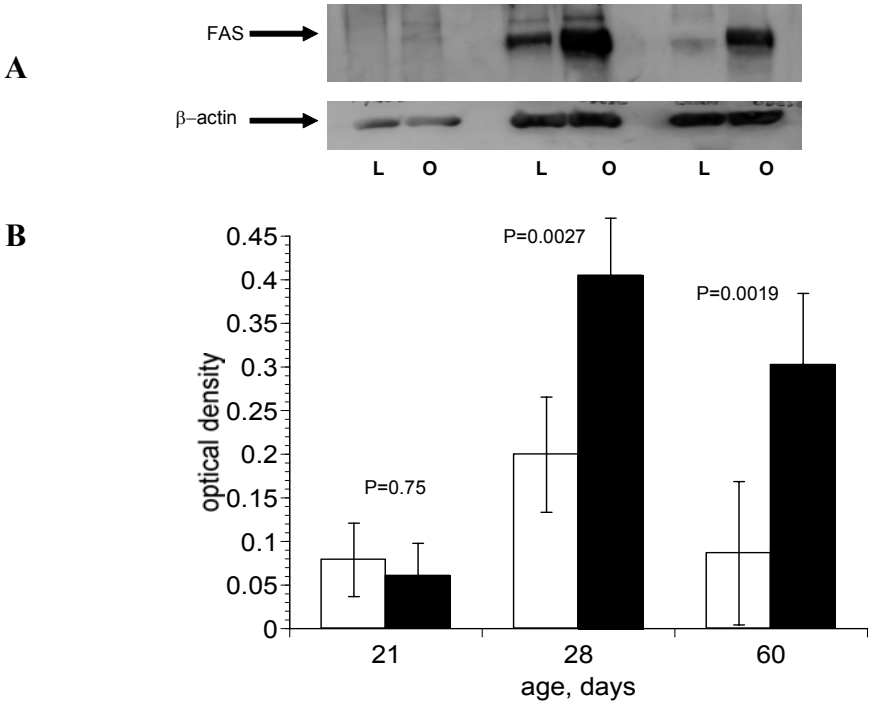

Figure 10. FAS expression at 21, 28 and 60 days of age. 18 μg of inguinal adipose protein extracts were prepared at each of the 3 ages, separated by 8% SDS-PAGE, and analyzed using a Western blot technique. FAS migrated as a 250 kilodalton (KD) protein on a 8% SDS-PAGE gel. Blots were quantified and mean optical densities of 5 blots containing sex matched lean and obese pairs at each age were analyzed. β-actin is shown to demonstrate equal loading of samples at each age. (B) Expression of FAS increases in all animals from 21 to 28 days of age and is highest in fatties at 28 days of age. This elevated level of protein expression begins to decrease by 60 days of age. The age by phenotype interaction was significant at 28 and 60 days of age (P=0.0027 and P=0.0019, respectively).

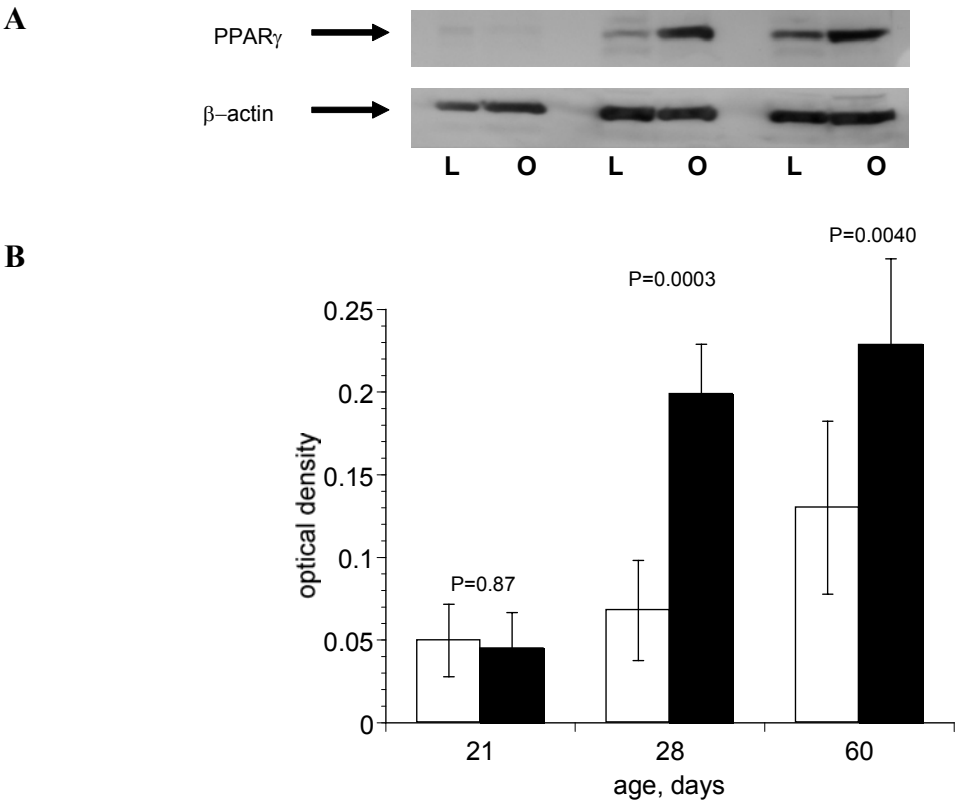

Figure 11. PPARγ expression in relation to insulin resistance at 21, 28 and 60 days of age. 18 μg of inguinal adipose protein extracts were prepared at each of the 3 ages, separated by 10% SDS-PAGE, and analyzed using a Western blot technique. (A) PPARγ was detected as a 54 kD band. Blots were quantified and mean optical densities of 7 blots were analyzed and quantified. β-actin is shown to demonstrate equal loading of samples at each age. (B) Expression of PPARγ increases in all animals from 21 to 28 days of age and is highest in fatties at 28 days of age (P=0.0003). These levels remained elevated at 60 days of age (P=0.0040).

the same level as at 21 days of age, but FAS expression remains high in fatties. By 60 days of age PPARγ expression has increased further in leans, but PPARγ expression remains at about the same high level in fatties, at a level that is still higher than the leans. Thus PPARγ expression seems to be driven up rapidly after the onset of hyperphagia in fatties and remains high. In lean rats, PPARγ expression increases more gradually with age. These results were consistent with our hypothesis that PPARγ is upregulating lipogenic enzymes that may in turn be increasing insulin sensitivity after the onset of hyperphagia.

Expression of FAS and PPARγ relative to the onset of hyperphagia.

There is clearly a dramatic induction of both FAS and PPARγ proteins during the fourth week of life. To determine the timing of these changes in expression relative to the onset of hyperphagia, rats were weighed daily through this interval, and killed by decapitation at days 21 through 28 for analysis of FAS and PPARγ expression in inguinal adipose tissue.

FAS expression increases gradually from 21 to 28 days of age (Fig. 12). FAS is significantly increased in fatties by 25 days of age and remains elevated through 28 days of age. The induction of PPARγ followed a similar pattern to the induction of FAS from 21 to 28 days of age, but is slightly delayed (Fig. 13). PPARγ protein expression begins to increase in both genotype groups at 27 days of age and became significantly higher in fatties at 28 days of age. These data indicate that changes in FAS and PPARγ expression follow a similar pattern, increasing in lean rats and in fatties within a few days of the

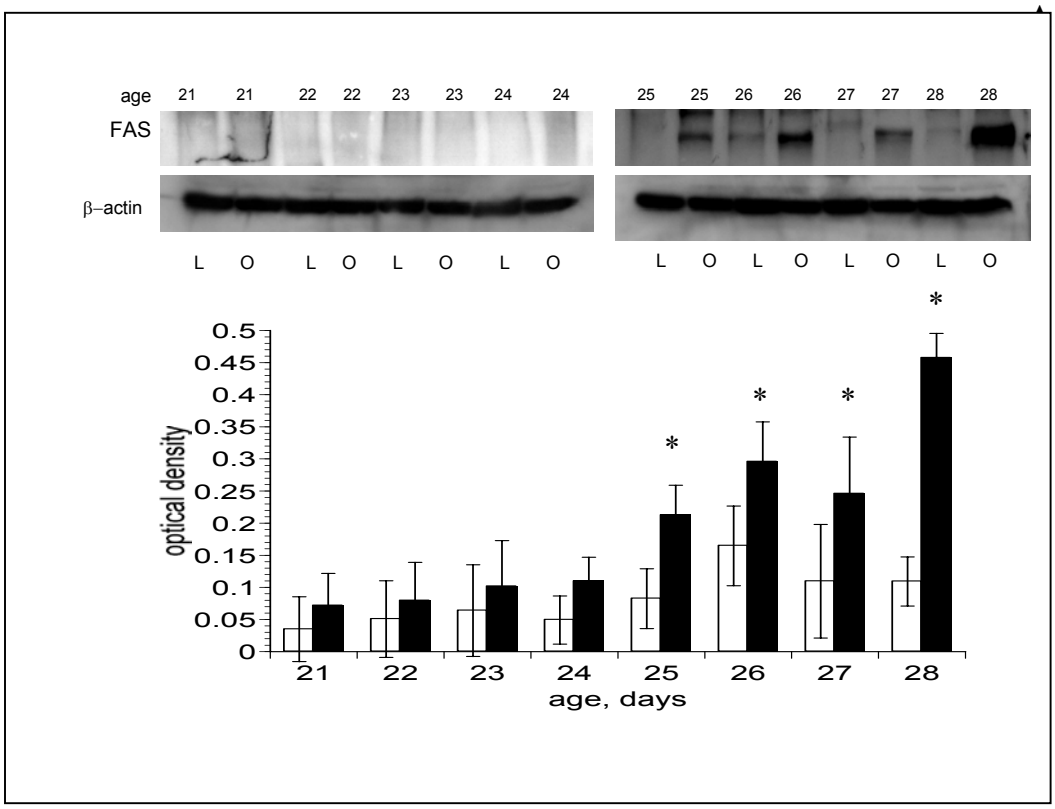

Figure 12. FAS expression relative to the onset of hyperphagia. FAS expression relative to the onset of hyperphagia. (A) 18 μg of inguinal adipose protein extracts was prepared from each age between 21 to 28 days of age, separated by 8% SDS-PAGE, and analyzed using a Western blot technique. β-actin is shown to demonstrate equal loading of samples at each age. (B) A picture of the Western blot was taken and bands were quantified densitometrically. The data were analyzed as a degree of increase in optical density (OD) above zero. Expression of FAS increases in all animals from 21 to 28 days of age and is highest in fatties, with the exception of day 27 were more data is needed. The age by phenotype interaction was significant at 25, 26, 27, and 28 days of age ($P<0.0001$, $P<0.0001$ $P=0.0092$, $P<0.0001$ respectively).

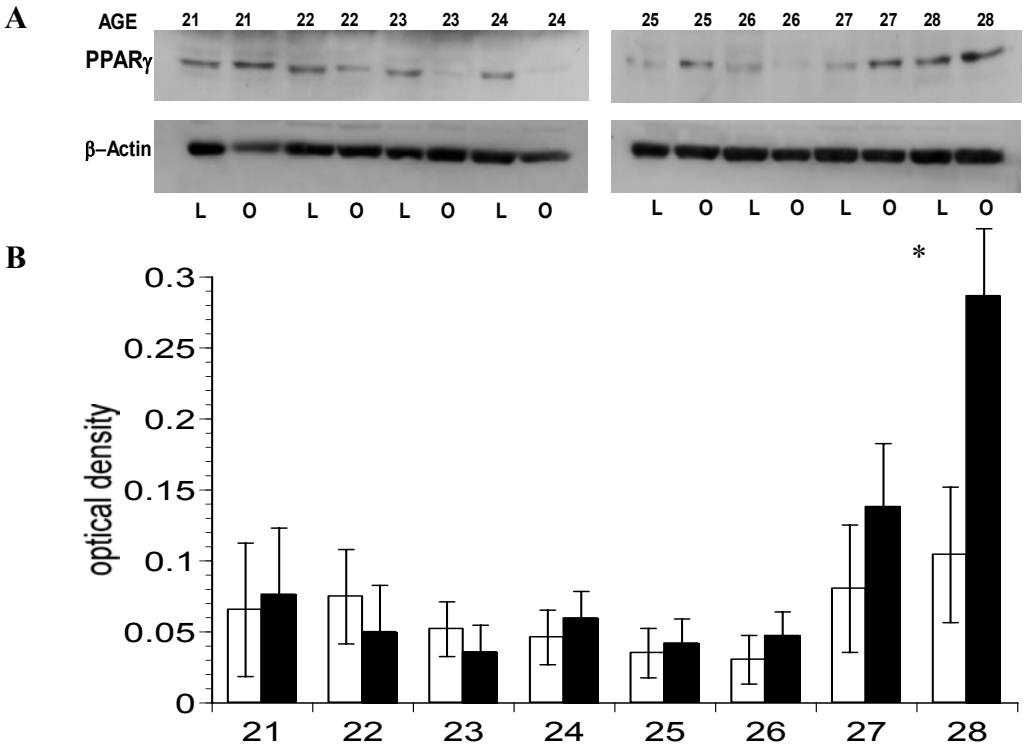

Figure 13. PPARγ expression relative to the onset of hyperphagia. PPARγ expression relative to the onset of hyperphagia. (A) 18 μg of inguinal adipose protein extracts was prepared from each age between 21 to 28 days of age, separated by 10% SDS-PAGE, and analyzed using a Western blot technique. β-actin is shown to demonstrate equal loading of samples at each age (B) A picture of the Western blot was taken and bands were quantified using densitometric quantitation. The data was analyzed as a degree of increase in optical density (OD) above zero. Expression of PPARγ corresponds to insulin tolerance test previously conducted in the lab. At 23 days of age, when animals reach a peak in insulin resistance, PPARγ expression is the lowest. After the onset of hyperphagia when fatties suddenly become insulin sensitive, PPARγ induction begins to increase and peaks at 28 days of age when fatties also reach a peak in insulin sensitivity. The age by phenotype interaction was significant by 28 days of age ($P<0.0001$). PPARγ expression appears to be rising later than FAS suggesting that it is responding to insulin but not determining the onset of hyperphagia.

onset of hyperphagia. This pattern is also similar to changes in insulin sensitivity that occur in this interval.

Discussion

In insulin resistant adult fatties, activation of PPARγ by thiazolidinediones increases insulin sensitivity by stimulating the expression of genes for lipogenesis in adipose tissue, leading to an increase in food intake and fat mass (4; 7). We propose that a similar PPARγ mechanism is involved in activating hyperphagia and increasing insulin sensitivity in fatties early in life.

Prior to this investigation, the best evidence to support this hypothesis derived from data on the effects of high and low fat diets on *PPARγ* and *FAS* mRNA expression. *FAS* mRNA is low during the suckling period and increases when pups are weaned onto a high carbohydrate diet (8; 132; 186; 187). This rise in *FAS* mRNA is driven by a sharp increase in insulin; when animals are weaned onto a high fat diet, insulin levels remain low, and *FAS* mRNA is undetectable (8). In contrast, adipose tissue *PPARγ* mRNA expression increases during the suckling period and reaches a plateau after weaning. Levels of *PPARγ* mRNA increase whether rats are weaned onto a high fat or high carbohydrate diet. Therefore, it appears that expression of *PPARγ* mRNA is regulated by developmental mechanisms rather than diet composition (8; 9). The combination of increasing insulin and increasing PPARγ expression could promote lipogenesis and insulin sensitivity in concert.

Rousseau et al found *PPARγ* mRNA levels increase during the third week of life, and reached its peak at 23 days of age, which corresponds with the age at which robust hyperphagia emerges in fatties (8). Our data show that neither *FAS* or *PPARγ* mRNA are effected by the fatty genotype before the onset of hyperphagia. However, both *FAS* and *PPARγ* mRNA are induced in fatty rats after the onset of hyperphagia. This pattern occurs in rats that remain with their dams and are consuming solid food and nursing. Therefore, the induction of *FAS* and *PPARγ* mRNA are triggered by developmental factors and not by investigator initiated weaning. The developmental factor that drives *FAS* expression is the increase in consumption of carbohydrates, which drives insulin secretion. The developmental factor that drives *PPARγ* expression is unknown, but is apparently not the change in diet. Leptin promotes insulin sensitivity by enhancing the expression of PPARγ in the adipose tissue of young and old rats (188). However, the leptin signaling defect in fatties clearly is not resolved in this interval. Furthermore, there are no differences in PPARγ expression prior to the onset of hyperphagia, even though leptin signaling is already impaired at this age (104). Therefore changes in leptin signaling are not involved in the induction of PPARγ expression in this interval.

Differences in glucose regulation are apparent across the weaning transition. Glucose infusion studies in suckling animals show that, despite higher glycemia and insulinemia, glucose utilization rate by tissues is much lower than in weaned animals, indicating that suckling rats are insulin resistant relative to weaned rats. Glucose clamp studies also indicate insulin fails to stimulate whole-body glucose utilization during the

suckling period (177; 178). When weaned onto a high carbohydrate diet these adaptations are reversed (189), as demonstrated by increased FAS expression.

These differences in glucose regulation seen in wildtype rats are further exaggerated in fatty rats. We have shown elsewhere that insulin sensitivity changes around the onset of hyperphagia in a complex pattern (Thesis Chapter 3). Fatties are more insulin resistant than their lean littermates at 3 weeks of age, before the onset of hyperphagia, hyperinsulinemia and overt obesity. Fatties and lean rats become increasingly insulin resistant from 21 to 23 days of age. At 23 days of age, insulin resistance reaches a peak and fatties become hyperphagic at the same day. After 23 days of age, fatties and leans become more insulin sensitive over the next couple of days, and they are as insulin sensitive as their lean littermates by 25 days of age. Fatties and leans gradually become more insulin resistant over the next few weeks, with fatties becoming insulin resistant at a higher rate as they become obese. The complex changes in insulin sensitivity around the onset of hyperphagia suggest that a developmental perturbation in insulin signaling occurs in this interval (Thesis Chapter 3).

When animals are insulin resistant at 3 weeks of age, FAS and PPARγ expression levels are similar between lean and fatty rats. It is likely that the early phase of insulin resistance that exists prior to the onset of hyperphagia primarily reflects minimal leptin signaling. The role of leptin in insulin resistance is apparent in animals that lack adipose tissue. These models also produce very little leptin and are insulin resistant. Adipose tissue implants or exogenous leptin injections improve their insulin sensitivity (91). Exogenous leptin injections also improve insulin resistance in people with congenital

generalized lipodystrophies (80; 82). These observations support the hypothesis that fatty rats develop insulin resistance early in life, before the onset of hyperinsulinemia and obesity, because of the reduction in leptin signaling.

After the onset of hyperphagia both FAS and PPARγ protein expression are induced and fatties become as insulin sensitive as their lean littermates. The time course of this change in FAS and PPARγ protein expression closely follows the time course of the change in insulin sensitivity. These data suggest that changes in FAS and PPARγ are closely linked to change in insulin sensitivity and to each other. FAS expression seems to increase earlier than PPARγ expression. FAS levels are higher in fatties by 25 days of age, while PPARγ levels become higher in fatties by 28 days of age. Several factors contribute to this delay. First, the range of variation in FAS expression is greater than that of PPARγ. Second, PPARγ levels rise in lean rats across this interval, making it more difficult to detect differences in expression. It is also likely that changes in PPARγ ligand binding and signaling occur before changes in expression begin. For these reasons, it is not possible to determine from these data whether increased PPARγ signaling occurs before or after the increase in FAS expression. It should be noted that, because receptor defect in fatties is not resolved during this period, these changes must be mediated by a mechanism that is independent of leptin signaling.

The portion of the leptin signal transduction pathway that regulates food intake does affect food intake during early postnatal development (121; 122). However, recent work indicates that leptin affects the maturation of the arcuate nucleus of the hypothalamus (ARH) during this period (119). Leptin normally functions to stimulate

the ARH neurons, reducing food intake later in life. These data suggest leptin not only provides signals to the brain to mediate food intake, but also serves as a vital component in brain development associated with leptin signaling. It is possible that the onset of hyperphagia is driven primarily by the maturation of these central pathways regulating food intake. However, fatty rats can be induced to overeat as early as 12, 15 and 18 days of age under some experimental conditions, which suggests that other factors are also required to stimulate hyperphagia (123). We propose that an interaction between the maturation of the central pathways for the regulation of food intake, and peripheral mechanisms regulating insulin signaling determines the onset of hyperphagia in fatties.

The events occurring around the onset of hyperphagia can be depicted as a developmental cascade (Fig. 14). During the first three weeks of age, fatties accumulate excess adipose tissue by thermoregulatory mechanisms. From 21 to 23 days of age, fatties become increasingly insulin resistant, and at 23 days of age, fatties develop robust hyperphagia, hyperinsulinemia and increased growth rate. After the onset of hyperphagia, *FAS* and *PPARγ* mRNA levels increase first, insulin sensitivity improves soon after, and FAS and PPARγ protein expression gradually increase. These data strongly suggest that PPARγ is involved in the development of insulin sensitivity after the onset of hyperphagia. The key to understanding the onset of hyperphagia could be found in the increasing insulin resistance that precedes it.

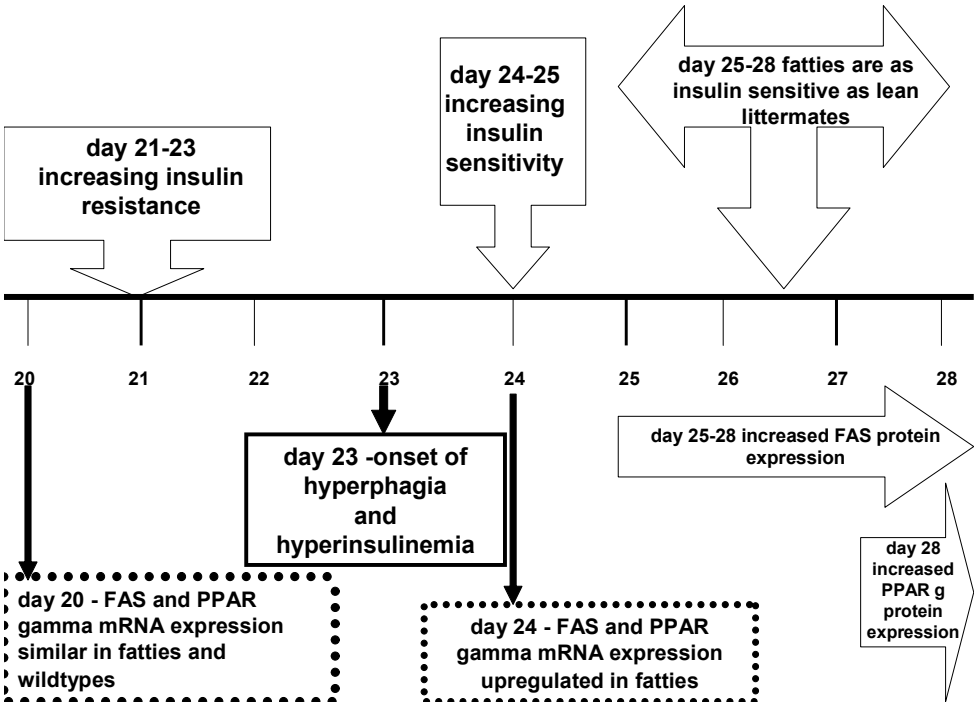

Figure 14. Developmental cascade.

PART 5. SUMMARY AND CONCLUSIONS

The goal of this research was to determine the relationship between insulin sensitivity and the development of hyperphagia in the fatty rat. Because fatties develop hyperphagia on a single day in postnatal development, this model system can be used to identify cellular and molecular mechanisms that contribute to the development of obesity in children.

These experiments show a complex relationship between insulin sensitivity and hyperphagia. Fatties experience two phases of insulin resistance, separated by a brief period of insulin sensitivity that follows the onset of hyperphagia. The early phase of insulin resistance occurs before fatties are obese and probably represents the lack of leptin signaling. During the later phase, insulin resistance becomes progressively greater as fatties become obese. Fatties also become increasingly insulin resistant in the few days prior to the onset of hyperphagia, and insulin resistance peaks on the first day of hyperphagia. After this peak fatties become as insulin sensitive as their littermates within two days. These data suggest that some developmental perturbation of insulin signaling is related to the onset of hyperphagia.

Several studies indicate the activation of pathways regulated by PPARγ promote insulin sensitivity and hyperphagia. In insulin resistant adult fatties, activation of PPARγ by thiazolidinediones increases insulin sensitivity by stimulating the expression of genes for lipogenesis in adipose tissue, leading to an increase in food intake and fat mass (4; 7). Rousseau *et al* further demonstrated that the expression of *PPARγ* mRNA is regulated by developmental mechanisms rather than diet composition (8). We proposed that a similar

PPARγ mechanism was involved in activating hyperphagia and increasing insulin sensitivity in fatties early in life.

We examined the expression of PPARγ in inguinal adipose tissue during the development of hyperphagia. PPARγ expression was low before the onset of hyperphagia. *PPARγ* mRNA increased in fatties within two days of the onset of hyperphagia. PPARγ protein levels increased more gradually after the onset of hyperphagia, becoming greater in fatties by 28 days of age. The changes in PPARγ expression occur in parallel with the changes in insulin sensitivity in the period. It is likley that changes in PPARγ signaling are more directly related to these changes.

In summary we propose that an interaction between the maturation of the central pathways for the regulation of food intake, and peripheral mechanisms regulating insulin signaling determines the onset of hyperphagia is fatties. The events occurring around the onset of hyperphagia can be depicted as a developmental cascade (Fig. 14). During the first three weeks of life, fatties accumulate excess adipose tissue by thermoregulatory mechanisms. From 21 to 23 days of age, fatties become increasingly insulin resistant, and at 23 days of age, fatties develop robust hyperphagia, hyperinsulinemia and increased growth rate. After the onset of hyperphagia, *FAS* and *PPARγ* mRNA levels increase first, insulin sensitivity improves soon after, and FAS and PPARγ protein expression gradually increase.

The prevalence of pediatric obesity in the United States has increased dramatically over the past two decades. Our research shows that the development of hyperphagia in early life is dependent not only on central nervous systems pathways, but

also on peripheral metabolic pathways that mediate insulin sensitivity. The fact that hyperphagia does not occur before the increase in PPARγ expression suggests that it is crucial that pathways involved in developmental alterations in insulin signaling and increased food intake be understood to develop effective interventions for the obesity epidemic.

REFERENCE LIST

Reference List

1. **Strauss RS and Pollack HA**. Epidemic increase in childhood overweight, 1986-1998. *JAMA* 286: 2845-2848, 2001.

2. **Warden NA and Warden CH**. Biological influences on obesity. *Pediatr Clin North Am* 48: 879-891, 2001.

3. **Truett GE, Walker JA and Harris RB**. A developmental switch affecting growth of fatty rats. *Am J Physiol Regul Integr Comp Physiol* 279: R1956-R1963, 2000.

4. **Wang Q, Dryden S, Frankish HM, Bing C, Pickavance L, Hopkins D, Buckingham R and Williams G**. Increased feeding in fatty Zucker rats by the thiazolidinedione BRL 49653 (rosiglitazone) and the possible involvement of leptin and hypothalamic neuropeptide Y. *Br J Pharmacol* 122: 1405-1410, 1997.

5. **Murakami K, Tobe K, Ide T, Mochizuki T, Ohashi M, Akanuma Y, Yazaki Y and Kadowaki T**. A novel insulin sensitizer acts as a coligand for peroxisome proliferator-activated receptor-alpha (PPAR-alpha) and PPAR-gamma: effect of PPAR-alpha activation on abnormal lipid metabolism in liver of Zucker fatty rats. *Diabetes* 47: 1841-1847, 1998.

6. **Tontonoz P, Hu E and Spiegelman BM**. Stimulation of adipogenesis in fibroblasts by PPAR gamma 2, a lipid-activated transcription factor. *Cell* 79: 1147-1156, 1994.

7. **Nolan JJ, Ludvik B, Beerdsen P, Joyce M and Olefsky J**. Improvement in glucose tolerance and insulin resistance in obese subjects treated with troglitazone. *N Engl J Med* 331: 1188-1193, 1994.

8. **Rousseau V, Becker DJ, Ongemba LN, Rahier J, Henquin JC and Brichard SM**. Developmental and nutritional changes of ob and PPAR gamma 2 gene expression in rat white adipose tissue. *Biochem J* 321 (Pt 2): 451-456, 1997.

9. **Gorla-Bajszczak A, Siegrist-Kaiser C, Boss O, Burger AG and Meier CA**. Expression of peroxisome proliferator-activated receptors in lean and obese Zucker rats. *Eur J Endocrinol* 142: 71-78, 2000.

10. **Bray GA**. Evaluation of obesity. Who are the obese? *Postgrad Med* 114: 19-27, 38, 2003.

11. **Flegal KM, Carroll MD, Ogden CL and Johnson CL**. Prevalence and trends in obesity among US adults, 1999-2000. *JAMA* 288: 1723-1727, 2002.

12. **WHO**.2003 **Controlling the global obesity epidemic The challenge** [Online]. 2003.

13. **Stunkard AJ**. Current views on obesity. *Am J Med* 100: 230-236, 1996.

14. **Pi-Sunyer FX**. Medical hazards of obesity. *Ann Intern Med* 119: 655-660, 1993.

15. **Deitel M**. The Surgeon-General's call to action to prevent an increase in overweight and obesity. Released Dec. 13, 2001. *Obes Surg* 12: 3-4, 2002.

16. **Jackson Y, Dietz WH, Sanders C, Kolbe LJ, Whyte JJ, Wechsler H, Schneider BS, McNally LA, Charles-Azure J, Vogel-Taylor M, Starke-Reed P, Hubbard VS, Johnson-Taylor WL, Troiano RP, Donato K, Yanovski S, Kuczmarski RJ, Haverkos L, McMurry K, Wykoff RF, Woo V, Noonan AS, Rowe J, McCarty K and Spain CB**. Summary of the 2000 Surgeon General's listening session: toward a national action plan on overweight and obesity. *Obes Res* 10: 1299-1305, 2002.

17. **Surgeon General**.2001 The Surgeon General's Call To Action To Prevent and Decrease Overweight and Obesity 2001 [Online]. 2001.

18. **Styne DM**. Childhood and adolescent obesity. Prevalence and significance. *Pediatr Clin North Am* 48: 823-54, vii, 2001.

19. **Kuczmarski RJ, Ogden CL, Guo SS, Grummer-Strawn LM, Flegal KM, Mei Z, Wei R, Curtin LR, Roche AF and Johnson CL**. 2000 CDC Growth Charts

for the United States: methods and development. *Vital Health Stat 11* 1-190, 2002.

20. **Ogden CL, Flegal KM, Carroll MD and Johnson CL**. Prevalence and trends in overweight among US children and adolescents, 1999-2000. *JAMA* 288: 1728-1732, 2002.

21. **Must A**. Morbidity and mortality associated with elevated body weight in children and adolescents. *Am J Clin Nutr* 63: 445S-447S, 1996.

22. **Must A and Strauss RS**. Risks and consequences of childhood and adolescent obesity. *Int J Obes Relat Metab Disord* 23 Suppl 2: S2-11, 1999.

23. **Garn SM, LaVelle M, Rosenberg KR and Hawthorne VM**. Maturational timing as a factor in female fatness and obesity. *Am J Clin Nutr* 43: 879-883, 1986.

24. **Wang Y**. Is obesity associated with early sexual maturation? A comparison of the association in American boys versus girls. *Pediatrics* 110: 903-910, 2002.

25. **Kaplowitz P**. Delayed puberty in obese boys: comparison with constitutional delayed puberty and response to testosterone therapy. *J Pediatr* 133: 745-749, 1998.

26. **Janssen I, Craig WM, Boyce WF and Pickett W**. Associations between overweight and obesity with bullying behaviors in school-aged children. *Pediatrics* 113: 1187-1194, 2004.

27. **Wadden TA, Brownell KD and Foster GD**. Obesity: responding to the global epidemic. *J Consult Clin Psychol* 70: 510-525, 2002.

28. **Must A, Spadano J, Coakley EH, Field AE, Colditz G and Dietz WH**. The disease burden associated with overweight and obesity. *JAMA* 282: 1523-1529, 1999.

29. **Guo SS, Wu W, Chumlea WC and Roche AF**. Predicting overweight and obesity in adulthood from body mass index values in childhood and adolescence. *Am J Clin Nutr* 76: 653-658, 2002.

30. **Steinberger J and Daniels SR**. Obesity, insulin resistance, diabetes, and cardiovascular risk in children: an American Heart Association scientific statement from the Atherosclerosis, Hypertension, and Obesity in the Young Committee (Council on Cardiovascular Disease in the Young) and the Diabetes Committee (Council on Nutrition, Physical Activity, and Metabolism). *Circulation* 107: 1448-1453, 2003.

31. **Farooqi IS and O'Rahilly S**. Recent advances in the genetics of severe childhood obesity. *Arch Dis Child* 83: 31-34, 2000.

32. **Vogler GP, Sorensen TI, Stunkard AJ, Srinivasan MR and Rao DC**. Influences of genes and shared family environment on adult body mass index assessed in an adoption study by a comprehensive path model. *Int J Obes Relat Metab Disord* 19: 40-45, 1995.

33. **Stunkard AJ, Sorensen TI, Hanis C, Teasdale TW, Chakraborty R, Schull WJ and Schulsinger F**. An adoption study of human obesity. *N Engl J Med* 314: 193-198, 1986.

34. **Bray GA**. Progress in understanding the genetics of obesity. *J Nutr* 127: 940S-942S, 1997.

35. **Guo SS, Roche AF, Chumlea WC, Gardner JD and Siervogel RM**. The predictive value of childhood body mass index values for overweight at age 35 y. *Am J Clin Nutr* 59: 810-819, 1994.

36. **Rolland-Cachera MF, Deheeger M, Guilloud-Bataille M, Avons P, Patois E and Sempe M**. Tracking the development of adiposity from one month of age to adulthood. *Ann Hum Biol* 14: 219-229, 1987.

37. **Dietz WH**. Periods of risk in childhood for the development of adult obesity--what do we need to learn? *J Nutr* 127: 1884S-1886S, 1997.

38. **Whitaker RC and Dietz WH**. Role of the prenatal environment in the development of obesity. *J Pediatr* 132: 768-776, 1998.

39. **Mistry AM, Swick A and Romsos DR**. Leptin alters metabolic rates before acquisition of its anorectic effect in developing neonatal mice. *Am J Physiol* 277: R742-R747, 1999.

40. **MCCANCE RA**. Food, growth, and time. *Lancet* 2: 621-626, 1962.

41. **MCCANCE RA**. Food, growth, and time. *Lancet* 2: 671-676, 1962.

42. **Hahn P**. Effect of litter size on plasma cholesterol and insulin and some liver and adipose tissue enzymes in adult rodents. *J Nutr* 114: 1231-1234, 1984.

43. **Patel MS and Srinivasan M**. Metabolic programming: causes and consequences. *J Biol Chem* 277: 1629-1632, 2002.

44. **Lucas A**. Programming by early nutrition: an experimental approach. *J Nutr* 128: 401S-406S, 1998.

45. **Ravelli GP, Stein ZA and Susser MW**. Obesity in young men after famine exposure in utero and early infancy. *N Engl J Med* 295: 349-353, 1976.

46. **Pettitt DJ, Baird HR, Aleck KA, Bennett PH and Knowler WC**. Excessive obesity in offspring of Pima Indian women with diabetes during pregnancy. *N Engl J Med* 308: 242-245, 1983.

47. **Gillman MW, Rifas-Shiman S, Berkey CS, Field AE and Colditz GA**. Maternal gestational diabetes, birth weight, and adolescent obesity. *Pediatrics* 111: e221-e226, 2003.

48. **Plagemann A, Harder T, Melchior K, Rake A, Rohde W and Dorner G**. Elevation of hypothalamic neuropeptide Y-neurons in adult offspring of diabetic mother rats. *Neuroreport* 10: 3211-3216, 1999.

49. **Barker DJ, Hales CN, Fall CH, Osmond C, Phipps K and Clark PM**. Type 2 (non-insulin-dependent) diabetes mellitus, hypertension and hyperlipidaemia (syndrome X): relation to reduced fetal growth. *Diabetologia* 36: 62-67, 1993.

50. **Dietz WH and Gortmaker SL**. Preventing obesity in children and adolescents. *Annu Rev Public Health* 22: 337-353, 2001.

51. **Wisemandle W, Maynard LM, Guo SS and Siervogel RM**. Childhood weight, stature, and body mass index among never overweight, early-onset overweight, and late-onset overweight groups. *Pediatrics* 106: E14, 2000.

52. **Whitaker RC, Pepe MS, Wright JA, Seidel KD and Dietz WH**. Early adiposity rebound and the risk of adult obesity. *Pediatrics* 101: E5, 1998.

53. **Guo SS, Huang C, Maynard LM, Demerath E, Towne B, Chumlea WC and Siervogel RM**. Body mass index during childhood, adolescence and young adulthood in relation to adult overweight and adiposity: the Fels Longitudinal Study. *Int J Obes Relat Metab Disord* 24: 1628-1635, 2000.

54. **Johnson SL and Birch LL**. Parents' and children's adiposity and eating style. *Pediatrics* 94: 653-661, 1994.

55. **van Lenthe FJ, Kemper CG and van Mechelen W**. Rapid maturation in adolescence results in greater obesity in adulthood: the Amsterdam Growth and Health Study. *Am J Clin Nutr* 64: 18-24, 1996.

56. **Braddon FE, Rodgers B, Wadsworth ME and Davies JM**. Onset of obesity in a 36 year birth cohort study. *Br Med J (Clin Res Ed)* 293: 299-303, 1986.

57. **Must A, Jacques PF, Dallal GE, Bajema CJ and Dietz WH**. Long-term morbidity and mortality of overweight adolescents. A follow-up of the Harvard Growth Study of 1922 to 1935. *N Engl J Med* 327: 1350-1355, 1992.

58. **Knoll JH, Nicholls RD, Magenis RE, Graham JM, Jr., Lalande M and Latt SA**. Angelman and Prader-Willi syndromes share a common chromosome 15 deletion but differ in parental origin of the deletion. *Am J Med Genet* 32: 285-290, 1989.

59. **Nicholls RD and Knepper JL**. Genome organization, function, and imprinting in Prader-Willi and Angelman syndromes. *Annu Rev Genomics Hum Genet* 2: 153-175, 2001.

60. **Prader A LAWH**. Ein syndrom von adipositas, kleinwuchs, kryyplorchimus, and oligophrenic nach myalonieartigen, zustand im neugeborenenalter. *Schweiz Medical Wochenschr* 86: 1260-1261, 1956.

61. **Goldstone AP**. Prader-Willi syndrome: advances in genetics, pathophysiology and treatment. *Trends Endocrinol Metab* 15: 12-20, 2004.

62. **Goldstone AP, Unmehopa UA, Bloom SR and Swaab DF**. Hypothalamic NPY and agouti-related protein are increased in human illness but not in Prader-Willi syndrome and other obese subjects. *J Clin Endocrinol Metab* 87: 927-937, 2002.

63. **Haig D and Wharton R**. Prader-Willi syndrome and the evolution of human childhood. *Am J Human Biol* 15: 320-329, 2003.

64. **Holland AJ, Treasure J, Coskeran P, Dallow J, Milton N and Hillhouse E**. Measurement of excessive appetite and metabolic changes in Prader-Willi syndrome. *Int J Obes Relat Metab Disord* 17: 527-532, 1993.

65. **Krude H, Biebermann H, Luck W, Horn R, Brabant G and Gruters A**. Severe early-onset obesity, adrenal insufficiency and red hair pigmentation caused by POMC mutations in humans. *Nat Genet* 19: 155-157, 1998.

66. **Krude H and Gruters A**. Implications of proopiomelanocortin (POMC) mutations in humans: the POMC deficiency syndrome. *Trends Endocrinol Metab* 11: 15-22, 2000.

67. **HERVEY GR**. The effects of lesions in the hypothalamus in parabiotic rats. *J Physiol* 145: 336-352, 1959.

68. **Coleman DL and Hummel KP**. Effects of parabiosis of normal with genetically diabetic mice. *Am J Physiol* 217: 1298-1304, 1969.

69. **Coleman DL**. Effects of parabiosis of obese with diabetes and normal mice. *Diabetologia* 9: 294-298, 1973.

70. **Harris RB, Hervey E, HERVEY GR and Tobin G**. Body composition of lean and obese Zucker rats in parabiosis. *Int J Obes* 11: 275-283, 1987.

71. **Zhang Y, Proenca R, Maffei M, Barone M, Leopold L and Friedman JM**. Positional cloning of the mouse obese gene and its human homologue. *Nature* 372: 425-432, 1994.

72. **Vaisse C, Halaas JL, Horvath CM, Darnell JE, Jr., Stoffel M and Friedman JM**. Leptin activation of Stat3 in the hypothalamus of wild-type and ob/ob mice but not db/db mice. *Nat Genet* 14: 95-97, 1996.

73. **Ahima RS and Flier JS**. Leptin. *Annu Rev Physiol* 62: 413-437, 2000.

74. **da Silva BA, Bjorbaek C, Uotani S and Flier JS**. Functional properties of leptin receptor isoforms containing the gln-->pro extracellular domain mutation of the fatty rat. *Endocrinology* 139: 3681-3690, 1998.

75. **Tartaglia LA, Dembski M, Weng X, Deng N, Culpepper J, Devos R, Richards GJ, Campfield LA, Clark FT, Deeds J and .** Identification and expression cloning of a leptin receptor, OB-R. *Cell* 83: 1263-1271, 1995.

76. **Chen H, Charlat O, Tartaglia LA, Woolf EA, Weng X, Ellis SJ, Lakey ND, Culpepper J, Moore KJ, Breitbart RE, Duyk GM, Tepper RI and Morgenstern JP**. Evidence that the diabetes gene encodes the leptin receptor: identification of a mutation in the leptin receptor gene in db/db mice. *Cell* 84: 491-495, 1996.

77. **Lee GH, Proenca R, Montez JM, Carroll KM, Darvishzadeh JG, Lee JI and Friedman JM**. Abnormal splicing of the leptin receptor in diabetic mice. *Nature* 379: 632-635, 1996.

78. **Farooqi IS, Matarese G, Lord GM, Keogh JM, Lawrence E, Agwu C, Sanna V, Jebb SA, Perna F, Fontana S, Lechler RI, DePaoli AM and O'Rahilly S**. Beneficial effects of leptin on obesity, T cell hyporesponsiveness, and

neuroendocrine/metabolic dysfunction of human congenital leptin deficiency. *J Clin Invest* 110: 1093-1103, 2002.

79. **Arioglu E, Rother KI, Reitman ML, Premkumar A and Taylor SI**. Lipoatrophy syndromes: when 'too little fat' is a clinical problem. *Pediatr Diabetes* 1: 155-168, 2000.

80. **Garg A**. Lipodystrophies. *Am J Med* 108: 143-152, 2000.

81. **Oral EA, Ruiz E, Andewelt A, Sebring N, Wagner AJ, Depaoli AM and Gorden P**. Effect of leptin replacement on pituitary hormone regulation in patients with severe lipodystrophy. *J Clin Endocrinol Metab* 87: 3110-3117, 2002.

82. **Oral EA, Simha V, Ruiz E, Andewelt A, Premkumar A, Snell P, Wagner AJ, Depaoli AM, Reitman ML, Taylor SI, Gorden P and Garg A**. Leptin-replacement therapy for lipodystrophy. *N Engl J Med* 346: 570-578, 2002.

83. **Unger RH and Zhou YT**. Lipotoxicity of beta-cells in obesity and in other causes of fatty acid spillover. *Diabetes* 50 Suppl 1: S118-S121, 2001.

84. **Unger RH and Zhou YT**. Lipotoxicity of beta-cells in obesity and in other causes of fatty acid spillover. *Diabetes* 50 Suppl 1: S118-S121, 2001.

85. **Unger RH, Zhou YT and Orci L**. Regulation of fatty acid homeostasis in cells: novel role of leptin. *Proc Natl Acad Sci U S A* 96: 2327-2332, 1999.

86. **Unger RH, Zhou YT and Orci L**. Regulation of fatty acid homeostasis in cells: novel role of leptin. *Proc Natl Acad Sci U S A* 96: 2327-2332, 1999.

87. **Unger RH and Zhou YT**. Lipotoxicity of beta-cells in obesity and in other causes of fatty acid spillover. *Diabetes* 50 Suppl 1: S118-S121, 2001.

88. **Seip M and Trygstad O**. Generalized lipodystrophy, congenital and acquired (lipoatrophy). *Acta Paediatr Suppl* 413: 2-28, 1996.

89. **Bjornstad PG, Foerster A and Ihlen H**. Cardiac findings in generalized lipodystrophy. *Acta Paediatr Suppl* 413: 39-43, 1996.

90. **Unger RH and Zhou YT**. Lipotoxicity of beta-cells in obesity and in other causes of fatty acid spillover. *Diabetes* 50 Suppl 1: S118-S121, 2001.

91. **Shimomura I, Hammer RE, Ikemoto S, Brown MS and Goldstein JL**. Leptin reverses insulin resistance and diabetes mellitus in mice with congenital lipodystrophy. *Nature* 401: 73-76, 1999.

92. **Petersen KF, Oral EA, Dufour S, Befroy D, Ariyan C, Yu C, Cline GW, Depaoli AM, Taylor SI, Gorden P and Shulman GI**. Leptin reverses insulin resistance and hepatic steatosis in patients with severe lipodystrophy. *J Clin Invest* 109: 1345-1350, 2002.

93. **Forman BM, Tontonoz P, Chen J, Brun RP, Spiegelman BM and Evans RM**. 15-Deoxy-delta 12, 14-prostaglandin J2 is a ligand for the adipocyte determination factor PPAR gamma. *Cell* 83: 803-812, 1995.

94. **Burant CF, Sreenan S, Hirano K, Tai TA, Lohmiller J, Lukens J, Davidson NO, Ross S and Graves RA**. Troglitazone action is independent of adipose tissue. *J Clin Invest* 100: 2900-2908, 1997.

95. **Arioglu E, Duncan-Morin J, Sebring N, Rother KI, Gottlieb N, Lieberman J, Herion D, Kleiner DE, Reynolds J, Premkumar A, Sumner AE, Hoofnagle J, Reitman ML and Taylor SI**. Efficacy and safety of troglitazone in the treatment of lipodystrophy syndromes. *Ann Intern Med* 133: 263-274, 2000.

96. **Reaven GM**. Role of insulin resistance in human disease (syndrome X): an expanded definition. *Annu Rev Med* 44: 121-131, 1993.

97. **Reaven GM**. Pathophysiology of insulin resistance in human disease. *Physiol Rev* 75: 473-486, 1995.

98. **Bultman SJ, Michaud EJ and Woychik RP**. Molecular characterization of the mouse agouti locus. *Cell* 71: 1195-1204, 1992.

99. **Dubern B, Clement K, Pelloux V, Froguel P, Girardet JP, Guy-Grand B and Tounian P**. Mutational analysis of melanocortin-4 receptor, agouti-related protein, and alpha-melanocyte-stimulating hormone genes in severely obese children. *J Pediatr* 139: 204-209, 2001.

100. **Zucker TF and Zucker LM**. Fat accretion and growth in the rat. *J Nutr* 80: 6-19, 1963.

101. **Truett GE, Jacob HJ, Miller J, Drouin G, Bahary N, Smoller JW, Lander ES and Leibel RL**. Genetic map of rat chromosome 5 including the fatty (fa) locus. *Mamm Genome* 6: 25-30, 1995.

102. **Friedman JM and Halaas JL**. Leptin and the regulation of body weight in mammals. *Nature* 395: 763-770, 1998.

103. **Halaas JL and Friedman JM**. Leptin and its receptor. *J Endocrinol* 155: 215-216, 1997.

104. **Phillips MS, Liu Q, Hammond HA, Dugan V, Hey PJ, Caskey CJ and Hess JF**. Leptin receptor missense mutation in the fatty Zucker rat. *Nat Genet* 13: 18-19, 1996.

105. **Wang MY, Zhou YT, Newgard CB and Unger RH**. A novel leptin receptor isoform in rat. *FEBS Lett* 392: 87-90, 1996.

106. **Zucker LM and Antoniades HN**. Insulin and obesity in the Zucker genetically obese rat "fatty". *Endocrinology* 90: 1320-1330, 1972.

107. **Kortner G, Petrova O, Vogt S and Schmidt I**. Sympathetically and nonsympathetically mediated onset of excess fat deposition in Zucker rats. *Am J Physiol* 267: E947-E953, 1994.

108. **Planche E, Joliff M and Bazin R**. Energy expenditure and adipose tissue development in 2- to 8-day-old Zucker rats. *Int J Obes* 12: 353-360, 1988.

109. **Kaul R, Heldmaier G and Schmidt I**. Defective thermoregulatory thermogenesis does not cause onset of obesity in Zucker rats. *Am J Physiol* 259: E11-E18, 1990.

110. **Markewicz B, Kuhmichel G and Schmidt I**. Onset of excess fat deposition in Zucker rats with and without decreased thermogenesis. *Am J Physiol* 265: E478-E486, 1993.

111. **Rosen ED and Spiegelman BM**. Molecular regulation of adipogenesis. *Annu Rev Cell Dev Biol* 16: 145-171, 2000.

112. **Rangwala SM and Lazar MA**. Transcriptional control of adipogenesis. *Annu Rev Nutr* 20: 535-559, 2000.

113. **Lowell BB, Susulic V, Hamann A, Lawitts JA, Himms-Hagen J, Boyer BB, Kozak LP and Flier JS**. Development of obesity in transgenic mice after genetic ablation of brown adipose tissue. *Nature* 366: 740-742, 1993.

114. **Pujol A, Berthault MF, Picon L and Penicaud L**. Regulation of glucose homeostasis in pre-obese Zucker rats before and after weaning. *Int J Obes* 15: 505-511, 1991.

115. **Henning SJ**. Postnatal development: coordination of feeding, digestion, and metabolism. *Am J Physiol* 241: G199-G214, 1981.

116. **Cramer CP, Thiels E and Alberts JR**. Weaning in rats: I. Maternal behavior. *Dev Psychobiol* 23: 479-493, 1990.

117. **Stern JS and Johnson PR**. Spontaneous activity and adipose cellularity in the genetically obese Zucker rat (fafa). *Metabolism* 26: 371-380, 1977.

118. **Ahima RS, Prabakaran D and Flier JS**. Postnatal leptin surge and regulation of circadian rhythm of leptin by feeding. Implications for energy homeostasis and neuroendocrine function. *J Clin Invest* 101: 1020-1027, 1998.

119. **Bouret SG, Draper SJ and Simerly RB**. Trophic action of leptin on hypothalamic neurons that regulate feeding. *Science* 304: 108-110, 2004.

120. **Godbole VY, Grundleger ML, Pasquine TA and Thenen SW**. Composition of rat milk from day 5 to 20 of lactation and milk intake of lean and preobese Zucker pups. *J Nutr* 111: 480-487, 1981.

121. **Buchberger P and Schmidt I**. Is the onset of obesity in suckling fa/fa rats linked to a potentially larger milk intake? *Am J Physiol* 271: R472-R476, 1996.

122. **Kraeft S, Schwarzer K, Eiden S, Nuesslein-Hildesheim B, Preibisch G and Schmidt I**. Leptin responsiveness and gene dosage for leptin receptor mutation (fa) in newborn rats. *Am J Physiol* 276: E836-E842, 1999.

123. **Kowalski TJ, Ster AM and Smith GP**. Ontogeny of hyperphagia in the Zucker (fa/fa) rat. *Am J Physiol* 275: R1106-R1109, 1998.

124. **McLaughlin CL and Baile CA**. Ontogeny of feeding behavior in the Zucker obese rat. *Physiol Behav* 26: 607-612, 1981.

125. **Vasselli JR and Maggio CA**. Diet composition determines course of hyperphagia in developing Zucker obese rats. *Physiol Behav* 48: 805-811, 1990.

126. **Turkenkopf IJ, Johnson PR and Greenwood MR**. Development of pancreatic and plasma insulin in prenatal and suckling Zucker rats. *Am J Physiol* 242: E220-E225, 1982.

127. **Albright ALSJL**. Adipose tissue. In: Encyclopedia of Sports Medicine and Science, edited by Farley TD. 1998.

128. **Hopfer U**. Digestion and Absorption of Basic Nutritional Constituents. In: Textbook of Biochemistry with Clinical Correlations, New York: Wiley-Liss, 2002, p. 1082-1110.

129. **Semenkovich CF**. Regulation of fatty acid synthase (FAS). *Prog Lipid Res* 36: 43-53, 1997.

130. **Wakil SJ**. Fatty acid synthase, a proficient multifunctional enzyme. *Biochemistry* 28: 4523-4530, 1989.

131. **Volpe JJ and Vagelos PR**. Regulation of mammalian fatty-acid synthetase. The roles of carbohydrate and insulin. *Proc Natl Acad Sci U S A* 71: 889-893, 1974.

132. **Penicaud L, Ferre P, Assimacopoulos-Jeannet F, Perdereau D, Leturque A, Jeanrenaud B, Picon L and Girard J**. Increased gene expression of lipogenic enzymes and glucose transporter in white adipose tissue of suckling and weaned obese Zucker rats. *Biochem J* 279 (Pt 1): 303-308, 1991.

133. **Clarke SD and Jump DB**. Dietary polyunsaturated fatty acid regulation of gene transcription. *Annu Rev Nutr* 14: 83-98, 1994.

134. **Magana MM and Osborne TF**. Two tandem binding sites for sterol regulatory element binding proteins are required for sterol regulation of fatty-acid synthase promoter. *J Biol Chem* 271: 32689-32694, 1996.

135. **Kim JB, Sarraf P, Wright M, Yao KM, Mueller E, Solanes G, Lowell BB and Spiegelman BM**. Nutritional and insulin regulation of fatty acid synthetase and leptin gene expression through ADD1/SREBP1. *J Clin Invest* 101: 1-9, 1998.

136. **Paulauskis JD and Sul HS**. Hormonal regulation of mouse fatty acid synthase gene transcription in liver. *J Biol Chem* 264: 574-577, 1989.

137. **Moustaid N, Beyer RS and Sul HS**. Identification of an insulin response element in the fatty acid synthase promoter. *J Biol Chem* 269: 5629-5634, 1994.

138. **Sul HS, Latasa MJ, Moon Y and Kim KH**. Regulation of the fatty acid synthase promoter by insulin. *J Nutr* 130: 315S-320S, 2000.

139. **Blennemann B, Leahy P, Kim TS and Freake HC**. Tissue-specific regulation of lipogenic mRNAs by thyroid hormone. *Mol Cell Endocrinol* 110: 1-8, 1995.

140. **Chawla A, Repa JJ, Evans RM and Mangelsdorf DJ**. Nuclear receptors and lipid physiology: opening the X-files. *Science* 294: 1866-1870, 2001.

141. **Rosen ED and Spiegelman BM**. PPARgamma : a nuclear regulator of metabolism, differentiation, and cell growth. *J Biol Chem* 276: 37731-37734, 2001.

142. **Tontonoz P, Hu E, Devine J, Beale EG and Spiegelman BM**. PPAR gamma 2 regulates adipose expression of the phosphoenolpyruvate carboxykinase gene. *Mol Cell Biol* 15: 351-357, 1995.

143. **Houseknecht KL, Cole BM and Steele PJ**. Peroxisome proliferator-activated receptor gamma (PPARgamma) and its ligands: a review. *Domest Anim Endocrinol* 22: 1-23, 2002.

144. **Dreyer C, Keller H, Mahfoudi A, Laudet V, Krey G and Wahli W**. Positive regulation of the peroxisomal beta-oxidation pathway by fatty acids through activation of peroxisome proliferator-activated receptors (PPAR). *Biol Cell* 77: 67-76, 1993.

145. **Peters JM, Hennuyer N, Staels B, Fruchart JC, Fievet C, Gonzalez FJ and Auwerx J**. Alterations in lipoprotein metabolism in peroxisome proliferator-activated receptor alpha-deficient mice. *J Biol Chem* 272: 27307-27312, 1997.

146. **Michalik L, Desvergne B, Tan NS, Basu-Modak S, Escher P, Rieusset J, Peters JM, Kaya G, Gonzalez FJ, Zakany J, Metzger D, Chambon P, Duboule D and Wahli W**. Impaired skin wound healing in peroxisome proliferator-activated receptor (PPAR)alpha and PPARbeta mutant mice. *J Cell Biol* 154: 799-814, 2001.

147. **Chawla A, Lee CH, Barak Y, He W, Rosenfeld J, Liao D, Han J, Kang H and Evans RM**. PPARdelta is a very low-density lipoprotein sensor in macrophages. *Proc Natl Acad Sci U S A* 100: 1268-1273, 2003.

148. **Tontonoz P, Hu E, Graves RA, Budavari AI and Spiegelman BM**. mPPAR gamma 2: tissue-specific regulator of an adipocyte enhancer. *Genes Dev* 8: 1224-1234, 1994.

149. **Tontonoz P, Graves RA, Budavari AI, Erdjument-Bromage H, Lui M, Hu E, Tempst P and Spiegelman BM**. Adipocyte-specific transcription factor ARF6 is a heterodimeric complex of two nuclear hormone receptors, PPAR gamma and RXR alpha. *Nucleic Acids Res* 22: 5628-5634, 1994.

150. **Barak Y, Nelson MC, Ong ES, Jones YZ, Ruiz-Lozano P, Chien KR, Koder A and Evans RM**. PPAR gamma is required for placental, cardiac, and adipose tissue development. *Mol Cell* 4: 585-595, 1999.

151. **Kubota N, Terauchi Y, Miki H, Tamemoto H, Yamauchi T, Komeda K, Satoh S, Nakano R, Ishii C, Sugiyama T, Eto K, Tsubamoto Y, Okuno A, Murakami K, Sekihara H, Hasegawa G, Naito M, Toyoshima Y, Tanaka S, Shiota K, Kitamura T, Fujita T, Ezaki O, Aizawa S, Kadowaki T and .** PPAR gamma mediates high-fat diet-induced adipocyte hypertrophy and insulin resistance. *Mol Cell* 4: 597-609, 1999.

152. **Rosen ED, Sarraf P, Troy AE, Bradwin G, Moore K, Milstone DS, Spiegelman BM and Mortensen RM**. PPAR gamma is required for the differentiation of adipose tissue in vivo and in vitro. *Mol Cell* 4: 611-617, 1999.

153. **Xu HE, Lambert MH, Montana VG, Parks DJ, Blanchard SG, Brown PJ, Sternbach DD, Lehmann JM, Wisely GB, Willson TM, Kliewer SA and Milburn MV**. Molecular recognition of fatty acids by peroxisome proliferator-activated receptors. *Mol Cell* 3: 397-403, 1999.

154. **Kliewer SA, Sundseth SS, Jones SA, Brown PJ, Wisely GB, Koble CS, Devchand P, Wahli W, Willson TM, Lenhard JM and Lehmann JM**. Fatty acids and eicosanoids regulate gene expression through direct interactions with peroxisome proliferator-activated receptors alpha and gamma. *Proc Natl Acad Sci U S A* 94: 4318-4323, 1997.

155. **Storlien LH, Baur LA, Kriketos AD, Pan DA, Cooney GJ, Jenkins AB, Calvert GD and Campbell LV**. Dietary fats and insulin action. *Diabetologia* 39: 621-631, 1996.

156. **Jiang C, Ting AT and Seed B**. PPAR-gamma agonists inhibit production of monocyte inflammatory cytokines. *Nature* 391: 82-86, 1998.

157. **Kliewer SA, Lenhard JM, Willson TM, Patel I, Morris DC and Lehmann JM**. A prostaglandin J2 metabolite binds peroxisome proliferator-activated receptor gamma and promotes adipocyte differentiation. *Cell* 83: 813-819, 1995.

158. **Harris PK and Kletzien RF**. Localization of a pioglitazone response element in the adipocyte fatty acid-binding protein gene. *Mol Pharmacol* 45: 439-445, 1994.

159. **Lehmann JM, Moore LB, Smith-Oliver TA, Wilkison WO, Willson TM and Kliewer SA**. An antidiabetic thiazolidinedione is a high affinity ligand for peroxisome proliferator-activated receptor gamma (PPAR gamma). *J Biol Chem* 270: 12953-12956, 1995.

160. **Hallakou S, Doare L, Foufelle F, Kergoat M, Guerre-Millo M, Berthault MF, Dugail I, Morin J, Auwerx J and Ferre P**. Pioglitazone induces in vivo adipocyte differentiation in the obese Zucker fa/fa rat. *Diabetes* 46: 1393-1399, 1997.

161. **Vidal-Puig AJ, Considine RV, Jimenez-Linan M, Werman A, Pories WJ, Caro JF and Flier JS**. Peroxisome proliferator-activated receptor gene expression in human tissues. Effects of obesity, weight loss, and regulation by insulin and glucocorticoids. *J Clin Invest* 99: 2416-2422, 1997.

162. **Martin G, Schoonjans K, Staels B and Auwerx J**. PPARgamma activators improve glucose homeostasis by stimulating fatty acid uptake in the adipocytes. *Atherosclerosis* 137 Suppl: S75-S80, 1998.

163. **Randle PJ**. Regulatory interactions between lipids and carbohydrates: the glucose fatty acid cycle after 35 years. *Diabetes Metab Rev* 14: 263-283, 1998.

164. **Okuno A, Tamemoto H, Tobe K, Ueki K, Mori Y, Iwamoto K, Umesono K, Akanuma Y, Fujiwara T, Horikoshi H, Yazaki Y and Kadowaki T**. Troglitazone increases the number of small adipocytes without the change of white adipose tissue mass in obese Zucker rats. *J Clin Invest* 101: 1354-1361, 1998.

165. **Suter SL, Nolan JJ, Wallace P, Gumbiner B and Olefsky JM**. Metabolic effects of new oral hypoglycemic agent CS-045 in NIDDM subjects. *Diabetes Care* 15: 193-203, 1992.

166. **Hofmann C, Lorenz K, Braithwaite SS, Colca JR, Palazuk BJ, Hotamisligil GS and Spiegelman BM**. Altered gene expression for tumor necrosis factor-

alpha and its receptors during drug and dietary modulation of insulin resistance. *Endocrinology* 134: 264-270, 1994.

167. **De Vos P, Lefebvre AM, Miller SG, Guerre-Millo M, Wong K, Saladin R, Hamann LG, Staels B, Briggs MR and Auwerx J**. Thiazolidinediones repress ob gene expression in rodents via activation of peroxisome proliferator-activated receptor gamma. *J Clin Invest* 98: 1004-1009, 1996.

168. **Uysal KT, Wiesbrock SM, Marino MW and Hotamisligil GS**. Protection from obesity-induced insulin resistance in mice lacking TNF-alpha function. *Nature* 389: 610-614, 1997.

169. **Uysal KT, Wiesbrock SM and Hotamisligil GS**. Functional analysis of tumor necrosis factor (TNF) receptors in TNF-alpha-mediated insulin resistance in genetic obesity. *Endocrinology* 139: 4832-4838, 1998.

170. **Qian H, Hausman GJ, Compton MM, Azain MJ, Hartzell DL and Baile CA**. Leptin regulation of peroxisome proliferator-activated receptor-gamma, tumor necrosis factor, and uncoupling protein-2 expression in adipose tissues. *Biochem Biophys Res Commun* 246: 660-667, 1998.

171. **Gregoire FM, Smas CM and Sul HS**. Understanding adipocyte differentiation. *Physiol Rev* 78: 783-809, 1998.

172. **Hu E, Tontonoz P and Spiegelman BM**. Transdifferentiation of myoblasts by the adipogenic transcription factors PPAR gamma and C/EBP alpha. *Proc Natl Acad Sci U S A* 92: 9856-9860, 1995.

173. **Wu Z, Bucher NL and Farmer SR**. Induction of peroxisome proliferator-activated receptor gamma during the conversion of 3T3 fibroblasts into adipocytes is mediated by C/EBPbeta, C/EBPdelta, and glucocorticoids. *Mol Cell Biol* 16: 4128-4136, 1996.

174. **MacDougald OA and Lane MD**. Transcriptional regulation of gene expression during adipocyte differentiation. *Annu Rev Biochem* 64: 345-373, 1995.

175. **Brown MS and Goldstein JL**. The SREBP pathway: regulation of cholesterol metabolism by proteolysis of a membrane-bound transcription factor. *Cell* 89: 331-340, 1997.

176. **Tontonoz P, Kim JB, Graves RA and Spiegelman BM**. ADD1: a novel helix-loop-helix transcription factor associated with adipocyte determination and differentiation. *Mol Cell Biol* 13: 4753-4759, 1993.

177. **Issad T, Coupe C, Pastor-Anglada M, Ferre P and Girard J**. Development of insulin-sensitivity at weaning in the rat. Role of the nutritional transition. *Biochem J* 251: 685-690, 1988.

178. **Issad T, Coupe C, Ferre P and Girard J**. Insulin resistance during suckling period in rats. *Am J Physiol* 253: E142-E148, 1987.

179. **Issad T, Ferre P, Pastor-Anglada M, Baudon MA and Girard J**. Development of insulin sensitivity in white adipose tissue during the suckling-weaning transition in the rat. Involvement of glucose transport and lipogenesis. *Biochem J* 264: 217-222, 1989.

180. **Unger RH and Orci L**. Diseases of liporegulation: new perspective on obesity and related disorders. *FASEB J* 15: 312-321, 2001.

181. **Truett GE, Heeger P, Mynatt RL, Truett AA, Walker JA and Warman ML**. Preparation of PCR-quality mouse genomic DNA with hot sodium hydroxide and tris (HotSHOT). *Biotechniques* 29: 52, 54, 2000.

182. **Walker JA and Truett GE**. An assay for the Cpefat obesity mutation created with a modified primer. *Mamm Genome* 8: 783-784, 1997.

183. **Chawla A, Schwarz EJ, Dimaculangan DD and Lazar MA**. Peroxisome proliferator-activated receptor (PPAR) gamma: adipose-predominant expression and induction early in adipocyte differentiation. *Endocrinology* 135: 798-800, 1994.

184. **Wilfinger WW, Mackey K and Chomczynski P**. Effect of pH and ionic strength on the spectrophotometric assessment of nucleic acid purity. *Biotechniques* 22: 474-481, 1997.

185. **Harris RB**. Parabiosis between db/db and ob/ob or db/+ mice. *Endocrinology* 140: 138-145, 1999.

186. **Girard J, Ferre P, Pegorier JP and Duee PH**. Adaptations of glucose and fatty acid metabolism during perinatal period and suckling-weaning transition. *Physiol Rev* 72: 507-562, 1992.

187. **Girard J, Perdereau D, Foufelle F, Prip-Buus C and Ferre P**. Regulation of lipogenic enzyme gene expression by nutrients and hormones. *FASEB J* 8: 36-42, 1994.

188. **Qian H, Hausman GJ, Compton MM, Azain MJ, Hartzell DL and Baile CA**. Down-regulation of CCAAT/enhancer binding proteins alpha, beta and delta in adipose tissue by intracerebroventricular leptin in rats. *Biochim Biophys Acta* 1442: 245-251, 1998.

189. **Ferre P, Decaux JF, Issad T and Girard J**. Changes in energy metabolism during the suckling and weaning period in the newborn. *Reprod Nutr Dev* 26: 619-631, 1986.

APPENDIX

A1. Protocol: Western Blot Analysis for Adipose Tissue

I. Preparation of Homogenizing Buffer and Protease Inhibitors

A. 2 X Homogenizing Buffer Salts

1. Mix these salts in 45 ml water.
    a. Add 0.7882 grams of 100 mM Tris HCl (M.W. 157.64).
    b. Add 8.732 grams of 510 mM sucrose (M.W. 342.30).
    c. Add 0.0292 grams of 2 mM EDTA (F.W. 292.25).
2. Adjust pH to 7.4.
3. Adjust volume to 50 ml.
4. Store at 4 degrees C.

B. Protease Inhibitors

1. Prepare stock solutions and store in 2 ml µl aliquots at -20ºC. 2 ml Protease Inhibitor aliquots should be discarded after approximately 3 uses to minimize breakdown after thawing multiple times.

    a. 2 µM phenylmethylsulfonyl fluoride (100X, Sigma P7626, F.W33. 174.20)
    b. 1 µM pepstatin (250X, Sigma P4205, F.W. 685.90)
    c. 80 trypsin inhibitory units per ml of aprotinin or 200 µg/ml (100X, Sigma A1153)
    d. 10 µM leupeptin (100X, Sigma L2884, F.W. 475.60)
    e. 2 mM sodium orthovanadate (100X, Sigma S6508, F.W. 183.90)

C. Prepare homogenizing buffer with protease inhibitors.

1. Prepare immediately before processing tissues. Place all reagents on ice. Some reagents degrade rapidly when added to an aqueous solution.

2. Mix protease inhibitors with homogenizing buffer with the following volumes per 10 ml of final volume.

a. 5 ml 2X homogenizing buffer

b. 0.05 ml 1 mM PMSF

c. aprotinin

HOMOGENIZING BUFFER

Volume to Make	10	Completed
1X Homogenizing Salts	5	
Water	4.66	
2 uM PMSF	0.05	
80 trypsin inhibitory millii	0.05	
1 uM pepstatin	0.04	
10 uM leupeptin	0.1	
2 mM sodium orthovanadate	0.1	
Final Volume	10	

II. Preparation of adipose tissue extracts

1. Chill homogenizer generator in an ice bath prior to using it.

2. Prepare homogenizing buffer immediately before processing tissues. All reagents should be on ice. Some reagents degrade rapidly when added to an aqueous solution.

3. Add 1 ml of homogenization buffer per 1 gram of fat pad to each centrifuge tube.

4. Homogenize fat pad in the homogenization buffer while on ice.

5. Centrifuge homogenates at 5000 x g for 2 minutes at 4ºC. Remove debris and insoluble material; save the supernatant containing fat pad extracts.

6. Centrifuge homogenates at 13000 x g for 15 minutes at 4ºC. Remove any debris and insoluble material, save the supernatant containing fat pad extracts.

7. Continue to centrifuge homogenates at 13000 x g for 15 minutes at 4ºC until all debris and insoluble material is removed, save the supernatant containing fat pad extracts.

8. Freeze tissue extracts at -80 ºC or quantify amount of protein in sample using the Bradford method.

IV. Quantify adipose tissue extracts using the Bradford Method

1. Components:

   a. 1.5 ml BSA (0.5 mg/ml)

   b. 200 ml 0.15N NaCl

   c. 1 L Bradford Reagent

   d. Store reagents at 4º C.

2. Into 5 separate microcuvette tubes, aliquot 0, 5, 10, 15 and 20 µl of 0.5 mg/ml BSA solution.

3. Pipette 5 µl of each unknown into microcuvettes.

4. Bring the volume of each to 100 µl with 0.15N NaCl.

5. Add to each tube, 1 ml Bradford Reagent and vortex. Incubate at room temperature for 2 minutes.

5. Measure Abs_{595} nm, using a 1 ml microcuvette. Generate a standard curve by plotting Abs_{595} nm versus protein concentration.

6. For the unknown sample, use the standard curve as a reference to determine the concentration of the unknown sample. If after the initial assay, the unknown protein concentration is too high, dilute the protein or assay a smaller aliquot of the unknown.

X. SDS-PAGE Electrophoresis.

Gel electrophoresis

Separate proteins using a 10% polyacrylamide gel according to the BioRad protocol for SDS– PAGE Laemmli (for discontinuous gels). Load 21, 28 and 60 day old lean and obese sex matched animals onto gel.

1.) Pour 10% resolving gel (follow BioRad Mini-Protean 3 Cell instruction manual). Wait 45 minutes- 1 hour for gel to harden.

2.) Pour stacking gel, and place 1.5mm combs into gel. (Follow BioRad Mini-Protean 3 Cell instruction manual.) Wait 30 minutes for gel to harden.

3.) Load equal amounts of protein from 14, 21, and 28 day old sample extracts. Calculate appropriate amount of 5X sample loading buffer and water to mix with sample extract for equal loading volumes for each sample. Run samples on gel for 4 hours at 50 volts.

XI. Western Blot Analysis

Follow BioRad Mini TransBlot Electrophoretic Transfer Cell instruction manual to conduct transfer.

After samples have run to completion, carefully remove gel and soak in transfer buffer. Proceed to pour transfer buffer containers with the filter pad and filter paper. Equilibrate the membrane for 1 minute in methanol, wash in DDI water for 5 minutes, and then place in container with transfer buffer. Soak all items for 15-30 minutes. After 15-30 minutes, immerse in tank with ice chest. Place tank on 4ºC. Transfer gel to membrane for 5 hours at 100 volts, 400mAMP or overnight (11-15 hours) at 30 volts, 90 mAMP. Overnight transfers are preferred to ensure proteins are completely transferred and box does heat.

XII. PPARγ and Fatty Acid Synthase antibody

Follow Cell Signal Protocol for PPARγ antibody #2492. Follow Santa Cruz Protocol for FAS antibody. Add a 1: 500 dilution of primary antibody to membrane and a 1: 2000 dilution of secondary antibody to membrane.

XIII. Western Blotting Luminol Reagent

Follow Santa Cruz Protocol sc-2048.

SAS code

Program 1. Daily gain data analysis.

```
libname XX 'c:/SASRUNS/XX';
  options linesize = 110; options pagesize = 20000;
  options nocenter; options nodate; options nonumber; run;
  data one;
infile 'c:/SASRUNS/ArrayGroup/youngunsgain.txt' FIRSTOBS=2;
input id lit sex geno G15-G24 ;
*Change data to univariate format;
y = G19; age = 19; output;
y = G20; age = 20; output;
y = G21; age = 21; output;
y = G22; age = 22; output;
y = G23; age = 23; output;
y = G24; age = 24; output;
drop G15-G24;
run;
proc sort;
by id age;
run;
proc mixed method=ml scoring=150 info update itdetails;
 class id lit sex geno age;
 model y=geno|sex|age/noint;
 random lit;
 repeated age/ subject=id group=sex type=un;
 parms/OLS;
 lsmeans geno*age /diff slice=age;
run;
```

Dataset 1. Daily gains.

id	lit	sex	geno	G15	G16	G17	G18	G19	G20	G21	G22	G23	G24
12380	1142	0	0	1.66	2.94	2.22	1.58	2.07	1.49	1.13	0.57	3.46	2.87
12381	1142	0	2	1.59	2.24	2.02	1.66	2.05	0.90	1.43	1.56	3.66	4.98
12382	1142	0	2	1.95	3.52	1.52	1.78	2.08	0.88	1.44	3.79	4.67	3.31

12383	1142	0	0	1.79	3.10	2.64	1.46	1.68	0.77	2.08	3.00	2.79	3.10
12384	1142	0	2	1.89	2.43	2.13	1.60	1.80	1.08	1.07	0.66	5.89	2.96
12385	1142	0	0	2.41	2.36	2.23	1.44	1.53	1.10	1.70	2.66	1.88	3.25
12386	1142	0	2	1.60	3.15	1.98	1.73	1.79	0.93	1.25	3.94	3.42	3.92
12387	1142	0	0	2.08	2.82	2.64	1.03	1.44	1.29	1.84	2.58	2.67	3.65
12418	1146	0	0	3.12	1.70	1.65	1.61	2.18	1.94	1.70	2.50	2.34	3.32
12419	1146	0	2	2.92	2.24	2.22	2.10	2.02	2.51	1.91	4.94	5.25	3.13
12420	1146	0	2	2.87	1.79	1.65	1.86	2.37	1.88	2.34	4.03	4.77	4.11
12421	1146	0	0	3.25	1.83	1.94	1.89	2.19	1.53	2.54	1.63	3.83	2.90
12423	1146	0	2	2.72	2.02	1.75	1.51	2.03	1.30	2.84	3.21	3.79	4.18
12425	1146	0	0	2.85	2.20	1.15	2.42	2.51	1.61	1.62	2.64	2.43	2.77
12427	1146	1	2	3.64	2.08	1.51	1.67	2.25	1.94	2.14	1.07	6.57	5.05
12428	1146	1	0	2.84	2.02	2.24	2.25	1.80	2.15	2.13	3.07	3.63	3.31
12433	1147	0	2	2.23	2.23	1.11	1.91	1.37	2.14	0.77	0.37	3.04	4.60
12434	1147	0	0	2.25	2.23	1.55	2.04	1.54	2.15	1.74	2.80	2.31	3.95
12435	1147	0	2	2.29	1.93	1.53	1.78	0.91	1.58	1.13	1.22	4.17	4.94
12436	1147	0	2	2.30	1.76	1.12	2.01	1.02	2.09	0.98	4.73	3.61	4.54
12437	1147	0	0	1.99	2.27	1.40	2.35	1.61	2.03	1.69	2.66	2.07	4.09
12438	1147	0	0	2.08	1.51	1.60	1.91	1.11	2.10	2.02	2.45	1.92	4.34
12439	1147	0	1	2.20	2.45	1.52	2.02	1.54	2.12	1.86	2.17	1.75	3.92
12440	1147	1	1	2.13	1.35	2.02	1.97	1.21	2.40	1.93	2.42	1.83	4.50
12460	1149	0	1	2.82	1.12	2.39	0.92	1.88	1.58	2.21	2.24	3.23	4.30
12461	1149	0	0	2.65	1.10	2.12	0.78	2.43	1.32	2.52	2.40	3.67	3.96
12462	1149	0	1	2.91	1.29	2.28	1.31	2.10	1.61	2.32	2.86	2.03	4.63
12464	1149	0	1	2.52	1.26	2.36	1.18	2.15	1.82	2.27	1.99	4.31	3.58
12465	1149	0	1	2.95	1.26	1.74	1.35	2.27	1.84	2.02	2.60	3.46	3.54
12467	1149	1	1	2.43	1.22	2.28	1.02	2.09	1.61	2.31	2.18	3.69	3.63
12468	1149	1	1	2.70	1.52	2.27	1.30	2.64	1.41	3.16	2.61	4.21	4.65
12469	1149	0	2	2.96	1.62	2.00	0.93	2.01	1.38	1.58	3.31	4.88	4.34
12474	1150	0	2	2.46	2.23	1.40	1.96	1.05	1.83	1.14	3.11	4.73	4.79
12475	1150	0	1	2.12	1.44	1.90	1.39	1.24	1.22	2.81	2.69	2.87	4.53
12476	1150	0	1	2.40	2.29	1.81	2.19	1.13	2.08	2.39	2.94	2.64	5.10
12477	1150	0	1	2.56	2.45	0.75	2.03	1.37	2.48	2.83	3.06	3.34	3.79
12478	1150	1	0	2.79	2.27	1.61	2.08	1.52	1.70	2.46	3.26	2.95	3.45
12481	1150	0	0	2.62	2.04	1.74	1.57	1.54	1.83	1.68	2.48	3.25	4.10
12483	1150	1	1	3.04	2.63	1.57	1.71	1.63	2.29	2.52	3.15	3.86	4.22
12485	1150	1	2	2.78	1.15	1.49	1.93	1.38	2.03	0.89	1.58	5.72	4.88
12487	1151	0	0	3.05	1.41	1.56	1.88	1.61	1.85	.	.	.	.
12488	1151	0	1	2.36	1.40	1.86	1.85	2.03	1.77	.	.	.	.
12489	1151	0	1	2.58	1.63	2.22	1.73	2.18	1.25	.	.	.	.
12490	1151	0	1	3.12	1.22	2.08	2.05	1.37	1.46	.	.	.	.
12492	1151	0	2	2.21	1.51	1.71	1.67	1.03	1.98	.	.	.	.
12494	1151	1	0	2.51	1.45	1.58	1.86	1.70	1.85	.	.	.	.
12495	1151	1	2	2.48	1.49	2.09	1.90	1.23	1.88	.	.	.	.

12496	1151	1	0	2.07	1.75	1.95	2.35	1.77	2.05	.	.	.	.
12498	1152	0	2	1.49	2.08	1.25	1.36	1.36	1.70	.	.	.	.
12499	1152	0	0	1.51	1.19	1.79	1.70	1.15	2.38	.	.	.	.
12500	1152	0	1	1.82	1.18	1.61	1.93	1.35	2.59	.	.	.	.
12504	1152	1	2	2.12	1.52	1.67	1.53	0.89	1.83	.	.	.	.
12505	1152	1	1	1.56	1.16	1.79	1.80	1.48	2.19	.	.	.	.
12506	1152	1	0	2.22	1.27	1.68	2.69	0.88	3.01	.	.	.	.
12507	1152	1	0	2.07	1.31	1.62	2.23	1.25	3.16	.	.	.	.
12509	1152	1	2	2.21	0.89	1.82	1.75	1.29	1.53	.	.	.	.
12511	1153	0	2	1.62	0.90	0.65	0.66	1.00	0.68	.	.	.	.
12512	1153	0	1	1.71	0.75	0.80	1.45	0.92	1.05	.	.	.	.
12513	1153	0	0	1.35	0.87	1.23	0.93	2.02	2.18	.	.	.	.
12514	1153	0	2	1.52	1.05	1.01	1.42	1.24	1.42	.	.	.	.
12515	1153	0	1	1.56	0.48	0.82	1.05	1.88	2.00	.	.	.	.
12516	1153	0	1	1.30	1.16	0.84	0.93	1.33	1.43	.	.	.	.
12517	1153	0	0	1.17	0.87	1.19	1.52	2.77	1.81	.	.	.	.
12519	1153	1	1	1.00	1.09	0.63	1.57	1.30	1.57	.	.	.	.
12554	1157	0	2	1.78	1.71	2.12	1.50	1.19	1.34	.	.	.	.
12556	1157	0	1	2.01	1.38	1.87	1.21	1.02	2.36	.	.	.	.
12557	1157	0	0	2.67	1.46	1.36	1.65	0.87	2.20	.	.	.	.
12563	1157	1	0	1.52	2.08	2.19	1.53	1.22	2.30	.	.	.	.
12564	1157	0	0	1.88	1.65	1.77	1.44	1.43	1.50	.	.	.	.
12565	1157	1	2	1.65	2.03	1.71	1.91	0.96	1.85	.	.	.	.
12566	1157	1	0	2.19	1.87	1.79	1.74	1.11	1.73	.	.	.	.
12567	1157	1	0	2.09	1.69	1.97	1.46	1.47	1.97	.	.	.	.
12608	1162	0	0	1.64	1.78	0.67	1.46	1.40	1.47	.	.	.	.
12609	1162	0	0	2.23	1.11	1.29	1.30	1.39	1.87	.	.	.	.
12610	1162	0	2	2.16	2.04	0.80	1.49	1.21	2.06	.	.	.	.
12611	1162	0	0	2.15	2.00	0.70	1.55	0.79	1.76	.	.	.	.
12614	1162	1	0	1.85	2.15	1.07	1.67	1.22	2.15	.	.	.	.
12616	1162	1	2	1.89	2.31	0.59	1.46	0.89	1.38	.	.	.	.
12617	1162	1	0	2.27	1.56	0.98	1.46	1.32	1.51	.	.	.	.
12618	1162	1	2	2.49	1.73	1.18	1.30	1.24	1.76	.	.	.	.
12633	1164	0	1	2.84	1.50	0.95	2.76	1.74	1.64	3.64	2.48	2.79	4.49
12635	1164	0	1	2.75	1.44	1.28	2.42	1.94	1.42	2.28	2.32	2.77	3.42
12637	1164	0	1	2.67	1.89	1.46	2.24	1.71	2.91	2.36	2.72	3.74	4.24
12638	1164	1	1	2.58	1.67	1.43	2.49	1.92	2.09	3.05	2.03	3.43	3.67
12639	1164	1	2	2.23	1.52	1.41	2.50	1.69	1.33	2.70	2.09	3.54	3.54
12640	1164	1	1	3.01	1.87	1.52	2.40	2.09	2.51	3.64	2.86	3.61	3.55
12641	1164	1	0	2.55	1.82	1.47	2.17	2.03	1.90	3.32	2.39	2.35	4.54
12644	1165	0	1	3.47	1.66	2.25	1.37	2.12	2.63	3.06	2.15	2.03	3.54
12646	1165	1	0	3.02	2.06	2.05	2.29	3.38	2.48	4.08	2.50	2.83	3.10
12647	1165	0	0	2.78	2.33	2.40	1.57	2.44	2.37	3.27	2.35	3.06	2.95
12649	1165	1	1	3.29	2.35	2.34	1.97	2.53	3.15	3.62	2.32	3.26	3.52

```
12650 1165 1 2 2.76 2.37 1.94 2.05 2.16 2.95 4.25 4.14 3.68 4.45
12651 1165 1 2 2.63 2.01 1.82 2.28 1.97 2.79 3.80 4.65 3.43 4.07
12652 1165 1 0 3.30 2.21 2.18 1.97 2.76 3.05 3.04 1.98 2.72 2.50
12655 1166 0 1 2.76 1.35 1.81 2.42 1.57 1.55 2.96 2.10 3.64 3.51
12656 1166 0 2 2.12 1.29 1.96 2.13 1.69 1.80 3.68 2.95 4.71 3.21
12657 1166 0 1 2.78 1.36 1.73 2.47 1.23 2.35 2.88 1.54 3.88 3.51
12658 1166 0 1 2.78 1.28 2.14 1.91 0.80 2.64 2.35 1.77 3.49 2.52
12662 1166 1 0 2.96 1.63 1.68 2.54 2.28 2.03 2.95 1.76 4.24 2.71
12663 1166 1 0 2.39 1.81 0.96 2.43 1.36 1.95 3.20 1.92 3.06 3.13
12664 1166 1 1 2.36 1.58 2.14 1.62 2.26 1.87 3.12 2.08 3.50 3.28
12665 1166 1 2 2.75 1.48 1.90 1.93 1.37 2.27 3.06 3.56 5.62 3.86
12667 1167 0 2 1.62 1.13 2.19 1.92 1.41 1.84 2.20 3.79 4.11 .
12668 1167 0 2 2.09 1.45 2.45 2.17 1.78 1.74 2.92 4.91 3.64 .
12669 1167 1 1 1.83 1.57 1.70 2.59 1.74 2.04 3.51 3.36 3.23 .
12670 1167 1 0 1.45 1.70 2.59 2.48 2.53 2.98 3.08 2.85 2.69 .
12671 1167 1 1 2.00 1.35 2.90 2.56 2.21 3.50 3.62 3.58 3.68 .
12672 1167 1 1 2.24 1.80 2.90 2.49 2.56 2.24 3.12 3.03 3.01 .
12675 1167 1 0 1.82 1.24 2.79 2.14 1.77 2.31 3.71 2.83 3.79 2.79
12676 1167 1 2 2.29 1.92 2.82 1.93 1.67 2.12 3.20 3.20 5.33 3.23
12789 1178 0 1 1.97 1.40 1.02 1.74 1.00 3.53 1.87 3.53 4.69 2.57
12790 1178 0 0 1.84 2.08 0.76 1.40 0.96 2.23 3.10 3.02 4.05 3.06
12791 1178 0 1 2.04 0.99 1.03 1.53 0.47 2.15 2.22 3.08 4.51 3.04
12792 1178 0 2 2.40 1.21 1.10 1.54 1.17 1.78 1.68 2.07 6.03 4.20
12793 1178 0 2 1.54 1.19 0.72 1.43 0.51 2.11 0.83 4.43 2.74 4.31
12795 1178 0 0 1.84 1.23 1.14 1.37 0.73 2.11 1.66 2.22 4.39 3.45
12797 1178 1 0 2.58 1.63 0.62 1.66 1.08 2.27 2.52 3.54 3.63 3.82
12799 1178 1 2 2.04 1.31 1.14 1.83 0.08 1.69 0.37 2.42 4.32 4.71
```

Program 2. Tissue weights data analysis.

```
libname XX 'c:/SASRUNS/XX';
   options linesize = 110; options pagesize = 20000;
   options nocenter;  options nodate; options nonumber; run;
   data one;
infile 'c:/SASRUNS/ArrayGroup/tissues.txt' FIRSTOBS=2;
input id lit sex geno age body ing stom empty contents liver;
run;
proc mixed method=ml scoring=150 info update itdetails;
  class id lit sex geno age;
  model body=geno|sex|age/noint;
  random lit/ group=sex;
  lsmeans geno*age /diff slice=age;
run;
proc mixed method=ml scoring=150 info update itdetails;
```

```
  class id lit sex geno age;
  model ing=geno|sex|age/noint;
  random lit/ group=sex;
  lsmeans geno*age /diff slice=age;
run;
proc mixed method=ml scoring=150 info update itdetails;
  class id lit sex geno age;
  model stom=geno|sex|age/noint;
  random lit/ group=sex;
  lsmeans geno*age /diff slice=age;
run;
proc mixed method=ml scoring=150 info update itdetails;
  class id lit sex geno age;
  model empty=geno|sex|age/noint;
  random lit/ group=sex;
  lsmeans geno*age /diff slice=age;
run;
proc mixed method=ml scoring=150 info update itdetails;
  class id lit sex geno age;
  model contents=geno|sex|age/noint;
  random lit/ group=sex;
  lsmeans geno*age /diff slice=age;
run;
proc mixed method=ml scoring=150 info update itdetails;
  class id lit sex geno age;
  model liver=geno|sex|age/noint;
  random lit/ group=sex;
  lsmeans geno*age /diff slice=age;
run;
```

Dataset 3. Tissue weights.

id	litter	sex	genotype	age	body	inguinal	stom	empty	content	liver
12380	1142	0	0	24	45.71	0.600	0.639	0.372	0.267	1.630
12381	1142	0	2	24	48.97	1.261	2.100	0.344	1.756	2.034
12382	1142	0	2	24	50.25	1.397	1.852	0.569	1.283	2.086
12383	1142	0	0	24	47.51	0.623	1.406	0.317	1.089	1.617
12384	1142	0	2	24	44.81	0.965	2.183	0.337	1.846	1.956
12385	1142	0	0	24	45.43	0.603	1.194	0.313	0.881	1.550
12386	1142	0	2	24	48.04	1.170	2.542	0.342	2.200	2.027
12387	1142	0	0	24	47.57	0.530	1.515	0.466	1.049	1.648
12418	1146	0	0	24	48.44	0.315	1.846	0.396	1.450	1.411
12419	1146	0	2	24	57.03	1.467	3.185	0.502	2.683	2.741
12420	1146	0	2	24	54.92	1.276	2.316	0.520	1.796	2.268

12421	1146	0	0	24	51.22	0.735	1.453	0.406	1.047	1.946
12423	1146	0	2	24	53.40	1.385	2.428	0.387	2.041	2.371
12425	1146	0	0	24	50.26	0.765	1.628	0.420	1.208	1.760
12427	1146	1	2	24	56.92	1.354	3.353	0.423	2.930	2.604
12428	1146	1	0	24	53.81	0.666	1.258	0.388	0.870	2.181
12433	1147	0	2	24	40.76	0.904	1.313	0.343	0.970	1.780
12434	1147	0	0	24	47.32	0.610	1.259	0.460	0.799	1.884
12435	1147	0	2	24	47.10	1.264	2.071	0.357	1.714	2.160
12436	1147	0	2	24	50.80	1.198	2.218	0.370	1.848	2.333
12437	1147	0	0	24	48.59	0.610	1.326	0.378	0.948	1.997
12438	1147	0	0	24	45.41	0.555	1.399	.394	1.005	1.600
12461	1149	0	0	24	50.54	0.542	1.690	0.399	1.291	2.017
12469	1149	0	2	24	52.29	1.352	2.043	0.398	1.645	2.464
12474	1150	0	2	24	50.50	1.183	1.302	0.392	0.910	2.376
12478	1150	1	0	24	49.13	0.540	0.961	0.650	0.311	1.903
12481	1150	0	0	24	48.01	0.225	1.658	0.388	1.270	2.011
12485	1150	1	2	24	49.44	0.988	2.767	0.389	2.378	2.376
12487	1151	0	0	20	36.42	0.316	1.284	0.266	1.018	1.212
12492	1151	0	2	20	32.86	0.770	0.975	0.130	0.845	1.026
12494	1151	1	0	20	36.30	0.302	1.222	0.234	0.988	1.300
12495	1151	1	2	20	37.70	0.838	0.750	0.251	0.499	1.220
12498	1152	0	2	20	35.30	0.829	1.055	0.452	0.603	1.244
12499	1152	0	0	20	37.12	0.322	1.013	0.275	0.738	1.270
12504	1152	1	2	20	38.90	0.856	1.122	0.275	0.847	1.330
12506	1152	1	0	20	38.43	0.339	1.258	0.379	0.879	1.359
12507	1152	1	0	20	38.52	0.361	1.318	0.268	1.050	1.368
12509	1152	1	2	20	36.31	0.834	1.215	0.268	0.947	1.324
12511	1153	0	2	20	30.13	0.558	0.728	0.210	0.518	0.939
12513	1153	0	0	20	32.89	0.217	1.117	0.254	0.863	1.079
12514	1153	0	2	20	32.99	0.660	1.118	0.256	0.862	0.987
12517	1153	0	0	20	32.92	0.296	1.433	0.267	1.166	1.206
12554	1157	0	2	20	35.52	0.820	0.641	0.230	0.411	1.242
12557	1157	0	0	20	33.76	0.367	1.004	0.260	0.744	1.189
12563	1157	1	0	20	34.32	0.360	1.116	0.228	0.888	1.197
12564	1157	0	0	20	33.30	0.314	0.913	0.238	0.675	1.141
12565	1157	1	2	20	32.59	0.616	0.970	0.204	0.766	1.107
12566	1157	1	0	20	34.14	0.337	1.119	0.493	0.626	1.108
12567	1157	1	0	20	34.88	0.350	1.088	0.224	0.864	1.241
12610	1162	0	2	20	35.26	0.755	1.169	0.224	0.945	1.224
12611	1162	0	0	20	32.55	0.292	1.056	0.268	0.788	0.199
12614	1162	1	0	20	33.68	0.244	1.384	0.241	1.143	1.178
12616	1162	1	2	20	35.28	0.758	1.079	0.291	0.788	1.279
12617	1162	1	0	20	35.04	0.293	1.395	0.259	1.136	1.257
12618	1162	1	2	20	34.62	0.706	1.048	0.209	0.839	1.102

12639	1164	1	2	24	48.95	1.406	1.869	0.326	1.543	1.824
12641	1164	1	0	24	48.18	0.566	1.831	0.380	1.451	1.977
12646	1165	1	0	24	55.86	0.903	2.059	0.375	1.684	2.073
12650	1165	1	2	24	59.25	1.824	2.833	0.415	2.418	2.640
12651	1165	1	2	24	58.33	1.816	2.331	0.443	1.888	2.584
12652	1165	1	0	24	53.97	0.849	1.669	0.384	1.285	2.131
12662	1166	1	0	24	48.95	0.679	1.876	0.365	1.511	2.009
12665	1166	1	2	24	56.04	1.459	2.853	0.406	2.447	2.558
12675	1167	1	0	24	52.06	0.630	0.853	0.426	0.427	1.960
12676	1167	1	2	24	55.34	1.358	0.851	0.396	0.455	2.387

Program 3. RT-PCR expression data analysis.

```
libname XX 'c:/SASRUNS/XX';
   options linesize = 110; options pagesize = 20000;
   options nocenter;  options nodate; options nonumber; run;
   data one;
infile 'c:/SASRUNS/ArrayGroup/RTPCRdata.txt' FIRSTOBS=2;
input id  lit  geno  age  FAS PPARG;
run;
proc mixed;
  class id lit geno age;
  model FAS=geno|age/noint;
  lsmeans geno*age /diff slice=age;
run;
proc mixed;
  class id lit geno age;
  model PPARG=geno|age/noint;
  lsmeans geno*age /diff slice=age;
run;
```

Dataset 3. RTPCR data.

id	litter	sex	genotype	age	FAS	PPAR
12380	1142	F	0	24	0.0819	0.9250
12381	1142	F	2	24	4.0527	3.0227
12382	1142	F	2	24	7.3692	2.5564
12383	1142	F	0	24	0.1063	1.0940
12384	1142	F	2	24	4.9019	2.3369
12385	1142	F	0	24	0.2579	1.0060
12418	1146	F	0	24	0.1255	0.8071
12419	1146	F	2	24	4.9180	2.7210
12420	1146	F	2	24	4.7169	1.7536

12421	1146	F	0	24	0.3735	1.4516
12423	1146	F	2	24	4.4104	2.8401
12425	1146	F	0	24	0.1835	1.2530
12427	1146	M	2	24	1.0747	1.3852
12428	1146	M	0	24	0.3864	1.0693
12478	1150	M	0	24	0.6639	3.1742
12485	1150	M	2	24	4.0651	2.6825
12487	1151	F	0	20	0.0602	0.6076
12492	1151	F	2	20	0.1301	1.1572
12494	1151	M	0	20	0.0630	0.6706
12495	1151	M	2	20	0.1018	0.8918
12498	1152	F	2	20	0.3995	1.0887
12499	1152	F	0	20	0.1347	0.6801
12504	1152	M	2	20	0.1049	0.5714
12506	1152	M	0	20	0.2102	0.5209
12507	1152	M	0	20	0.1627	0.7350
12509	1152	M	2	20	0.1832	0.9311
12511	1153	F	2	20	0.0905	0.4755
12513	1153	F	0	20	0.1316	0.5074
12514	1153	F	2	20	0.1483	0.6598
12554	1157	F	2	20	0.2139	0.6687
12557	1157	F	0	20	0.1286	0.4705
12610	1162	F	2	20	.	0.2239
12611	1162	F	0	20	0.0707	0.4720
12614	1162	M	0	20	0.0943	0.6170
12616	1162	M	2	20	0.1068	1.2944
12617	1162	M	0	20	0.0806	0.4510
12618	1162	M	2	20	0.1405	1.0534
12639	1164	M	2	24	0.8485	1.1207
12641	1164	M	0	24	0.4359	0.7876
12646	1165	M	0	24	0.0842	0.4380
12651	1165	M	2	24	2.8248	1.4908
12652	1165	M	0	24	0.1927	1.1696
12662	1166	M	0	24	0.2481	0.6819
12665	1166	M	2	24	2.1343	1.1220
12675	1167	M	0	24	0.3783	0.5053
12676	1167	M	2	24	1.7615	0.5233

Program 4. Data analysis of developmental changes in FAS protein expression.

```
libname XX 'c:/SASRUNS/XX';
```

```
   options linesize = 110; options pagesize = 20000;
   options nocenter;  options nodate; options nonumber; run;
   data one;
infile 'c:/SASRUNS/WESTERNS/FAS3day2.txt' FIRSTOBS=2;
input age sex $ pheno $ blot OD;
run;
proc mixed;
  class age pheno blot;
  model OD=age|pheno;
  random blot/group= age;
  lsmeans pheno*age/slice = age;
run;
```

Dataset 4. Optical densities.

21	M	L	1	0.0118044
21	M	O	1	0
21	M	L	2	0.056297
21	M	O	2	0.079956
21	F	L	3	0.0129866
21	F	O	3	0
21	M	L	4	0.187917
21	M	O	4	0.0655556
21	F	L	5	0.0582721
21	F	O	5	0.156208
28	M	L	1	0.125252
28	M	O	1	0.365409
28	M	L	2	0.25652
28	M	O	2	0.55606
28	F	L	3	0.0807006
28	F	O	3	0.0977968
28	M	L	4	0.261495
28	M	O	4	0.511643
28	F	L	5	0.273566
28	F	O	5	0.491966
60	M	L	1	0.270552
60	M	O	1	0.646922
60	M	L	2	0.0744017
60	M	O	2	0.314155
60	F	L	3	0.0215855
60	F	O	3	0.0844441
60	M	L	4	0
60	M	O	4	0.0482319

60	F	L	5	0.0656534
60	F	O	5	0.416643

Program 5. Data analysis of developmental changes in PPAR γ protein expression.

```
libname XX 'c:/SASRUNS/XX';
   options linesize = 110; options pagesize = 20000;
   options nocenter;  options nodate; options nonumber; run;
   data one;
infile 'c:/SASRUNS/WESTERNS/PPAR3ages.txt' FIRSTOBS=1;
input age sex $ pheno $ blot OD;
run;
proc mixed;
  class age pheno blot;
  model OD=age|pheno;
  random blot/group= age;
  lsmeans pheno*age/slice = age;
run;
```

Dataset 5. Optical densities.

21	M	L	1	0
21	M	O	1	0
21	F	L	2	0
21	F	O	2	0
21	F	L	3	0.104963
21	F	O	3	0.124392
21	F	L	4	0.123372
21	F	O	4	0.052176
21	F	L	5	0.084525
21	F	O	5	0.005445
21	F	L	6	0.01687
21	F	O	6	0.023101
21	M	L	6	0
21	M	O	6	0.111621
21	M	L	7	0.0682472
21	M	O	7	0.041754
28	M	L	1	0.047868
28	M	O	1	0.3406
28	F	L	2	0
28	F	O	2	0.072445
28	F	L	3	0.112018

28	F	O	3	0.342657
28	F	L	4	0.040115
28	F	O	4	0.089164
28	F	L	5	0
28	F	O	5	0.17479
28	F	L	6	0.111621
28	F	O	6	0.114884
28	M	L	6	0.171476
28	M	O	6	0.226076
28	M	L	7	0.07749
28	M	O	7	0.246342
60	M	L	1	0.279382
60	M	O	1	0.406315
60	F	L	2	0.022131
60	F	O	2	0.121289
60	F	L	3	0.228799
60	F	O	3	0.23975
60	F	L	4	0.050606
60	F	O	4	0.07755
60	F	L	5	0
60	F	O	5	0.1232
60	F	L	6	0.038552
60	F	O	6	0.068248
60	M	L	6	0.107077
60	M	O	6	0.264347
60	M	L	7	0.257442
60	M	O	7	0.469979

Program 5. Data analysis of FAS protein expression relative to the onset of hyperphagia.

```
libname XX 'c:/SASRUNS/XX';
   options linesize = 110; options pagesize = 20000;
   options nocenter;  options nodate; options nonumber; run;
   data one;
infile 'c:/SASRUNS/WESTERNS/FASinterval2.txt' FIRSTOBS=1;
input age sex $ pheno $ blot OD;
run;
proc mixed;
  class age pheno blot;
  model OD=age|pheno;
  random blot/group= age;
  lsmeans pheno*age/slice = age;
```

run;

Dataset 5. Optical densities.

21	M	L	1	0.0141041
21	M	O	1	0.0226174
21	M	L	2	0.00829203
21	M	O	2	0
21	F	L	3	0.0222799
21	F	O	3	0.00403722
21	M	L	4	0
21	M	O	4	0
21	M	L	5	0.12937
21	M	O	5	0.330576
22	M	L	1	0.0257382
22	M	O	1	0.0325572
22	M	L	2	0
22	M	O	2	0.00594738
22	F	L	3	0
22	F	O	3	0
22	M	L	4	0
22	M	O	4	0.0203361
22	M	L	5	0.22741
22	M	O	5	0.337275
23	M	L	1	0.0215845
23	M	O	1	0.0250187
23	M	L	2	0.0113241
23	M	O	2	0.0606475
23	F	L	3	0.00531113
23	F	O	3	0
23	M	L	4	0
23	M	O	4	0.0015488
23	M	L	5	0.280486
23	M	O	5	0.419404
24	M	L	1	0.0325804
24	M	O	1	0.0404937
24	M	L	2	0.0746487
24	M	O	2	0.071483
24	F	L	3	0
24	F	O	3	0
24	M	L	4	0.0275443
24	M	O	4	0.266017
24	M	L	5	0.11132

24	M	O	5	0.169256
25	M	L	1	0.0299619
25	M	O	1	0.0719539
25	M	L	2	0.0363862
25	M	O	2	0.29378
25	F	L	3	0.0783712
25	F	O	3	0.0394461
25	M	L	4	0.087554
25	M	O	4	0.351943
25	M	L	5	0.179928
25	M	O	5	0.305041
26	M	L	1	0.0585733
26	M	O	1	0.122725
26	M	L	2	0.388048
26	M	O	2	0.470556
26	F	L	3	0.0584837
26	F	O	3	0.259117
26	M	L	4	0.116544
26	M	O	4	0.401669
26	M	L	5	0.201887
26	M	O	5	0.223659
27	M	L	1	0.0368975
27	M	O	1	0.0593664
27	M	L	2	0.130568
27	M	O	2	0.2877
27	M	L	4	0.00775261
27	M	O	4	0.0915266
27	M	L	5	0.262853
27	M	O	5	0.544017
28	M	L	1	0.0284616
28	M	O	1	0.328512
28	M	L	2	0.165723
28	M	O	2	0.4493
28	F	L	3	0.1544
28	F	O	3	0.367791
28	M	L	4	0.167394
28	M	O	4	0.480243
28	M	L	5	0.0299756
28	M	O	5	0.66078

Program 6. Data analysis of PPAR γ protein expression relative to the onset of hyperphagia.

```
libname XX 'c:/SASRUNS/XX';
```

```
   options linesize = 110; options pagesize = 20000;
   options nocenter;  options nodate; options nonumber; run;
   data one;
infile 'c:/SASRUNS/WESTERNS/PPARinterval2.txt' FIRSTOBS=1;
input age sex $ pheno $ blot OD;
run;
proc mixed;
  class age pheno blot;
  model OD=age|pheno;
  random blot/group= age;
  lsmeans pheno*age/slice = age;
run;
```

Dataset 6. Optical densities.

21	M	L	1	0
21	M	O	1	0.0247159
21	M	L	2	0
21	M	O	2	0
21	M	L	3	0.029348
21	M	O	3	0.00470846
21	F	L	4	0.000710212
21	F	O	4	0.0224767
21	F	L	5	0.269699
21	F	O	5	0.303174
21	F	L	6	0.0934748
21	F	O	6	0.101685
21	F	L	7	.
21	F	O	7	.
22	M	L	1	0
22	M	O	1	0.0141936
22	M	L	2	0
22	M	O	2	0.00132742
22	M	L	3	0.0342412
22	M	O	3	0.0432273
22	F	L	4	0.0574618
22	F	O	4	0.0496434
22	F	L	5	0.275433
22	F	O	5	0.141537
22	F	L	6	0.0816291
22	F	O	6	0.0479148
22	F	L	7	.
22	F	O	7	.
23	M	L	1	0.0425565

23	M	O	1	0.0291472
23	M	L	2	0.00166817
23	M	O	2	0.00794407
23	M	L	3	0.026836
23	M	O	3	0.076076
23	F	L	4	0.0126551
23	F	O	4	0.0122281
23	F	L	5	0.157849
23	F	O	5	0.0587771
23	F	L	6	0.0699772
23	F	O	6	0.0288646
23	F	L	7	.
23	F	O	7	.
24	M	L	1	0
24	M	O	1	0
24	M	L	2	0.0505263
24	M	O	2	0.108164
24	M	L	3	0.0623579
24	M	O	3	0.150528
24	F	L	4	0.0316361
24	F	O	4	0.0746361
24	F	L	5	0.0767623
24	F	O	5	0
24	F	L	6	0.0554279
24	F	O	6	0.0229594
24	F	L	7	.
24	F	O	7	.
25	M	L	1	0
25	M	O	1	0
25	M	L	2	0.0255029
25	M	O	2	0.0109833
25	M	L	3	0.0121242
25	M	O	3	0.0647869
25	F	L	4	0
25	F	O	4	0
25	M	L	5	0.0687502
25	M	O	5	0.121474
25	M	L	6	0.104195
25	M	O	6	0.0381804
25	F	L	7	0.0347957
25	F	O	7	0.0564576
26	M	L	1	0
26	M	O	1	0.0428774
26	M	L	2	0

26	M	O	2	0.00703042
26	M	L	3	0.0504534
26	M	O	3	0.0904056
26	F	L	4	0
26	F	O	4	0
26	M	L	5	0.116526
26	M	O	5	0.0677189
26	M	L	6	0.0355528
26	M	O	6	0.0735313
26	F	L	7	0.0102024
26	F	O	7	0.0468601
27	M	L	1	0.00298176
27	M	O	1	0.196812
27	M	L	2	0
27	M	O	2	0
27	M	L	3	0.0727615
27	M	O	3	0.0713798
27	F	L	4	0
27	F	O	4	0.0208481
27	M	L	5	0.133009
27	M	O	5	0.230111
27	M	L	6	0.307325
27	M	O	6	0.355454
27	F	L	7	0.0468935
27	F	O	7	0.0899623
28	M	L	1	0.00681094
28	M	O	1	0.338871
28	M	L	2	0.00310534
28	M	O	2	0.101991
28	M	L	3	0.0302741
28	M	O	3	0.244235
28	F	L	4	0.0501374
28	F	O	4	0.235733
28	M	L	5	0.275609
28	M	O	5	0.33179
28	M	L	6	0.286908
28	M	O	6	0.546089
28	F	L	7	0.0770787
28	F	O	7	0.205225

VITA

Holiday Durham was born in Fort Belvoir, Virginia. She graduated from Clemson University in 2001, earning a Bachelor of Science degree in Food Science and Technology with an emphasis in Nutrition Science. Subsequently, she attended Vanderbilt University dietetic internship and is a Registered Dietitian.

After earning her Master of Science degree in Nutrition Science at the University of Tennessee, Knoxville, Holiday will begin pursuing her doctorate in the fall of 2004.