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The Characterization of Two Differentially Expressed Plasma Proteins in Obese Versus Lean Rats in Two Rodent Models of Obesity

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Abstract

Zucker fa and La/N fa^f rats are widely studied models of genetic obesity and its complications. These two rodent models of obesity were utilized to search for a circulating protein marker for obesity.

Plasma samples from both of these models of obesity were collected and analyzed via SDS-PAGE analysis. Two proteins were found which demonstrate differential expression between lean and obese rats. Both proteins demonstrated increased expression in the obese rats compared to the lean. One differentially expressed protein migrated on SDS-PAGE gels at 116 KD while the second migrated at 22 KD compared to molecular weight markers.

The 22 KD protein also exhibited differential expression in plasma samples obtained from lean Zucker rats. Preliminary results from genetic backcrossing analysis point to a possible gene dosage effect with this protein. This coupled with the finding that the differential expression of this protein occurs before the onset of obesity in these rats may possibly make this an important circulating marker for obesity.

These proteins were subjected to sequencing by Edman degradation. The protein migrating at 116 KD was identified as complement component C3 α chain, a pivotal member of the complement cascade, which is vital to the body's immune system. The protein migrating at 22 KD was identified as apolipoprotein A-I, an important member of the body's lipid transport system. Antibodies to these two proteins were purchased and the identities confirmed through Western blot analysis.

Complement component C3 α chain may be important as it could serve as a circulating reservoir for the formation of acylation stimulating protein (ASP). Apolipoprotein A-I is an important protein component of high density lipoprotein particles which are important in cholesterol and lipid transport.

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Introduction

All species, no matter to what genus and phylum they belong, face the problem of energy storage and long term survival. In the animal kingdom adipose tissue seems to be a universal adaptation for storing and transporting energy reserves. However, the form of this lipid-filled tissue varies much depending on the branch of the animal kingdom investigated. The invertebrates store lipids in the form of oil droplets, but only Arthropods possess a distinctly differentiated adipose tissue. Adipose tissue takes on different forms depending on the stage of an insect's life cycle. It is composed of an almost fluid body, yellowish or dull white in color, and during the larval stage may account for up to 90% of an insect's weight, decreasing in the imago and varying by class of insect in larval and adult form. Many insects also use their adipose tissue for the storage of waste products through their entire lifespan, or at least until metamorphosis (Vague & Fenasse, 1965).

Among the fishes, the liver is an extremely important adipose storage organ, with the liver of some sharks making up 25% of their body weight and containing upwards of 75% oil (Van Gaver, et al, 1976). Among the fishes, the liver may or may not play a role in lipid storage in both fish with lean meat and fish with fatty meat (Vague & Fenasse, 1965).

Amphibians do not have a great deal of adipose tissue, especially in subcutaneous tissue, as the skin is mostly attached loosely by connective tissue. Salamanders may have some adipose tissue around the gonads. Frogs have some adipose tissue contained in subcutaneous tissue in the chest and abdomen. Bone marrow in frogs also contains

adipose tissue, but mostly during hibernation (Vague & Fenasse, 1965; Komoroski, 1998).

In birds, the adipose tissue is very distinct and very well developed. The amount of adipose tissue among sea birds may be very large, as in some barnyard fowl, namely ducks and chickens. The adipose tissue among migratory birds such as geese tends to vary according to timing of the migration, with pre-migration metabolism mostly geared to storing fat. This storage of fat results not only from efficient metabolism, but also from hyperphagia (Dorst, 1968; Vague & Fenasse, 1965).

Adipose tissue is particularly well developed in mammals. Whales which live in arctic waters generally have up to 35% fat tissue, which is mostly used for insulation (Stini, 1981). The fat tissue in whales consists of subcutaneous tissue and varies between 3 and 50 cm in thickness (Van Gaver, 1976; Vague & Fenasse, 1965). Ruminants tend to store fat in preparation for long periods of deprivation. For example, the hump in the camel and the withers of the zebu tend to shrink during dry seasons and increase during rainy seasons when food is plentiful (Vague & Fenasse, 1965). Carnivorous animals usually keep very little adipose tissue. In rodents the adipose tissue is contained within the abdominal cavity in most instances. Some rodents have little fat, while others may contain a great deal. Many of the genetic models used to study obesity are from the rodent family, although these are mostly not wild type strains (Vague & Fenasse, 1965 ; Tschop & Heiman, 2001). In primates other than humans, subcutaneous tissue is atrophied. Humans are among the fattest of mammals with the leanest humans having around 10% of total body weight in adipose tissue, while values as high as 35% exist among the obese (Pitts, et al, 1969; Stini, 1981).

Humans and Obesity:

Throughout the history of humankind, one problem has consistently plagued the human race, the specter of famine. During humanity's early history, when humans were hunter-gatherers and the availability of food was not always certain, the ability to store energy to sustain the body during lean times was important. Therefore, humans evolved methods of storing energy, one of the main tools being adipose tissue. Fat tissue is dense, energy rich and compact so that vast amounts of energy can be stored in a small weight and volume.

In many industrialized countries today, humans have achieved a control over the food supply that is unparalleled in history. The advent of agriculture, followed by mass production and large company-owned farms using modern herbicides, irrigation and equipment, has done much to eliminate starvation and malnutrition in the industrial nations of the world and in many developing nations. Today, we live in a time when the production of foodstuffs, along with their storage and transportation to faraway consumers, ensures the greatest abundance of food in history.

While hunger still exists in the modern world, diseases of hypernutrition and excess body weight in many industrialized countries have replaced the disease of malnutrition. Today, according to the World Health Organization (WHO), almost 1 billion people worldwide are considered to be overweight or obese. Additionally, obesity-related disorders and conditions constitute a considerable health expense and are considered to be the largest threat to healthy populations in the future (WHO, 2000).

Obesity:

Obesity is defined as a significant increase over one's ideal body weight. Obesity, in its simplest terms, is a chronic condition characterized by the excess accumulation of body fat. The first studies to track the complications of obesity and excessive weight were done by the major life insurance companies of the United States. Done in the interest of minimizing risk to the life insurance industry, these studies resulted in factors used to assess risk due to overweight. One of the most famous products of these studies was the Metropolitan Life height and weight tables (Metropolitan Life Insurance Co, 1983). These tables were developed from data compiled over many years to form a picture of what weights were of lower risk to the individual and the company and what weights were of higher risk of early death. Accordingly, these weight risk factors, in addition to factors such as age and smoking and drinking, were used to decide the rates charged an individual for life insurance.

Today, there are several methods which can be used to accurately determine one's body fat content: however, they are expensive and not widely available. Typically, indirect indicators of overweight and obesity are used in the clinical setting. The two most widely used indirect measures are Body Mass Index (BMI) and Waist Circumference (NHLBI, 2001). Waist circumference, a measure of abdominal circumference at the level of the iliac crest, is very useful in estimating abdominal fat content. Generally, waist circumferences above 40 in. in men and above 35 in. in women are considered to be high risk. When used with BMI, waist circumference can provide an independent estimate of risk beyond that predicted by the BMI alone. Body Mass Index is calculated by taking an individual's weight in kilograms and dividing it by the square

of his/her height in meters squared. It is the method most frequently used to classify overweight and obesity in the clinic and in epidemiological studies. Tables are available that allow an individual to take his or her weight and height and find the corresponding BMI (NHLBI, 2001).

Studies, including the Framingham study, the National Health Examination Study (NHES I), National Health and Nutrition Examination Study (NHANES) II and III in the United States, along with other worldwide studies conducted over the last 40 years, have led the World Health Organization to establish guidelines for healthy weight, overweight, and obesity (NHLBI, 2001) (WHO, 1998). Under these classifications, normal weight is considered to be a BMI between 18.5 and 24.9, overweight is considered to be a BMI between 25 and 29.9 and obesity is considered to be a BMI of 30 and above. Under the latest guidelines obesity is further subdivided into 3 separate categories: Class 1 (30-34.9 kg/m^2) Class 2 (35-39.9 kg/m^2) and Class 3 ($\geq$ 40 kg/m^2).

Prevalence of Obesity:

Many epidemiological studies have been conducted to assess the prevalence of obesity in the United States (Flegal, et al, 1998; Mokdad, et al, 1998; Kuczmarski, et al, 1994; Garrison, et al, 1985). While all of these studies provide useful data concerning the level of obesity in general, the levels of obesity in men and women, the prevalence of obesity among different racial and ethnic groups, the incidence of obesity in adolescents and children, etc., there is often some disparity in the numbers from study to study. When examined collectively, however, these studies allow one to discern the trends in weight gain in the U.S. population over time. What is apparent from these studies is that

between the years 1960 and 1980 there was less than a 2% overall increase in the number of people considered to be obese or having a BMI greater than 30. After 1980, however, the number of people with a BMI of 30 or above rose sharply with a gain of more than 7% in men and 8% in women in just 14 years.

Some researchers studying trends of overweight and obesity in the 1990's have begun to describe the increase in obesity and its associated complications as an epidemic (Mokdad, et al, 1999). There have been many studies that link the increase in obesity to an increase in leisure activity (MMWR, 2000), a decrease in manual labor at work (Kuczmarski, et al, 1994), or an increase in the consumption of high caloric value food (NHLBI, 2001), or that analyze the genetic predisposition of the U.S. population toward overweight and obesity (Naggert, et al, 1997). One thing from this epidemiological literature is clear: obesity is becoming more prevalent. Mokdad and his colleagues analyzed data from all states that participated in the Behavioral Risk Factor Surveillance System (BRFSS) to determine what the trends for the prevalence of obesity were in the 1990's. The BRFSS was a telephone survey of adults over the age of 18 conducted by the Centers for Disease Control and Prevention and State health departments. It is apparent from the data collected that obesity is increasing at epidemic rates in the U.S. The prevalence of obesity, (BMI $\geq$ 30), increased from 12% in 1991 to 17.9% in 1998. The prevalence of obesity increased in every state, in both sexes and across age groups and races. The increase varied greatly by region and state, with Delaware showing the smallest increase of 11.3% and Georgia having the largest at 101.8% (Mokdad, et al, 1999).

While the data above may be at odds somewhat with the data from the last NHANES study, (NHANES III), it must be remembered that the BRFSS study is dependent on self-reporting of health conditions and weight. Because underreporting of weight and food consumption, and over reporting of activity and exercise is an issue, the numbers presented are probably lower than the actual levels of obesity. Therefore, the real levels of obesity are probably more in line with those reported in 1994 during NHANES III. Despite the differences on specific details, the data from the BRFSS do point out the trend of an increasing prevalence of obesity through the 1990s.

Complications of Obesity:

Obesity, while carrying a social stigma and a health risk in and of itself, also increases the risk for developing a wide variety of other diseases. Table I summarizes many of the known medical complications of obesity (Endocrinology and Metabolism, 1995; NHLBI, 2001).

Using data from NHANES III, researchers have assessed the prevalence of major obesity-associated co-morbidities such as Type II diabetes mellitus, gallbladder disease, coronary heart disease, high blood pressure, high blood cholesterol level and osteoarthritis, with regard to obesity class and gender (Must, et al, 1999). The prevalence of type 2 diabetes mellitus increased sharply in both overweight and obese men and women; moreover, the incidence of type 2 diabetes increased with increasing weight class (Must, et al, 1999). Likewise, the incidence of high blood pressure increased with increasing weight class category in both genders (Must, et al, 1999). High blood cholesterol was found to be more prevalent in overweight individuals than in normal or

Table I:

Medical Complications of Obesity:

Cardiovascular

- Coronary artery disease
- Myocardial infarction
- Congestive heart failure
- Sudden death
- Cerebrovascular accidents
- Hypertension
- Left ventricular hypertrophy

Metabolic

- Hyperlipidemia
- Insulin resistance
- Non-insulin-dependent diabetes mellitus
- Cholesterol gallstones

Cancer

- Males: colon, rectum, prostate
- Females: breast, ovary, endometrium, cervix, gallbladder, bile ducts

Hormonal

- Menstrual abnormalities
- Hyperandrogenism
- Hirsutism
- Acanthosis nigricans
- Polycystic ovaries
- Decreased sex hormone-binding globulin
- Increased estrogens
- Decreased testosterone in males
- Decreased growth hormone
- Decreased prolactin responsiveness
- Enhanced cortisol production

Rheumatic

- Osteoarthritis

Taken from: Endocrinology and Metabolism, third edition, Felig, et al, editors. McGraw-Hill, New York, 1995.

underweight individuals; however, there was not a corresponding increase in the blood cholesterol level with increasing weight classes above overweight. Prevalence ratios for coronary heart disease were not elevated in the overweight category, but were elevated for men with class I obesity and for women in all 3 obesity classes (Must, et al, 1999).

There is a strong difference in gallbladder disease by sex according to Must, et al, (1999), gallbladder disease is twice as prevalent in obese women as it is among obese men. Another co-morbidity, which is very sex-specific, is osteoarthritis. While osteoarthritis was shown to increase steadily for both men and women with increasing weight class, women showed much more prevalence for osteoarthritis in all weight categories including normal and underweight (Must, et al, 1999).

Obviously, obesity has much more of an impact on health than the direct effects of weight gain per se. The indirect effects of obesity together with its ability to cause or predispose one to develop other significant diseases may be far more damaging than obesity itself.

Deaths Due to Obesity:

How many obesity-related deaths occur in the United States each year? It is not enough to look at premature deaths due to obesity itself to answer this question. One has to consider also the premature deaths due to co-morbidities of obesity such as coronary heart disease, cancer, and diabetes. When the co-morbidities due to obesity are taken into account, the number becomes not only hard to calculate, but also very large. The number of deaths due to obesity has been cited as 300,000 per year by studies done on premature deaths from the year 1980 (Amler and Eddins, 1987). Allison and his

colleagues analyzed data from 6 different studies, (NHANES I, American Cancer Society Cancer Prevention Study I, the Tecumseh Community Health Study, the Framingham Heart Study, the Alameda Community Health Study, and the Nurses Health Study) to try to provide a more definitive answer to the question. The annual number of deaths which were estimated to be due to obesity varied by the study, but an average mean of the deaths from all 6 studies was 280,184, with a range of 236,111 to 341,153 (Allison, et al, 1999). The Nurses Health Study most closely followed the mean of the 6 studies. It demonstrates that at BMI's below 26, an individual's weight does not appear to be a factor in the death of the individual; however, at BMI's of 27 and above, an individual's weight class begins to affect length of life. This further strengthens the importance of controlling body weight in a population.

Obesity Worldwide:

Obesity isn't just a disease of the United States. Almost every industrialized or developing country is being affected by the obesity epidemic (WHO, 1998). Much of this is due to the increased stability and availability of high caloric value foodstuffs in these countries and to the increased automation and decreased manual labor of an industrialized economy. The average daily intake of energy in the world in 1963 was 2300 kcal per capita. This number increased to 2720 kcal in 1992 and is projected to increase to 2900 kcal per capita by 2010 (Seidell, 2000). While there are still many undernourished individuals in Africa, India and Asia, these numbers are declining in developing areas within these countries and many more people are gaining access to the nutritional levels required to become obese.

A World Health Organization (WHO) report has called obesity one of the most important contributors to ill health facing the world today (WHO, 1998). In addition, the World Health Organization has predicted a worldwide epidemic of type II diabetes mellitus: the number of people with this condition is expected to double from 143 million in 1997 to more than 300 million in 2025 (WHO, 1998). Additionally, it is predicted that the number of obese people worldwide will increase to roughly 300 million by the year 2025 (Seidell, 2000).

Economic Impact of Obesity:

Health risks associated with obesity have led to increased medical care and disability costs both in the US and abroad. In 1995, the total cost attributed to medical care and disability related to obesity was 99.2 billion dollars in the U.S. Of these $51.6 billion were direct costs, representing 5.7 percent of the total national health expenditure of the United States in 1995 (NHLBI, 2001). The indirect cost of obesity (lost worker output) was estimated to be $47.6 billion in 1995 in the United States alone (NHLBI, 2001). The overall drain on society is growing each year as the prevalence of obesity in the United States increases. There is no end in sight. In addition to cost, obesity leads to social discrimination, loss of performance and opportunities, as well as a decreased life span.

Background:

In order to maintain a constant body weight once growth has ceased, there must be a balance between energy intake and energy output. The weight gain in an average woman over a 40 year time period is roughly 11 kilograms, which calculates to an average error in energy balance of just 350 milligrams in daily food intake and represents an error of less than 0.03% (Martin, et al, 1991). To understand how the body can manage this balance, it is necessary to study how the body handles energy.

The body has three main sources of stored energy, carbohydrates stored in the form of glycogen, proteins stored in the form of muscle tissue and fat stored as triacylglycerols in adipose tissue. Fat is the most important of these. Metabolic oxidation of carbohydrates and proteins yields about 17 kJ/g, whereas the oxidation of triacylglycerols yields around 37 kJ/g (Matthews & Van Holde, 1995). This difference in energy yield is made even more disparate by the fact that triacylglycerol stored in fat tissue is very dense and anhydrous, allowing much greater amounts to be stored in small volumes. By contrast, glucose polymers are very hydrophilic with each gram of glycogen binding about 2 grams of water. Thus it is estimated that fat has 6 times the caloric content by weight compared to carbohydrate (Williams & Van Holde, 1995). The average 70 kg human has fat reserves of about 400,000 kJ and about 100,000 kJ in protein.

Fat in the body originates from two sources: fat ingested in the diet and fat manufactured by *de novo* biosynthesis in times of caloric excess. Both of these are important in the development of obesity and will therefore be explored in detail below.

Fat Digestion and Absorption:

The digestion and absorption of fat is hindered by the hydrophobic nature of triacylglycerols. In the lumen of the small intestine, lipids are emulsified by the action of bile salts, which have both a hydrophobic and a hydrophilic region. This makes lipids vulnerable to breakdown by water-soluble enzymes, the most important of which is pancreatic lipase. Through the action of this enzyme, lipids are hydrolyzed to a mixture of free fatty acids, β-monoacylglycerols, diacylglycerols and glycerol. These products are absorbed through the intestinal mucosal cells, where the triacylglycerols are resynthesized in the golgi complex and endoplasmic reticulum. Before secretion into the lymph system, the triacylglycerols are complexed with proteins to form chylomicrons. The hydrophobic triacylglycerols are on the inside of the chylomicron, whereas the proteins, phosphoglycerides, and free cholesterol form a hydrophilic skin which helps the chylomicron stay soluble in the blood and lymph systems while traveling to tissues in the body for metabolism or storage.

Fat Transport:

There are several groups of lipoproteins involved in the transport of lipids to different tissues and their compositions vary accordingly (Table II). Because lipids are of much lower density than proteins, the lipid content of a lipoprotein class is inversely related to its density. The standard lipoprotein classification includes, in increasing order of density: chylomicrons, very low-density lipoprotein (VLDL), low-density lipoprotein (LDL), intermediate-density lipoprotein (IDL), and high-density lipoprotein (HDL). All of these classes share some common features. Fats are transported in the bloodstream in

the form of lipoproteins. The apoproteins or free protein components of these complexes are manufactured mainly in the liver with some 20% being manufactured in the intestinal mucosal cells. There are distinct families of lipoproteins, each with a unique function in lipid transport. Lipoproteins in each class contain characteristic apoproteins and have distinctive lipid compositions (Table II).

Chylomicrons transport exogenously-derived lipids from the intestine to peripheral tissues, most notably heart, muscle, and adipose tissue. VLDL performs a comparable function for triacylglycerols that are synthesized in the liver. As lipids being carried in chylomicrons and VLDL are hydrolyzed on the capillary surfaces of peripheral organs, protein-rich remnants remain. This results in the formation of IDL's, which are taken up by the liver. These IDL's are then degraded in the liver with protein remnants such as apo-B-100 being used to synthesize LDL, the principle lipoprotein used to transport cholesterol synthesized in the liver to peripheral tissues. HDL's are the primary vehicle for the transport of excess cholesterol from the tissues back to the liver.

Fat Synthesis:

Triacylglycerols are synthesized in both the liver and in adipose cells. The triacylglycerols synthesized in adipose cells are mostly for storage while those synthesized in the liver are mostly for export to other tissues for energy utilization or to adipose tissue for storage. Failure to export lipid because of a reduced ability of the liver to synthesize apoproteins, as occurs in cirrhosis, results in fatty liver syndrome.

The human diet contains both fats and carbohydrates, however most of the stored energy in the human body is in the form of fat. The common link between carbohydrate

Table II:

Properties of major human plasma lipoprotein classes

	Chylomicron	VLDL	IDL	LDL	HDL
Density(s units)	<0.95	0.950-1.006	1.006-1.019	1.019-1.063	1.063-1.210
Components (% dry weight)					
Protein	2	8	15	22	40-50
Triacylglycerol	86	55	31	6	4
Free cholesterol	2	7	7	8	4
Cholesterol esters	3	12	23	42	12-20
Phospholipids	7	18	22	22	25-30
Apoprotein comp.	A-I, A-II B-48 C-I,C-II, C-III	B-100 C-I,C-II, C-III, E	B-100 C-I,C-II, C-III, E	B-100	A-I, A-II, C-I, C-II, C-III, D, E

Redrawn from: Biochemistry, Matthews and van Holde, 1996.

and fat metabolism is acetyl-CoA. While acetyl-CoA generated from pyruvate can be converted into fatty acids, significant net conversion of acetyl-CoA to carbohydrate does not occur.

The enzymes catalyzing the synthesis of fatty acids exist in a multifunctional complex composed of six molecules of two polypeptide chains each. This is a very efficient arrangement because substrates do not have to seek out catalytic sites but rather are brought into contact with them. This also prevents a buildup of intermediates in the cells which are actively synthesizing fatty acids.

The synthesis steps involved in fatty acid formation are remarkably similar to those involved in fatty acid digestion except that they occur in reverse with different

enzymes and coenzymes. The fatty acid synthesis reaction proceeds through stepwise additions of two-carbon units, with each step proceeding via condensation, reduction, dehydration, and another reduction (Figure 1). There is a need for an activated intermediate, malonyl-CoA, at each two-carbon addition step and the reductive enzymes require NADPH. Fatty acid synthesis exists in dynamic equilibrium with fatty acid oxidation in adipose tissue (Mathews, Van Holde, 1995).

Fat degradation:

The pathway for fatty acid oxidation was elucidated in 1904 in a set of experiments conducted by Franz Knoop. Dogs were fed fatty acids in which the terminal methyl group was attached to a phenyl group. When the dogs metabolized fatty acids, different products appeared in their urine depending on the number of carbons in the fatty acid chains. Even-numbered fatty acids formed phenylactic acid as a breakdown product while odd-numbered fatty acids formed benzoic acid as a breakdown product. This was interpreted to mean that the breakdown of fatty acids occurred in a stepwise manner at carbon 3 (the β carbon with respect to the carboxyl group) and released a two-carbon group with each oxidation cycle. In the 1940's it was discovered that fatty acid oxidation occurred in the mitochondria in an ATP-dependent reaction.

Fatty acids must be transported into the mitochondrial matrix for oxidation; however, the inner mitochondrial membrane is impermeable to free fatty acids. This is overcome by an ATP-powered transport mechanism carried out by fatty acyl-CoA ligases specific for long, medium or short-chain fatty acids. These ligases catalyze the formation of a fatty acyl thioester conjugate with coenzyme A in a two-step process involving the

cleavage of ATP to give AMP and pyrophosphate. The fatty acyl CoA is transferred to a carrier called carnitine on the outer membrane surface in a reaction catalyzed by carnitine acyltransferase I. This fatty-acyl-carnitine is transferred to the inner membrane in exchange for unbound carnitine by the enzyme carnitine acyltransferase II. Once inside the mitochondrion, the acyl carnitine is converted back to fatty acyl-CoA before oxidation by the β-oxidation pathway.

The β-oxidation pathway oxidizes fatty-acyl CoA's through a series of reactions which release a two carbon fragment in the form of acetyl-CoA with each cycle. Each cycle consists of four reactions: (1) dehydrogenation to an enoyl derivative; (2) hydration of the resultant double bond; (3) dehydrogenation of the hydroxyl group; and (4) cleavage by attack of a second molecule of coenzyme A on the β-carbon. With each cycle an acetyl –CoA is produced along with a fatty acyl-CoA which is shorter by two carbon units. The acetyl-CoA derived from the β-oxidation of fatty acids is further oxidized in the citric acid cycle, providing energy in the form of ATP and releasing CO_2 (Figure 2). The oxidation of unsaturated fatty acids requires additional steps and enzymes. Enoyl-CoA hydratase acts only on trans compounds while unsaturated fatty acids have double bonds which are in a cis configuration. The first additional enzyme, enoyl-CoA-isomerase must convert this cis double bond into a trans double bond before β-oxidation can continue. This enzyme comes into play when mono-unsaturated fatty acids are to be oxidized. When polyunsaturated fatty acids are to be oxidized, a different enzyme, 2,4-dienoyl-CoA-reductase, must be used to convert the cis double bonds to trans double bonds.

Figure 1: Fatty acid synthesis takes place in the liver and throughout the body in adipocytes. The reaction takes place on a multi-functional protein complex called acylation carrier protein (ACP). The fatty acid chain is built from acetyl-CoA units.

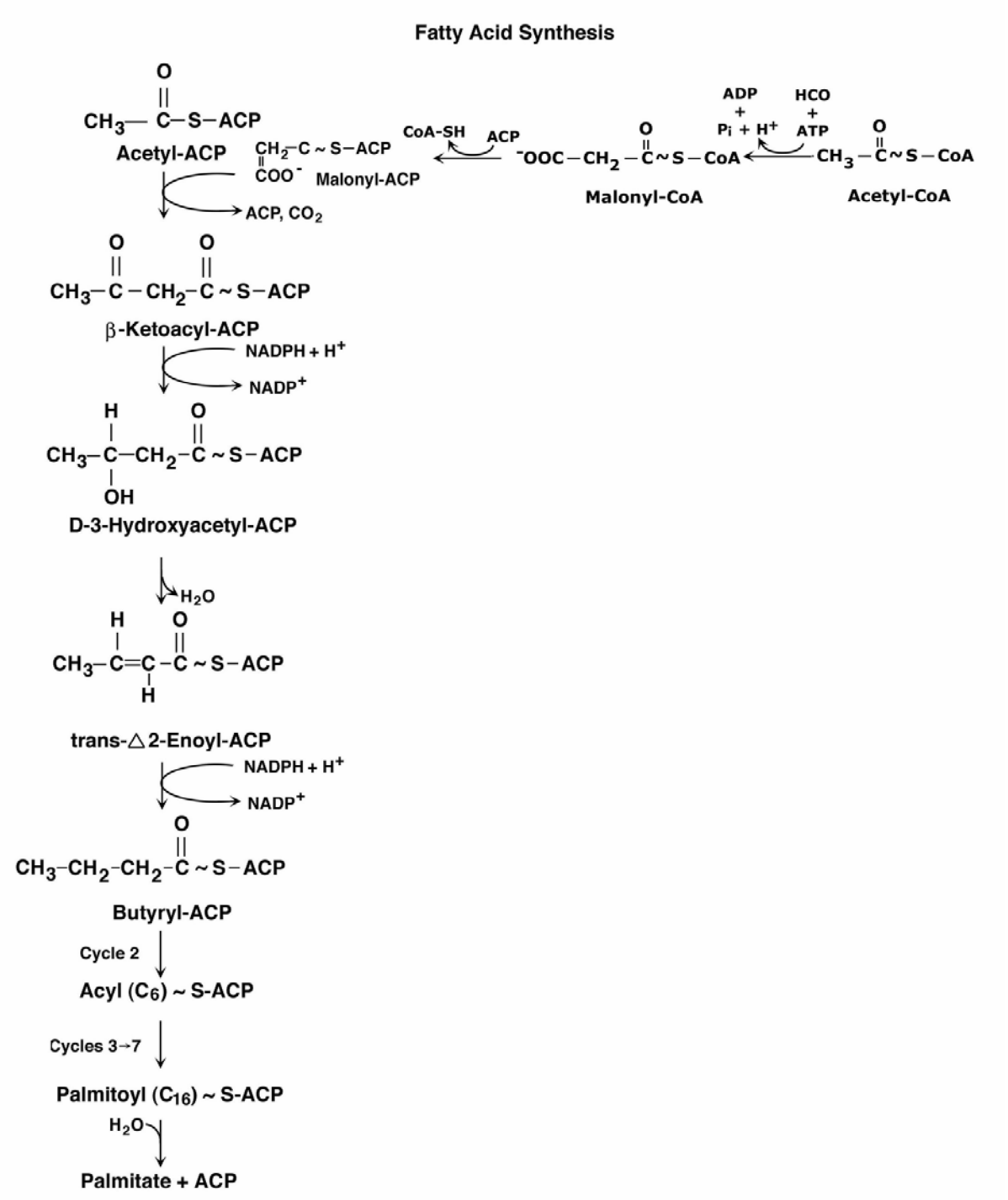
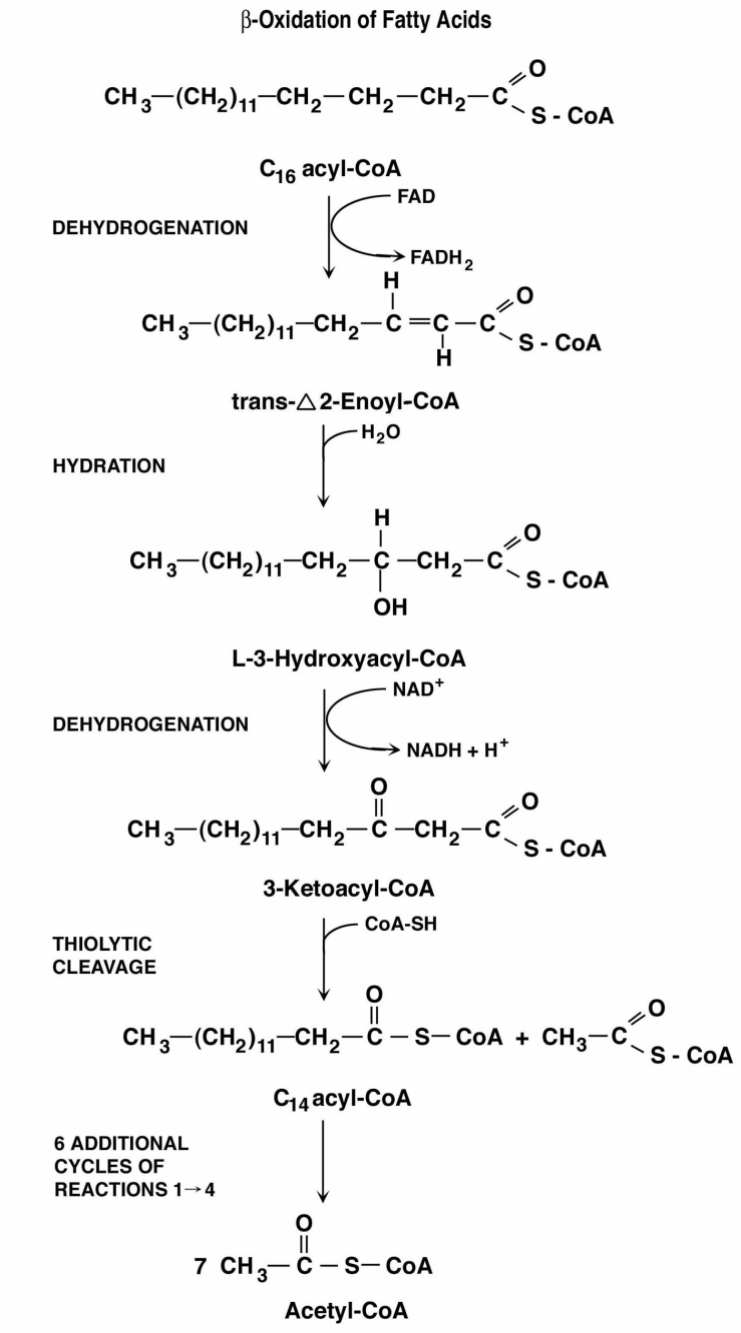


Figure 2: β-oxidation of fatty acids occurs inside mitochondria after the fatty acids have been transported into the mitochondria through an inter-membrane transport system using a carrier protein, carnitine acyl transferase. The final product of the oxidation of fatty acids is acetyl-CoA which is further metabolized to provide energy.



A small percentage of lipids contain odd-numbered carbon chains. In the β oxidation of these fatty acids, a 5-carbon homolog, β-ketopentanoyl-CoA, is formed at the last cycle and is subsequently split into an acetyl-CoA and a propionyl-CoA. Propionyl-CoA is further metabolized to succinyl-CoA at which point it enters the citric acid cyle and is metabolized for energy (Mathews, Van Holde, 1995).

The Adipocyte:

The triglyceride storage organ of the human body is composed of adipocytes. These specialized cells swell to hold triglycerides in droplike form inside their cytoplasm. The entire adipose organ in an average adult contains 30 billion adipocytes, each of which has a single droplet of triglyceride, roughly 0.5 ug in weight (Hirsch, et al, 1989). Studies have shown that the type of triglyceride stored in the adipocyte reflects the types of triglycerides being ingested in the diet. Over time, this has changed quite a bit. Before 1960, almost all the triglyceride stored in human adipocytes contained saturated fatty acids. Since 1960, however, there has been a 50% increase in the relative amount of the polyunsaturated fatty acid, linoleic acid, in adipose tissue (Berry, et al, 1986A; Hirsch, et al, 1960; Rizek, et al, 1974).

One objective of early studies of obesity was to compare the composition of triglycerides in adipocytes between obese and lean subjects. Results of these studies indicated that the fatty acid composition of adipose tissue was essentially the same in obese and non-obese individuals, suggesting that the selection of foods by obese people is not systematically different from lean individuals (Berry, et al, 1986B). This suggests

that obese subjects either eat more or metabolize less of the same foods than lean subjects.

Although the adipocyte is a storage organ, the activities of the adipocyte are hardly static. There is an active cycle of free or unesterified fatty acid uptake and esterification within the adipocyte and simultaneous lipolysis and release of free fatty acids. The cycle of lipolytic release and immediate re-esterification of fatty acids assures that there is an abundant supply of free fatty acid readily available as a metabolic fuel with a minimum expenditure of energy.

Metabolic Control:

Control mechanisms represent an exceedingly important area of obesity-related research. What mechanisms are responsible for the regulation of appetite? What mechanisms stimulate the production of fat from carbohydrates? What regulates the utilization of stored fat? And finally, what defects cause the malfunction of these processes that ultimately lead to the excess accumulation of fat?

There are several points of view regarding the control of body weight and energy stores in the body. Over the years, one of the most popular theories to explain weight control in subjects has been the set-point hypothesis, which suggests that a subject's body weight is controlled or maintained at a specified level. From this perspective, obesity is either the result of regulatory failure or the result of the body maintaining excess weight due to an elevated set point. Although we now know there are a multitude of causes for obesity in humans ranging from overeating to genetics, there is evidence that at least in some obesities, the elevated set-point hypothesis is valid (Keesey, 1981; Keesey, 1989).

The set point hypothesis is supported by the work of Cohn & Joseph, (1962) in which subjects of normal body weight were force-fed to elevate their body weight. Once the force-feeding was discontinued and the subjects were allowed to eat *ad libitum*, eating was sharply reduced. Moreover, when weight is lowered by caloric restriction the opposite happens: food intake is elevated (Levitsky, 1970). Another observation that seems to support the set-point hypothesis is that daily resting energy expenditure of animals, ranging in size from small birds and rodents to very large mammals, is a fixed function of metabolic mass or body weight raised to the 0.75 power ($BW^{.75}$). Another way of saying this is that all animals expend energy at comparable rates when expressed relative to their metabolic mass (Kleiber, 1947; Keesey, 1989).

There is evidence that both energy intake and energy expenditure are regulated in defense of the body weight set point. When the weight of rats is reduced by 15 per cent through caloric restriction, their rate of resting metabolism declines by 25 per cent (Corbett, et al, 1986). In response to forced feeding or over consumption there appears to be an increase in resting energy expenditure until the body weight returns to normal (Rothwell & Stock, 1982). If the body has a normal-weight set point, what happens in an obese subject? One theory proposes that the body's set point function is operational, however, in obesity the body operates at a higher set point, thereby maintaining a higher body weight. Data from experiments with force-fed rats support this hypothesis. Rats induced to overconsume palatable high fat foods generally tend to resist weight gain through increased energy expenditures; however, over time this diet regimen leads to the development of obesity (Sclafani, 1980). Additionally, after these animals are returned to a normal diet, their obesity persists (Rolls, et al, 1980). This suggests that prolonged

maintenance of a high fat diet causes changes in the set point to a higher level. In addition, prolonged weight gain is accompanied by changes in some hormones such as insulin and increases in adipocyte number (Faust, et al, 1978).

There are many factors that control energy metabolism in normal weight and obese subjects including circulating hormones such as insulin and leptin and genetic variables such as receptor defects. It is the interaction between these factors and the environment that helps determine weight gain or loss. A few of these factors are discussed in further detail below.

Uncoupling Mechanisms:

All of the food absorbed by mammals is converted to work, heat or excreta, with most of the energy actually being converted into heat. Even the energy used doing the physical work of muscles is mostly converted into heat. Until recently, it was thought that the generation of heat was mainly used for keeping the body's temperature at a normal state and, indeed, that is an important aspect of thermoregulation. However, several recent studies indicate that food intake can also regulate thermogenesis by a process referred to as diet induced or non-shivering thermogenesis. This concept says that increases in food consumption above that necessary to support basal metabolic functions are converted to heat. This could be an important regulatory tool for the body and might explain why it is so difficult to lose weight under food restricted diets and why weight comes back so easily after a prolonged fast.

Non-shivering thermogenesis is a vital process for most mammals at birth that takes place in the brown adipose tissues of the body. These cells are very rich in

mitochondria and have another distinguishing feature, uncoupling protein (UCP). In humans, brown adipose tissue is active at birth, but this decreases with age. Many scientists are investigating this in hopes that one day, brown adipose tissue could be up-regulated in adults to decrease weight gain.

The mechanism that is theorized to support this generation of heat is mitochondrial proton leak. That is to say that the generation of ATP by the cells does not exactly correspond to the amount of energy being released in the electron transport chain. The mediators of this wasting of energy are thought to be uncoupling proteins. These proteins transport H^+/OH^- ions across the mitochondrial membrane, thus dissipating the membrane potential. This dissipation of energy occurs in the form of heat (Dulloo & Samec, 2001).

These proteins are known as UCP1 (uncoupling protein 1), UCP2, UCP3 and UCP4 and are all from the same family. They are related to other mitochondrial translocators such as the P^-_i/OH^- exchanger. However, UCP is the only translocator capable of transporting only H^+. This was proven through inserting active UCP proteins into artificial liposomes. This transport also occurs regardless of pH, which is not the case with other H ion co-transporters. Additionally, UCPs are only found in brown adipose tissue mitochondria and are theorized to have arisen later in the development of eukaryotic cells. They are only found in mammals and must have evolved in conjunction with them (Klingenberg, 1990).

The activity of the UCP family of proteins has been found to be up regulated by fatty acids, which are being metabolized in the mitochondrion. They have been found to be down regulated by purine riboside di- or triphosphates (Klingenberg, 1990).

Cytokines:

Tumor Necrosis Factor α (TNF-α):

Tumor necrosis factor is a polypeptide cytokine produced primarily by mononuclear phagocytes, but also by adipocytes, and has as its primary role the initiation of the inflammatory response. TNF-α may inhibit glucose uptake in adipocytes by decreasing insulin induced auto-phosphorylation of the insulin receptor and insulin receptor substrate-1 and may also regulate leptin production (Chu, et al, 2001; Kern, et al, 2001: Kirchgessner, et al, 1997; Hotamisligil, et al, 1993; Mantzoros, et al, 1997; Uysal, et al, 1998). The action of resistin, another protein cytokine produced in adipocytes and thought to have a role in insulin resistance, is inhibited by treatment of 3T3-L1 adipocytes by TNF-α. This effect is reversed within 24 hr of the removal of TNF-α treatment. TNF-α has both a powerful autocrine effect on adipose tissue as well as a paracrine effect (Coppack, 2001). There appears to be a hierarchy of cytokines, so that some of the 'higher order' cytokines, such as TNF-α, orchestrate the synthesis, secretion, and activity of others (Coppack, 2001). TNF-α mRNA abundance is very low compared with that of other proteins in human white adipocytes (Montague, et al, 1998) and unstimulated adipose tissue releases relatively small amounts of TNF-α *in vitro* (Crawford, et al, 1997; Coppack, 2001). The synthesis of TNF-α has been shown to be responsive to both nutritional and immunological regulators (Coppack, 2001). There are also regional effects such that subcutaneous adipose tissue expresses mRNA for TNF-α and the receptors TNF-R1 and TNF-R2 at higher levels than omental tissue (Hube et al,

1998; Coppack, 2001). The synthesis of TNF-α has been shown to be related to both adiposity and fatty acid metabolism. Several studies demonstrate that obese animals have increased TNF-α mRNA and protein synthesis within adipose tissue (Hotamisligil, 1999; Coppack, 2001). While mRNA expression is higher in obese human subjects (Hotamisligil, et al, 1995; Hotamisligil, 1999; Hube, et al, 1999), it is not clear whether the protein release *in vitro* shows the same trend (Sewter, et al, 1999; Coppack, 2001). TNF-α which is able to induce its own synthesis is also a powerful regulator of other cytokines such as IL-6 (Stephens & Pekala, 1991). Insulin stimulates adipose tissue to produce more TNF-α, although the effect is less obvious in isolated adipocytes (Sewter et al, 1999; Coppack, 2001). The metabolic effects of TNF-α have been studied more extensively than those of the other cytokines. TNF-α can reduce expression of mRNA for glucose transporter 4 (Stephens & Pekala, 1991) as well as stimulate hormone-sensitive lipase expression to increase lipolysis. TNF-α has been reported to regulate cell size in mature adipocytes (Hotamisligil, et al, 1995), and cell number. It may regulate cell number by blocking differentiation of new adipocytes, promoting de-differentiation of existing adipocytes, or by inducing apoptosis of adipocytes or preadipocytes. TNF-α has been shown to do all three in *vitro (*Prins & O'Rahilly, 1997; Prins et al, 1997; Coppack, 2001).

IL-6:

Interleukin 6 (IL-6) levels have been found to be mildly elevated in obesity. Additionally, IL-6 has been found to inhibit lipoprotein lipase. IL-6 appears to act on the hypothalamus and the liver as a hormone. IL-6 probably acts to report the state of

adipose tissue stores to the body (Coppack, 2001). IL-6 is a pro-inflammatory cytokine secreted by adipose tissue in culture (Purohit et al, 1995; Fried et al, 1998), and its mRNA has also been demonstrated within adipocytes (Stephens et al, 1992; Purohit et al, 1995; Fried et al, 1998; Bastard et al, 2000). There is some dispute at this time about the degree of cytokine production by adipocytes. For example, Fried et al, (1998) reported that isolated adipocytes release only 10% as much IL-6 as adipose tissue in culture. Weight loss has been found to reduce plasma IL-6 concentrations (Kern et al, 1995; Bastard et al, 2000). TNF-α has been found to stimulate the production of IL-6 (Stephens & Pekala, 1991). IL-6 can also reduce adipose tissue lipoprotein lipase activity (Feingold & Grunfeld, 1992; Greenberg et al, 1992; Hardardottir et al, 1994). Exogenous administration of IL-6 is able to stimulate leptin secretion (Sarraf et al, 1997). Injections of IL-6 can increase circulating concentrations of fatty acids, C-reactive protein and fibrinogen (Strassmann et al. 1993). IL-6 is one of several cytokines which have been found to have anorectic effects on the brain (Plata-Salaman, 2000) and to induce gastric stasis.

Resistin:

Recently, a new protein was found to be secreted from adipocytes (Steppan et al, 2001). A murine fat cell line was treated with rosiglitazone and subtractive cloning was conducted to identify RNA's which were down regulated in the treated cells (Steppan, et al, 2001). An mRNA encoding a novel cysteine rich protein was identified. This protein has been found to play a part in insulin resistance and hence has been called resistin. Resistin mRNA has been found to be undetectable in pre-adipocytes and in many other

tissues of the body, but is readily found in murine white adipose tissue. Additionally resistin has been found to be expressed in human white adipose tissue.

Resistin is also associated with inflammatory states of tissue, being isolated previously by Holcomb, et al, (2000) in both humans and rodents from inflamed lung tissue and termed FIZZ 1. The thiazolidiones that were used to identify resistin also have anti-inflammatory effects by decreasing cytokines. Resistin is postulated to be a cytokine and possibly to work in concert with TNF-α, which could make it a very important molecule to obesity research.

Adiponectin:

Adiponectin is a protein that is structurally similar to cytokines which has been found to be expressed in adipocytes. Adiponectin is expressed inversely to total fat and is thought to play a role in mediating the obesity-related risk for coronary heart disease and type 2 diabetes mellitus (Comuzzie, et al, 2001; Arita, et al, 1999; Funahashi, et al, 1999; Okamoto, et al, 2000; Ouchi, et al, 1999; Hotta, et al, 2000; Fruebis, et al, 2001). Two groups have reported that replenishment of the adipocytokine adiponectin increases insulin sensitivity in different murine models of insulin resistance *in vivo* (Yamauchi, et al, 2001; Berg, et al, 2001). Recently Comuzzie and colleagues compared circulating levels of adiponectin to different genomic factors and established corresponding linkage to several obesity gene loci (Comuzzie, et al, 2001). While not much is known about this new circulating cytokine, intensive research is ongoing and it could prove to be a very important regulatory molecule for obesity.

Hormone Factors:

Insulin:

One of the complications of obesity mentioned in the introduction is diabetes. Indeed two characteristics of human obesity are hyperinsulinemia and insulin resistance. Most patients with non-insulin-dependent diabetes are obese. Hyperinsulinemia is apparent both in single sample measurements and in 24-hour profiles and in response to glucose, protein, arginine, leucine, glucagon, or other secretogogues that promote insulin release (Glass, 1989).

In spite of having high insulin levels, obese subjects do not become hypoglycemic, suggesting they are resistant to the action of insulin. This was demonstrated experimentally by Glass (1989) who observed a subnormal drop in plasma glucose in obese subjects after the exogenous administration of insulin (Glass, 1989). While insulin resistance and hyperinsulinemia could be caused by decreased or inactive insulin receptors, this does not appear to be the case (Glass, 1989). Hyperinsulinemia appears to be the primary factor. In a variety of models, hyperinsulinemia is known to cause a reduction in the number of insulin receptors on target tissues (downregulation) (Glass, 1989). Moreover, the reduction of hyperinsulinemia with diazoxide in obese subjects was shown to induce an increase in insulin receptor number on target tissues (Wigand, et al, 1979).

Growth Hormone:

Growth hormone works both directly and indirectly on tissues to promote growth. In some tissues growth hormone works directly, in others its effects are mediated by a polypeptide called somatomedin (Insulin-Like Growth Factor-I).

Growth hormone output is drastically reduced in obesity. In both basal state single measurements and 24 hr integrated measurements, growth hormone levels are markedly reduced in obese individuals (Ball, et al, 1972; Danowski, et al, 1969; Josefsburg, et al, 1976; Meistas, et al, 1982; Slavnov & Epstein, 1977). Also, the administration of synthetic GHRH (Growth Hormone Releasing Hormone) stimulates growth hormone output in normal subjects, but in obese subjects the response is either abolished or blunted (Davies, et al, 1985; Kopelman & Noonan, 1986; Kopelman, et al, 1985; Weinsier, et al, 1976). Additionally, the growth hormone increase normally seen with other physiologic stimuli, such as deep sleep (Copinschi, et al, 1978; Hunter, et al, 1966; Kalucy, et al, 1976; Laurian, et al, 1975; Newmark, 1980), fasting (Beck, et al, 1964), protein-containing meals (Rabinowitz, et al, 1967; Schultz, et al, 1970), or exercise (Garlaschi, et al, 1975; Hansen, 1973; Roth, et al, 1963) is blunted in obese subjects. Moreover, blunted growth hormone release is observed in obese subjects in response to a wide variety of stimuli, including administration of insulin, arginine, glucose, methoxamine, L-Dopa and glucagon (Barbarino, et al, 1978; Bell, et al, 1970; Cornelutti, et al, 1970; Copinschi, et al, 1967; Crockford & Salmon, 1969; Danowski, et al, 1969; El-Khodary, et al, 1971; Fingerhut & Krieger, 1974; Grzywa, 1986; Kalkhoff & Ferrow, 1971; Kalkhoff, et al, 1970; Kopelman, et al, 1979; Komorowski, 1977; Laurian, et al, 1975; Laurian, et al, 1977; Lessof & Yound, 1966; Mims, et al, 1973; Rabinowitcz,

et al, 1967; Roth, et al, 1963; Vandeweghe & Vermeulen, 1974; Williams, et al, 1984; Wold, et al, 1977).

The reduced or blunted output of growth hormone appears to be a consequence of obesity rather than the cause of obesity. The growth hormone output in obese subjects in response to hypoglycemia or GHRH administration is inversely related to the degree of obesity (Davies, et al, 1985; Josefsburg, et al, 1976; Komorowski & Paulikowski, 1979; Lessof, et al, 1966; Williams, et al, 1984). Normal-weight subjects who become obese through overfeeding demonstrate reduced growth hormone responses to deep sleep, exercise, and glucose or arginine administration (Sims, et al, 1973; Sims & Horton, 1968). Obese subjects who lose weight reverse this effect and demonstrate increased growth hormone release in response to exercise, deep sleep, hypoglycemia or GHRH administration (Ball, et al, 1972; Copinschi, et al, 1978; Crockford & Salmon, 1970; Lessof, et al, 1966; Londono, et al, 1969). Additionally, the reduced growth hormone output in obesity appears to be related to the increased adipose tissue mass rather than increased body size, as growth hormone output is not reduced in overweight subjects who are not obese, such as weightlifters (Kalkhoff & Ferrow, 1971).

How is it then that obese individuals with a low growth hormone output do not exhibit reduced growth? Several studies have indicated that somatomedin levels are normal in obese adults (Caufriez, et al, 1984) and elevated in obese children (Loche, et al, 1987; Rosskamp, et al, 1987; Van Vliet, et al, 1986). Normally, somatomedin levels would be reduced in a situation involving low growth hormone levels. The fact that they are not indicates that somatomedin release is stimulated by other factors. Insulin might

play a role because it can stimulate somatomedin production and obese subjects tend to be hyperinsulinemic. Increased levels of somatomedin may also explain the decrease in growth hormone levels through a negative feedback loop (Kelijman & Frohman, 1988; Loche, et al, 1987).

Prolactin:

Prolactin is a polypeptide hormone synthesized in the anterior pituitary gland, which promotes lactation in women (Glass, 1989). While prolactin deficiency results in a failure of lactation, prolactin excess usually leads to hypogonadism (Glass, 1989).

Studies have shown that both basal serum prolactin and 24-hour mean serum prolactin levels are normal in obese subjects (Carlson, et al, 1977; Copinschi, et al, 1978; Donders, et al, 1985; Grenman, et al, 1986; Hanna, et al, 1987; Kopelman, et al, 1979; Meistas, et al, 1982; Wilcox, 1977); however, a variety of stimuli which produce prolactin secretion in normal weight individuals, produce subnormal prolactin secretion in obese subjects (Glass, 1989; Cavagnini, et al, 1981; Sannia & Benna, 1983). This attenuated response is not improved by weight loss.

Testosterone:

Several studies have shown that obese men have low serum testosterone levels (Amatruda, et al, 1978; Glass, et al, 1977; Kley, et al, 1980a; Schneider, et al, 1979; Stanik, et al, 1981; Strain, et al, 1982; Valenti, et al, 1986). Serum testosterone levels in obese men seem to be inversely correlated with the degree of obesity (Amatruda, et al, 1978; Glass, et al, 1977; Kley, et al, 1980a; Schneider, et al, 1979; Stanik, et al, 1981;

Strain, et al, 1982; Valenti, et al, 1986; Kley, et al 1980b; Kley, et al, 1979). The low serum testosterone levels rise with weight reduction (Kley, et al, 1979; Stanik, et al, 1981; Strain, et al, 1982; Strain, et al, 1988).

In studies with Kleinfelter's syndrome and in men who are overweight, but not obese, the reduction in testosterone seems related directly to adipose tissue excess. Several studies have shown that obese men are not hypogonadal and have normal libido, potency, secondary sexual characteristics, testis size and spermatogenesis (Anatruda, et al, 1978; Anatruda, et al, 1982; Glass, et al, 1977; Kley, et al, 1979; Schneider, et al, 1979; Stanik, et al, 1981; Strain, et al, 1982). The reason for this appears to be low sex-hormone binding globulin, which is the major circulating protein that binds testosterone (Glass, et al, 1978; Kley, et al, 1981; Kley, et al, 1979; Strain, et al, 1988) with a corresponding decrease in bound testosterone. At the same time, there is an increase in free serum testosterone (Amatruda, et al, 1978; Glass, et al, 1978; Kley, et al, 1981; Strain, et al, 1982).

Other steroids have been investigated in obese men. Serum androstenedione is normal or slightly reduced in obese men and does not seem to be correlated with the degree of obesity (Glass, et al, 1977; Schindler, et al, 1975; Stanik, et al, 1981). Similarly, levels of dihydrotestosterone, a derivative of testosterone, appear to be normal in obese men (Strain, et al, 1981). Some studies have shown serum estrogen levels to be elevated in obese men even though they are not feminized. These estrogens appear to increase in proportion to body weight and decrease proportionally with weight loss (Kley, et al, 1981; Kley, et al, 1979; Kley, et al, 1980b; Komindr, et al, 1986; Stanik, et al, 1981; Strain, et al, 1988; Valenti, et al, 1986; Zumoff, et al, 1981). The fact that the

increased levels of estrogens are correlated with increasing body weight could mean that androgens are being converted to estrogens in peripheral tissues. Adipose tissue is one of the tissues which can convert androgens to estrogens, thus explaining why obese men have high serum estrogen levels (Frost, et al, 1980; Nimrod, et al, 1975; Perel, et al, 1970; Strain, et al, 1982). Studies involving LH (Leuteinizing Hormone) and FSH (Follicle Stimulating Hormone) have demonstrated normal serum levels of these two hormones except in the case of some extremely obese men that have been reported to have subnormal levels of LH and FSH (Strain, et al, 1982; Strain, et al, 1988; Schneider, et al, 1979; Kley, et al, 1980a).

Ovarian Steroids:

Obesity is associated with many clinical symptoms that appear to be due to abnormal ovarian function in women. Among these are irregular menses, hirsutism, early menarche, and delayed menopause (Hartz, et al, 1987; Rogers & Mitchell, 1952; Sherman, et al, 1981; Zacharias, et al, 1976). Assessment of these conditions in obese women is often difficult due to the normal fluctuations of hormones in the menstrual cycle. Therefore many of the studies of these hormones in obese women are done with post-menopausal obese subjects, in whom most circulating estrogens are derived from peripheral metabolism of circulating androgens.

These circulating androgens (androstenedione and testosterone) are secreted primarily by the adrenal gland in postmenopausal women. Adipose tissue can convert these androgens to estrogens, (Nimrod, et al, 1975; Schindler, et al, 1972) thus explaining why obese post-menopausal women have high circulating levels of estrogens (Davidson,

et al, 1981; Judd, et al, 1976; Noel, et al, 1981; Vermeulen & Verdonck, 1978). Additionally, some studies have shown that serum levels of androstenedione, the precursor for estrogen synthesis, may also be elevated in postmenopausal obese women, further facilitating estrogen production (Hagen, et al, 1982). These high levels of estrogen production may play a direct role in the low rates of osteoporosis in postmenopausal obese women (Aloia, et al, 1985).

In premenopausal obese women, the ovary produces most of the circulating estradiol. Low or normal serum estradiol levels in premenopausal obese women are not unheard of (Cunningham, et al, 1985; Evans, et al, 1983; Grenman, et al, 1986; Loughlin, et al, 1985; Plymate, et al, 1981). The estrogen produced by peripheral conversion is primarily estrone. Therefore obese premenopausal women tend to have elevated levels of circulating estrone along with higher rates of peripheral conversion of androstenedione due to their increased adipose tissue mass (Cunningham, et al, 1985; Kaufman, et al, 1981; Louglin, et al, 1985; Zhang, et al, 1984; Vermeulen, et al, 1978).

Adrenal Steroids:

In obese subjects, plasma cortisol, plasma-unbound cortisol, 24-hour mean plasma cortisol, and urinary free cortisol are normal as are the circadian rhythms for plasma and urine cortisol (Abou Samra, et al, 1985; Copinschi, et al, 1978; Galvao-Teles, et al, 1976; Genazzani, et al, 1978; Kalucy, et al, 1976; Kobberlign, et al, 1974; Kopelman, et al, 1988). Even though plasma cortisol levels remain normal in obesity, there is an enhanced metabolic clearance of this hormone. This means the body must maintain a higher level of cortisol production in order to maintain normal circulating

levels (Dunkelman, et al, 1964; Migeon, et al, 1963; O'Connell, et al, 1963; Schteingart, et al, 1963; Strain, et al, 1980).

Thyroid Hormone:

The major circulating thyroid hormones in humans are thyroxine (T_4) and triiodothyronine (T_3). Thyroid hormone is released in response to the action of a pituitary tropic hormone called thyroid-stimulating hormone (TSH). The release of TSH is stimulated by thyroid releasing hormone, a hypothalamic hormone, and inhibited by a negative feedback mechanism involving thyroid hormone. Since the thyroid plays a major role in energy metabolism in the body, many studies have been done to assess thyroid function in obesity. All of these studies have shown that serum levels of total and free T_4 are normal in obesity (Chomard, et al, 1985; deRosa, et al, 1983; Faber, et al, 1981; Feher, et al, 1975; Kvetny, 1985; Strata, et al, 1978; Glass, 1989); however, there has been confusion about the levels of serum T_3 in obesity. Some investigators have reported normal T_3 levels (deRosa, et al, 1983; Kvetny, 1985) while others have reported elevated T_3 levels (Faber, et al, 1981; Josefsberg, et al, 1976; Scriba, et al, 1979). There have also been disputes about the production rates of T_4 with investigators reporting decreased (Nicoloff, et al, 1966), normal (Vagenakis, et al, 1977), or increased (Benoit, et al, 1965) serum T_4 production in obese individuals. At this time there is no clear evidence suggesting that the rate of thyroxine production is a factor in obesity.

Leptin:

Leptin, a 16 kilodalton protein encoded by the obesity (ob) gene in mice, was identified in 1994 at Rockefeller University (Zhang, et al, 1994). When exogenous leptin is administered to animals there is a resultant loss of body fat (Pelleymounter, et al, 1995; Halaas, et al, 1995; Halaas, et al, 1997; Levin, et al, 1996; Pelleymounter, et al, 1998; Heymsfield, et al, 1999). Moreover, serum concentrations of leptin increase with body fat in very obese persons who do not have a genetic mutation in the leptin gene (Heymsfield, et al, 1999; Maffei, et al, 1995; Considine, et al, 1996; Considine, et al, 1995; Lonnqvist, et al, 1995; Hamilton, et al, 1995). Leptin appears to exert its effects mainly in the hypothalamus (Mercer, et al, 1996; Hoggard, et al, 1997). Animals with defects in this gene act as if they are in a perpetual state of starvation. Researchers have found that leptin is produced in adipocytes and secreted into the bloodstream in proportion to the fat stored in the adipocytes. When fat stores rise, adipocytes produce more leptin, and the hormone, in turn, tells the brain that it is time to stop eating and increase activity levels. As fat stores decline, leptin levels fall as well, signalling the brain to compensate by increasing food intake and lowering activity (Gura, 1997). Body fat appears to account for 50 to 60% of the variation in serum leptin concentrations among people (Ahren, et al, 1997).

So far, researchers have identified several rodent models of obesity involving leptin. Following the discovery that the ob/ob mouse was leptin deficient, researchers found that the db gene in diabetic mice codes for a defective leptin receptor (Leibel, 1997). Later researchers found that both the Zucker fatty rat and the corpulant rat strains

had leptin-receptor mutations, which prevented them from responding to this hormone (Kahle, et al, 1998; Chua, et al, 1996).

The initial speculation that leptin may be a cure for obesity was not supported by subsequent research. It has been shown, for example, that most obese human subjects, actually have high levels of leptin in their bloodstream (Considine, et al, 1996). Furthermore, most animal models studied thus far are unable to respond to the elevated levels of leptin due to defects in the receptor for leptin.

To exert its effects, leptin must cross the blood brain barrier to gain access to regions of the brain involved in regulating energy balance. In order to do this, it needs a transport mechanism, which appears to be receptor-mediated. In both humans and rodents, different leptin receptor (OB-R) isoforms have been found in lung, kidney, adipose tissue and brain (Mercer, et al, 1996; Hoggard, et al, 1997; Fei, et al, 1997; Tsuchiya, et al, 1999; Kutoh, et al, 1998). One leptin receptor isoform has been described as a short form (OB-Ra). It is present in the choroid plexus and seems to be involved mainly in the transport of leptin (Tartaglia, 1997). A second isoform, the long form (OB-Rb), is present in the choroid plexus and has a long intracellular domain that contains putative motifs for janus protein-tyrosine kinase and signal transducers and activators of transcription (Vaisse, et al, 1996; Burguera, et al, 2000). Additionally, the OB-Ra short form of the leptin receptor has been found to be the most abundant form in the blood brain barrier, further indicating its importance in the transport of leptin (Boado, et al, 1998). The biological actions of leptin are mediated through an interaction with a receptor that is expressed mainly in the hypothalamus, but also in other cerebral areas

(Burguera, et al, 2000). Studies comparing the permeability of leptin across the blood brain barrier in lean and obese rats demonstrated that although plasma leptin levels were higher in obese rats compared to lean rats, the leptin levels in cerebrospinal fluid were not significantly different between obese and lean rats. This would suggest that the leptin receptor in the blood brain barrier could easily be saturated (Burguera, et al, 2000).

Recently, researchers have found evidence that suggests the brain itself may produce leptin. Studies have revealed that there is a higher concentration of leptin in the internal jugular vein of both lean women and obese men compared to peripheral circulation (Weisner, et al, 1999) and that leptin is expressed in most anterior pituitary cell types (Jin, et al, 1999).

Neuropeptide Y:

The actions of leptin may be mediated indirectly through its effect on the production of other molecules. One such molecule, which may help to explain some of the phenomena surrounding obesity, is neuropeptide Y (NPY). Neuropeptide Y is a powerful appetite stimulating neurotransmitter which appears to be regulated by leptin. Production of NPY skyrockets in animals like the ob/ob mouse which lacks leptin. When exogenous leptin is administered to these mice, NPY production is suppressed with resulting weight loss (Gura, 1997).

The effects of NPY are mediated mainly through an intrahypothalamic pathway formed by the arcuate nucleus (ARC), where it is abundantly synthesized, and the paraventricular nucleus (PVN), where ARC neurons project their efferents (Kalra, et al, 1999). There are six types of the NPY receptor, two of which mediate feeding behavior,

the Y1 and Y5 subtypes (Inui, 1999). Infusion of NPY into brain ventricles has been shown to induce the development of overweight and adiposity (Beck, 1992), and cause hormonal and metabolic changes (Zarjevski, et al, 1993). Before the discovery of leptin in 1994 it was thought that perturbations in the NPY system were the main cause of obesity in certain animal models such as the Zucker rat.

Environmental Factors:

Changes in environment must be responsible for the large increase in obesity and overweight observed during the 1980's and 1990's. This dramatic increase in the prevalence of obesity suggests that the environment has changed in ways that promote the development of obesity. The current environment in the U.S. has shifted from one involving manual labor and outdoor sports to one of a sedentary lifestyle involving less time outdoors and a reduction in jobs requiring physical exertion. Time away from work involves less strenuous activities such as video games, the Internet, television and televised sports. Additionally, the American diet has changed with a shift toward higher caloric content fast foods. The environment of the U.S. is being exported around the world with the result of increasing obesity in the world's population (Hill, et al, 2000).

One area, which is only now beginning to be studied, is the effect of past environmental conditions. This might take the form of undernutrition during gestation, which has been shown to correlate with coronary heart disease later in life in humans (Barker, 1995). We are only just beginning to understand that past memories of environmental conditions may translate into pathology in hormonal secretion, metabolic activity, and organ structure. The interactions become more complex when we take into

account the current environment interacting with the gene pool as well as earlier environmental conditions to which a person is exposed (Hill, et al, 2000).

Genetics of Obesity:

Within human populations genetics appears to be a substantial factor in the amount and distribution of body fat. Although it is difficult to accurately estimate the genetic contribution to obesity in a human population, available data indicate that it may be between 25% and 70% depending on the population studied (Hill, et al, 2000). Familial studies have repeatedly demonstrated that BMI is highly correlated among first-degree relatives with obese parents producing a higher proportion of obese offspring (Bouchard, et al, 1988; Tambs, et al, 1991; Hill, et al, 2000). Even stronger support for a genetic component comes from twin studies in which the BMI is consistently similar between twins whether they were raised together in similar environments or separately (Allison, et al, 1996; Meyer, et al, 1994; Sorensen, 1995). Moreover, the BMI of an adoptee in childhood and adulthood is closely correlated with the BMI of the biological parents. In contrast, there is no correlation with the BMI of the adoptive parents in adulthood and only a weak correlation in childhood (Hill, et al, 2000). These observations suggest a strong genetic influence on body weight.

Most research in this area has focused on identifying individuals who may have genetic defects in metabolism that predispose them to obesity. Many studies have focused on abnormally low energy expenditures as an obesity risk. Although some have shown that a low resting metabolic rate may lead to obesity in humans (Segal, et al, 1989; Segal, et al, 1985), others have not (Goran, et al, 1995; Seidell, et al, 1992; Weinsier, et

al, 1995). Other studies have shown that the inability to oxidize fat may predispose some individuals to obesity (Zurlo, et al, 1990). Genetic factors may also influence behaviors which could lead to obesity, such as eating or sedentary activities; however, this has not been the focus of very many studies (Hill, et al, 2000).

In terms of causative factors, human obesities are very heterogeneous. They are influenced by behavior, one's social status, nutrition, psychology, energy expenditure and genetics (Bray, 1981; Bray, 1984). It cannot be denied that genetics plays some role in human obesity.

Leptin associated phenomena appear to cause obesity in several monogenic animal models including the ob/ob mouse, the Zucker fatty rat and the LA/N-cp rat. It has also been found to play a role in human metabolism and in a small number of human obesities. While there are several good single gene animal models for obesity very few of these appear to be relevant to human obesity. In table III, monogenetic models of rodent obesity are compared to their human counterpart.

Table III:

Human gene	Corresponding rodent model
Leptin	ob/ob
Leptin receptor	db/db, Zucker fa/fa, LA/N-cp
Proopiomelanocortin	Agouti
Prohormone convertase 1	fat/fat
Melanocortin 4 receptor	Mc4r-deficient mice

Of the animal models, the Zucker fatty rat is the model most studied for obesity. Obesity in this model appears to be due to a defective leptin receptor (Chua, et al, 1996; Chua, et al, 1996a; Phillips, et al, 1996; Iida, et al, 1996). This means that leptin cannot exert its inhibitory effect on food intake in these animals (Seeley, et al, 1996; Beck, 2000). The LA/N-cp fat rat also displays a leptin receptor defect against a slightly different genetic background. The obese ob/ob mouse is another classic rodent model of obesity. In these animals there is a nonsense mutation in the coding region of the leptin gene itself, which produces a nonfunctional peptide (Zhang, et al, 1994). When functional leptin is injected into these mice, it regulates food intake proving that the rest of the regulatory system is functional (Smith, et al, 1998). In the yellow Agouti mouse, the product of the Agouti gene interacts with melanocyte stimulating hormone (MSH) in the hypothalamus to produce obesity and with MSH at the level of type 1 melanocortin receptors to determine coat color (Graham, et al, 1997; Beck, 2000). There is also a fat mouse in which the gene for obesity maps to the Carboxypeptidase E gene. Although these mice have extremely low levels of carboxypeptidase E, the defect is not fatal (Fricker, et al, 1999; Beck, 2000). Finally, the tubby mouse is characterized by an autosomal recessive mutation that results in adult-onset obesity, which is not well understood at this time. It is known that when the expression of the tub gene in the brain is halted, it gives rise to the "tubby" phenomenon (Coleman, et al, 1990; Kleyn, et al, 1996; Noben-Trauth, et al, 1996; Beck, 2000).

In humans a great deal of interest is currently focused on the leptin gene. Several early studies have reported linkage of obesity with the leptin gene; however, causative mutations have not been identified (North, 1999; Clement, et al, 1996; Maffei,

et al, 1996). In recent studies, loss of function mutations for the human leptin gene have been identified (Clement, et al, 1998; Montague, et al, 1997; Strobel, et al, 1998; Arner, 2000). Even so, mutations in the coding region of the leptin gene are rare with only 5-10% of obese humans having low levels of leptin (Maffei, et al, 1995).

Rat Models of Obesity:

LA/N-cp rats:

The LA/N-cp fatty rat is a congenic strain, which was developed by C.T. Hansen at the NIH by crossing a Koletsky rat which was heterozygous for the cp gene with an LA/N rat. The LA/N strain is a normotensive strain developed at the NIH from a cross of the Albany (ALB/N) strain and a hooded strain of unknown origin. After twelve backcrossings the noncorpulent genes of the Koletsky were eliminated. Obesity in this model is inherited as an autosomal recessive trait (Michaelis, et al, 1983; Koletsky, 1973).

When compared to lean littermates, corpulent rats are hyperphagic, hyperlipidemic, hyperinsulinemic, and exhibit elevated levels of liver lipogenic enzymes. Corpulent rats are also more efficient than lean rats in converting equivalent amounts of food (g/unit body weight) into body fat (Michaelis, et al, 1983).

In corpulent rats, levels of serum insulin and triglyceride are markedly elevated (6 to 8 times lean), whereas the level of total serum cholesterol is only moderately elevated (2 times lean) (Michaelis, et al, 1983). Corpulent rats are essentially normoglycemic. Although genetic expression is similar between LA/N-cp and the Zucker rat strains, the magnitude of some metabolic parameters, e.g., insulin level, appears to be greater in

LA/N-cp rats. Corpulent rats consume more food and gain more weight than lean rats; however, food intake, when expressed as g per 100 g body weight, is not different between genotypes (Michaelis, et al, 1983). Body weight of obese homozygotes reaches approximately 950 g compared to 400 g for lean heterozygotes (Russell and Amy, 1986a). Total fat pad weight and size are greater in corpulent than in lean rats. Moreover, total fat pad weight is greater in heterozygous lean rats than in homozygous lean rats (Michaelis, et al, 1983).

The LA/N-cp rat, when homozygous for the corpulent gene, is prone to the development of vascular and myocardial lesions (Dolphin, et al, 1987). Both lean and obese rats have been shown by scanning electron microscopy to develop arterial lesions and occasional occlusive thrombi. These are significantly more frequent in the corpulent rats (Russell and Amy, 1986b). There has been a common perception that the rat is highly resistant to cardiovascular disease; however, rats over 400 days old have been shown to exhibit pathological lesions of the heart (Bernirschke, et al, 1978).

The hyperlipidemia is due to high concentrations of very low density lipoproteins (VLDL), which are relatively apolipoprotein B-poor, resulting in moderately elevated triglyceride concentrations (Russell and Amy, 1986b). In adult fat rats, plasma triglycerides are 330 mg/100 ml compared to 40 mg/100 ml, whereas total cholesterol is 215 mg/100 ml, compared to 80 mg/100 ml in heterozygotes (Russell and Amy, 1986b).

Sympathetically mediated thermogenesis is impaired in the LA/N-cp rat (Tulp, 1984). Several authors have proposed that an impaired mechanism for the development of non-shivering or diet-induced thermogenesis via brown adipose tissue might decrease an organism's capacity for energy expenditure, and thereby contribute to the

development of obesity (Rothwell and Stock, 1979; Himms-Hagen, 1984). Measurements of oxygen consumption were similar in both lean and corpulent rats. Isoproterenol stimulation resulted in a marked increase in oxygen consumption in lean but not in corpulent rats. Acute exposure to a 5^0C cold environment resulted in significant decreases in colonic and rectal temperatures in both genotypes, but body temperatures recovered more rapidly in lean than in corpulent rats (Tulp, 1984). Several authors have proposed that non-shivering or diet-induced thermogenesis is due largely, if not entirely, to brown adipose tissue (Rothwell and Stock, 1979; Himms-Hagen, 1984; Rothwell, et al, 1982).

Sympathomimetic stimulation of thermogenesis via the B-adrenergic agent isoproterenol, a proven activator of brown fat thermogenesis (Bukowieki, et al, 1980), and endogenous activation of thermogenesis mechanisms via cold exposure (Tulp, et al, 1982), have resulted in weight loss in these obese rats.

Zucker fa/fa rat:

The fatty (fa) mutation arose spontaneously in an outbred stock of rats designated 13M at the Laboratory of Comparative Pathology, Stow, Massachusetts. The fa gene was then transferred through breeding to outbred Wistar stock obtained from Kyoto University and animals from this strain were transferred to Indiana University Medical Center where inbreeding of Zucker rats was begun (Zucker, et al, 1961; Hansen, 1983).

Today, as noted earlier, the Zucker fatty rat is the most widely studied rodent model of obesity. It has several characteristics in common with human obesities including hyperphagia, hypertriacylglycerolemia, and hyperinsulinemia (Bray, 1977;

Argiles, 1989). Additionally, all metabolic changes are present between 3 and 5 weeks of age in these animals (Krief, et al, 1991).

The fatty mutation in the Zucker rat has been identified as a mutated leptin receptor (Chua, et al, 1996; Chua, et al, 1996a; Phillips, et al, 1996; Iida, et al, 1996). In these mutant animals, leptin cannot exert its inhibitory effects on food intake through its receptors in the central nervous system (Seeley, et al, 1996). This defect in leptin signalling in the Zucker fatty rat results in large changes in the functioning of many neuropeptidergic networks such as the signalling involving NPY (Beck, 2000).

Neuopeptide Y (NPY) is being intensively studied in the Zucker fatty rat because this peptide strongly stimulates feeding behavior and has strong effects on energy storage in adipose tissue (Stanley & Thomas, 1993; Billington, et al, 1994; Beck, et al, 1992; Zarjevski, et al, 1993).

Purpose of Investigation

Obesity has an economic cost to society as well as a human cost in terms of co-morbidities and loss of quality of life. Billions of dollars are being spent each year on weight loss programs and on reduction surgeries to combat the social stigma and loss of quality of life associated with obesity. However, one needs to look past the economic cost and focus instead on the human cost of obesity.

The purpose of this investigation was to evaluate the potential of circulating proteins in the bloodstream to serve as markers for obesity. If potential circulating markers for obesity could be found, it is possible that these could provide the basis for an

"early warning" blood test for obesity. When given to children, this test might be used to evaluate the potential they have for becoming obese later in life.

It was also hoped that the identification of these circulating markers of obesity might lead to a better understanding of obesity. These could be used for further studies into the metabolic changes that occur in obesity.

Materials and Methods

Animals:

Eight week old lean and obese Zucker rats were obtained from Charles River Laboratories. Lean and obese Zucker rats were housed in plastic cages with wood-chip bedding at the Marshall University School of Medicine Animal Resources Facility, Huntington, WV. The animals were maintained in a room with an ambient temperature of 23 ^{0}C and a 12 hr light/dark cycle and were given *ad libitum* access to Purina rat chow and water.

Lean and obese LAN/cp rats were graciously provided by Dr. Bowie Kahle and housed at the Marshall University Department of Biological Sciences, Huntington, WV. The rats had free access to Purina rat chow and tapwater and were kept in a room having an ambient temperature of 23 ^{0}C and a 12 hr light/dark cycle.

Reagents:

Methanol and pipetting supplies were obtained from Fisher (Houston, TX), acrylamide, bis-acrylamide, and temed from Bio-Rad laboratories (Richmond, CA), and

molecular weight markers from Sigma Chemical Company, (St. Louis, MO). BCA protein assay kits were purchased from Pierce Biochemicals (Rockford, IL), Immobilon-P, Immobilon-PSQ, and HA membranes from Millipore (New Bedford, MA), and ECL detection kits from Amersham Life Sciences, Inc. (Arlington Heights, IL). HRP-conjugated anti-complement C3 antibodies were obtained from Cappel (Durham, NC), and anti-apolipoprotein A1 primary antibodies and HRP conjugated secondary antibodies were obtained from Rockland (Gilbertsville, PA). All other reagents were obtained from Sigma Chemical Company (St. Louis, MO).

Sample Preparation:

Twelve week-old lean and obese LA/N-cp and Zucker rats were fasted overnight before blood samples were obtained. When large volumes of plasma were required, rats were anesthetized with Ketamine HCl/Xylazine (45:5 mg/kg) and then euthanized by exsanguination via cardiac puncture. Blood was collected in heparinized syringes and transferred to 15 ml centrifuge tubes. The samples were centrifuged at 4000 X g for 5 min. and plasma was removed and stored at –70 ^{0}C. When small volumes of serum/plasma were required for SDS-PAGE, a tail bleed was used to obtain the blood sample. Blood was collected in heparinized hematocrit tubes, sealed with Hemato-seal and centrifuged at 2000 X g for 5 min. The sections of the tubes containing the plasma were severed and the plasma collected in microcentrifuge tubes.

Immediately prior to use, plasma samples of both lean and obese animals were centrifuged at 12000 X g for 10 min. to remove lipids. This was done to prevent

smearing of the protein bands during SDS-PAGE due to a high lipid content in obese plasma.

Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis:

Plasma proteins were fractionated using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) by a modified method of Laemmli and O'Farrell (Laemmli, 1970; O'Farrell, et al. 1975). The acrylamide gel solution, (10% acrylamide, 373 mM Tris-HCl pH 8.7, 0.1% SDS) was prepared and degassed for 10 min. under vacuum. Cross-link polymerization was started by adding 0.01% N,N,N',N'-tetramethyl-ethylenediamine (TEMED) and 0.01% ammonium persulfate. The polymerizing gel solution was pipetted between paired BioRad Protean IIsi electrophoresis plates (15 cm x 15 cm x 1.5 mm). A layer of distilled water (1 ml) was gently pipetted onto the top of the polymerizing gels. Gels were allowed to polymerize for a minimum of 30 min. before the stacking gel was poured.

The stacking gel solution (4.8% acrylamide, 0.14% Bis, 125 mM Tris-HCl, pH 6.9, 0.1% SDS) was prepared, degassed under vacuum for 10 min., and polymerized by the addition of 0.05% TEMED and 0.05% ammonium persulfate. The water layer was removed from the tops of the separating gels and the stacking gel solution was carefully pipetted onto the tops of the separating gels. The formation of sample wells was accomplished by inserting 15 tooth (1.5 mm thick) combs into the polymerizing stacking gel. These were allowed to set a minimum of 30 min.

The gel sandwiches were then placed into a BioRad Protean IIsi electrophoresis apparatus filled with SDS running buffer (38 mM glycine, 5.0 mM Tris, 0.1% SDS). The sample combs were removed after addition of SDS running buffer to the upper chamber. Plasma protein concentrations were determined using the Pierce BCA protein assay kit. Sample volumes were adjusted so that each sample had the same concentration of protein. The plasma samples were diluted using SDS sample buffer (10% glycerol, 5% beta-mercaptoethanol, 62.5 mM Tris-HCl pH 6.8, 2.3% SDS), so that each gel lane received 600 μg of protein in both lean and obese samples on 10 % gels and 300 μg of protein per lane in 15% gels. The samples were then heated at 100 ^{0}C for 5 min to assure complete denaturation. The samples were then added to the wells and run alongside Sigma molecular weight markers for 18 hrs at 50V and 120V for an additional 6 hours. Upon completion, gels were removed and either stained to visualize proteins or transferred to membranes for sequencing or western blotting.

For separation of smaller plasma proteins, SDS-PAGE was used as described above with the following exceptions. Instead of being fractionated on 10% SDS-PAGE gels, these plasma samples were fractionated on 15% SDS-PAGE gels (15% acrylamide, 0.09% N,N-methylene-bis-acrylamide (BIS), 373 mM Tris-HCl pH 8.7, 0.1% SDS). Stacking gels were prepared as described above. Then the samples were added to the wells and run alongside Sigma molecular weight markers for 18 hours at 50V and 120V for an additional 4 hours. Upon completion of electrophoresis, gels were removed and either stained to visualize proteins or transferred to membranes for sequencing or western blotting.

Gel Staining:

SDS-PAGE gels were stained for protein by placing them in a solution of 0.25% coomassie brilliant blue R250 in 50% methanol and 7.5% acetic acid under gentle agitation for 20 min. The coomassie staining solution was removed and the removal of background staining was accomplished by the addition of 100 ml of high methanol destaining solution (50% methanol and 7.5% acetic acid) for 20 min. with gentle agitation. The high methanol destaining solution was removed and replaced with two consecutive 100 ml changes of low methanol destaining solution (5% methanol and 7.5% acetic acid). During this process, the gels were gently agitated and Kim-wipes were added as stain absorbers. All gels were stored in the low methanol destaining solution at 27 ^{0}C.

Electrophoretic Transfer of High Molecular Weight Proteins from SDS gels to Millipore sheets:

The fractionated plasma proteins were transferred from the SDS polyacrylamide gels to Millipore filters using a BioRad semi-dry Trans-Blot apparatus. The gels were taken from the BioRad Protean IIsi and the stacking gels removed. The separating gels were then rinsed with double deionized water and allowed to equilibrate in Bjerrum, Schafer-Nielsen transfer buffer solution (48 mM Tris, 39 mM glycine, 0.375% SDS, 20% methanol) for 20 min. A blot sandwich was assembled by first placing an extra thick pre-soaked filter paper next to the anode. The bubbles were rolled out using a pipet. Next, the pre-wetted blotting media (membrane) was placed onto the filter paper, after which the bubbles were removed by rolling with a pipet. Then, the pre-equilibrated gel was

placed onto the stack, the bubbles removed, and a final piece of pre-soaked filter paper added. The cathode plate was placed onto the transfer stack and the apparatus connected to a BioRad model 200/2.0 power supply and transferred at 20V and 0.6A for 20 min. Following the transfer, the safety cover was removed and the filter papers discarded. The membrane was dried by placing it between two clean, dry sheets of filter paper. The gel was stained with 0.20% Coomassie blue as described previously to determine transfer efficiency.

Sequence Analysis:

The N-terminal amino acid sequence of the 116 kDa protein was determined by repeated cycles of Edman degradation in the Marshall Universtiy School of Medicine Core Facility using an Applied Biosystems (ABI) Model 473A automated protein sequencer.

Plasma proteins were fractionated by SDS-PAGE and transferred electrophoretically to a Millipore Immobilon-P membrane, as described above. The membrane was stained with Ponceau S stain to visualize protein bands and then destained in double deionized water with multiple changes for 15 min.

The band of interest was excised from the membrane and applied to a pretreated glass sequencing filter, which was placed in the reaction chamber of the 473A Protein Sequencing System. The protein was sequenced from the N-terminal end by means of an automated process based on Edman degradation reactions. The phenylthiohydantoin (PTH)- amino acid standards and PTH-unknowns were analyzed via chromatography by HPLC using a reverse phase C18 column. The reverse phase column was equilibrated

with 90% buffer "A" (5% tetrahydrofuran/H_20, 1.9% 3 M sodium acetate pH 4.6, 0.4% 3 M sodium acetate, pH 3.8) and with 10% buffer "B" (100% acetonitrile). The column was eluted with a gradient of buffer B. The eluted PTH-amino acids were detected via their UV absorption at 280 nm. Elution times between the PTH-amino acids and the PTH-standards were compared using Applied Biosystems Model 610A 1.2.1 analysis program. The sequence obtained was compared to known protein sequences using DNASTAR software. The sequencing protocol was tested by sequencing beta-lactalbumin.

Western Blot Staining and Immunoprobing for Complement Component C3:

Plasma proteins, fractionated by SDS-PAGE as described above, were transferred electrophoretically to Millipore HA membranes and probed with anti-complement component C3 antibodies by the technique of Towbin, et al (1979). The HA membranes were incubated at 37 ^{0}C for 1 hour in phosphate buffered saline (PBS), pH 7.5, (80 mM Na_2HPO_4, 20 mM NaH_2PO_4, 100 mM NaCl) containing 5% non-fat dried milk as a blocking agent. At the end of 1 hour the blocking solution was decanted and the blot was rinsed 5 times in PBS with 5-min. periods of agitation. Next the blot was probed with HRP (Horse Radish Peroxidase)-conjugated polyclonal anti-complement C3 antibodies (Cappel, Durham, NC) dissolved in a solution of PBS containing 5% non-fat milk for a period of 1 hour. This was followed by 5 more rinses of PBS with periods of agitation.

Bands containing C3 were then detected using the ECL detection system (Amersham) according to the manufacturer's instructions. Briefly, the two ECL detection reagents were mixed 1:1 and allowed to sit on top of the membrane for 1 min.

The excess detection reagent solution was drained away and the membrane was wrapped in transparent film and placed in an autoradiography cassette with Fuji x-ray film. The film was exposed for 1 min. in a darkroom and immediately developed using an Alphatek AX390 SE automatic film developer. Quantification of C3 bands was accomplished by densitometry on a Molecular Dynamics personal densitometer. Results were expressed in arbitrary units.

Membranes were stained to check for total transfer of protein by soaking them in 0.1% Buffalo black stain for a period of 15 min. The backgrounds were destained by 3 rinses of 15 min. each with Buffalo black destaining solution (450 ml MeOH, 10 ml acetic acid, 40 ml DD/H_2O). The blots were then dried between two pieces of clean filter paper.

Complement Component C3 Assays:

Complement component C3 activity in sera from lean and obese rats was assessed using a complement component C3 assay kit (Sigma Chemical Co.). Fresh serum samples were prepared for each assay. Blood, obtained by a tail bleed, was allowed to clot at room temperature. Before the start of the assay, all serum samples were diluted 1:500 with gelatin veronal buffer (GVB). Antibody-sensitized sheep erythrocytes were washed and suspended in GVB at a density of $1x10^8$ cells/ml. Increasing amounts of the diluted serum were added to a series of siliconized tubes containing 200 ul of the erythrocyte mixture and 5 ul of complement component C3-deficient serum. Enough GVB was present in each tube to give a final volume of 0.5 ml. After a 30-min. incubation at 37 ^{0}C in a shaking water bath, 1 ml of cold GVB was added to each tube.

The tubes were then centrifuged at 4000 x g for 5 min. at 4 ^{0}C. A 1 ml aliquot of each supernatant was read in a Beckman spectrophotometer (model 450) at 415 nm. Hemolytic activity was expressed as C3H50 U/ul. (Boggs, et al. 1998)

Electrophoretic Transfer of Low Molecular Weight Proteins from SDS Gels to Millipore Sheets:

Electrophoretic transfer to membranes for either sequencing or Western blotting was performed as described above with a few differences. These proteins were transferred to Millipore Immobilon-P^{SQ} membranes for better binding and retention of proteins with a molecular weight below 40 KD. In addition these proteins were transferred for 25 min. at 25V and 0.75A. Following transfer the membranes were dried between two layers of clean dry filter paper.

Sequence Analysis of Low Molecular Weight protein:

Sequence analysis was performed by Dr. Carol Beach at the University of Kentucky, Combs Center for Molecular Studies. Briefly, plasma proteins were fractionated by 15% SDS-PAGE and stained with Coomassie brilliant blue as described previously. The bands of interest were excised from 8 lanes, each containing plasma from a different Zucker rat and stored in low MeOH destain. Four of these excised bands were picked at random and sent to the Combs Center for analysis.

Protein in these gel bands was digested with trypsin. The mixture of tryptic peptides was then analyzed by HPLC using a reverse phase column utilizing an acetonitrile/propanol system. Two peak fractions from the HPLC analysis were selected and subjected to automated Edman degradation analysis from the N-terminal end. The

resulting sequences were analyzed and compared to a national online protein database to obtain an identity.

Western Blot Staining and Immunoprobing of low molecular weight proteins:

Blot staining to determine the success of protein transfer was done as described above with the use of Buffalo black stain and destaining solution.

Western blot analysis was done as described above with a few exceptions. Plasma proteins were fractionated by SDS-PAGE and transferred electrophoretically to Immobilon-P^{SQ} membranes. The membranes were then blocked in a solution of PBS and 5% non-fat dried milk for 1 hr. to minimize nonspecific binding.

Polyclonal anti-mouse apo-lipoprotein A1 antibodies (Rockland) were diluted 1:4000 with PBS and non-fat dried milk and allowed to incubate at room temperature with the blocked membrane for 2 hours. After being washed with PBS as described earlier, the membrane with bound primary antibodies was incubated with HRP-conjugated secondary antibodies diluted in PBS 5% non-fat milk solution at a dilution of 1:4000. The blot was incubated in this secondary antibody solution for 1 hour. Following incubation, the blot was washed 5 times in PBS and then visualized with the ECL detection kit (Amersham, Arlington Heights, Ill), as described above.

Genetic Backcrossing:

Genetic backcrossing with non-related male and female breeder rats was done to determine possible gene dosage effects with apolipoprotein A1. Lean male and female Zucker rats obtained from litters containing an obese littermate were bred with unrelated known heterozygous lean male and female rats. The known heterozygous males were

obtained from Charles River Laboratories, (Wilmington, MA), and were certified heterozygous breeders. The female breeders were unrelated females from the Marshall University School of Medicine colony, which had previously produced litters containing one or more obese pups.

A determination of heterozygosity was made if one or more obese pups were obtained from a resulting litter. The determination of lean homozygosity for the obese gene was made after three pairings or 25 offspring resulting in no obese pups. Obese homozygosity was readily apparent as these rats began to rapidly gain weight after 5 weeks of age.

Statistics:

Statistical analysis was performed using Sigma Stat statistical software (Jandel Corporation, San Rafael, CA). In each experiment comparisons were made between the means of data from lean versus obese phenotypes. Following assumption testing to ensure that the data met the assumptions for the statistical test in question, significance was determined using either a t-test or a Mann-Whitney Rank Sum Test.

Results I

In order to search for circulating plasma markers associated with obesity, we analyzed and compared protein expression in plasma from lean and obese rats by SDS-polyacrylamide gel electrophoresis. We identified proteins demonstrating differential expression through sequencing. Finally we confirmed the identifications through the use of specific antibodies.

Analysis of the one-dimensional SDS-polyacrylamide gels used to fractionate plasma samples from twelve week old lean and obese Zucker fa rats revealed a band that was more heavily expressed in the obese phenotype. This band migrated approximately the same distance as the 116 KD molecular weight marker (Figure 3). Densitometric analysis indicated that there was approximately 1.8 times as much of this protein in the plasma of obese rats as there was in the plasma of age-matched lean animals (Figure 4).

To determine whether there was a similar difference in the electrophoretic profile of plasma proteins in another rodent model of obesity, plasma samples from lean and obese LA/N fa^f rats were subjected to SDS-PAGE. A differential expression of a protein in the 116 KD weight range similar to that observed with the Zucker fa rats was also evident in the LA/N fa^f strain (Figure 5). Densitometric analysis of the gel presented in Figure 3 indicated that there was 3.6 times as much of this protein present in the plasma of rats with the obese phenotype as there was in plasma of rats having a lean phenotype (Figure 6).

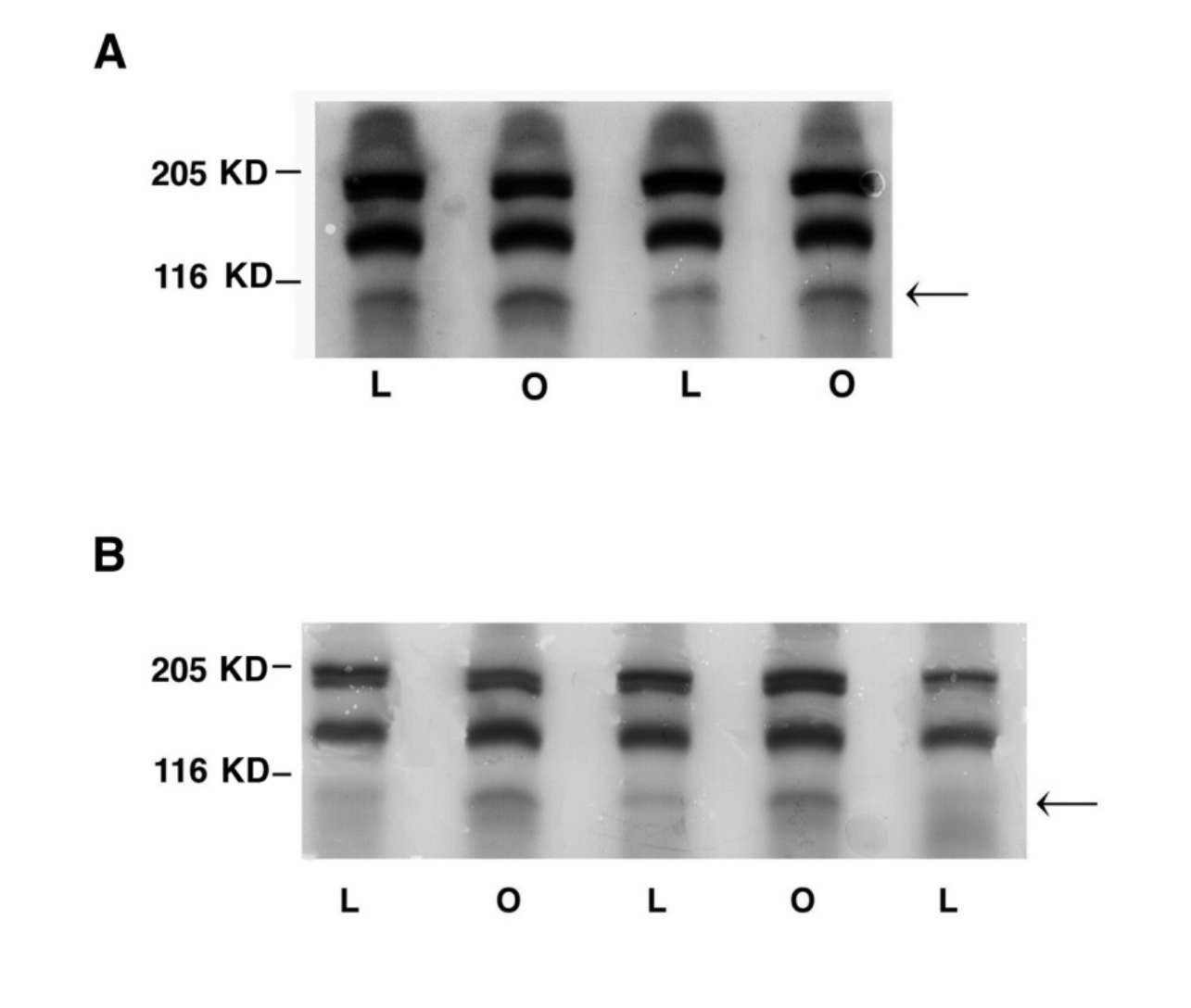


Figure 3. SDS-PAGE analysis of plasma from lean and obese Zucker fa rats. Plasma samples were heated in SDS sample buffer prior to being loaded onto the gels. Proteins were separated on 10% polyacrylamide gels and the resultant bands were stained with Coomassie Blue R-250 for visualization. A and B show two gels run under identical conditions. O = lanes containing plasma from obese rats; L= lanes containing plasma from lean rats; 205 and 116 indicate MW marker range.

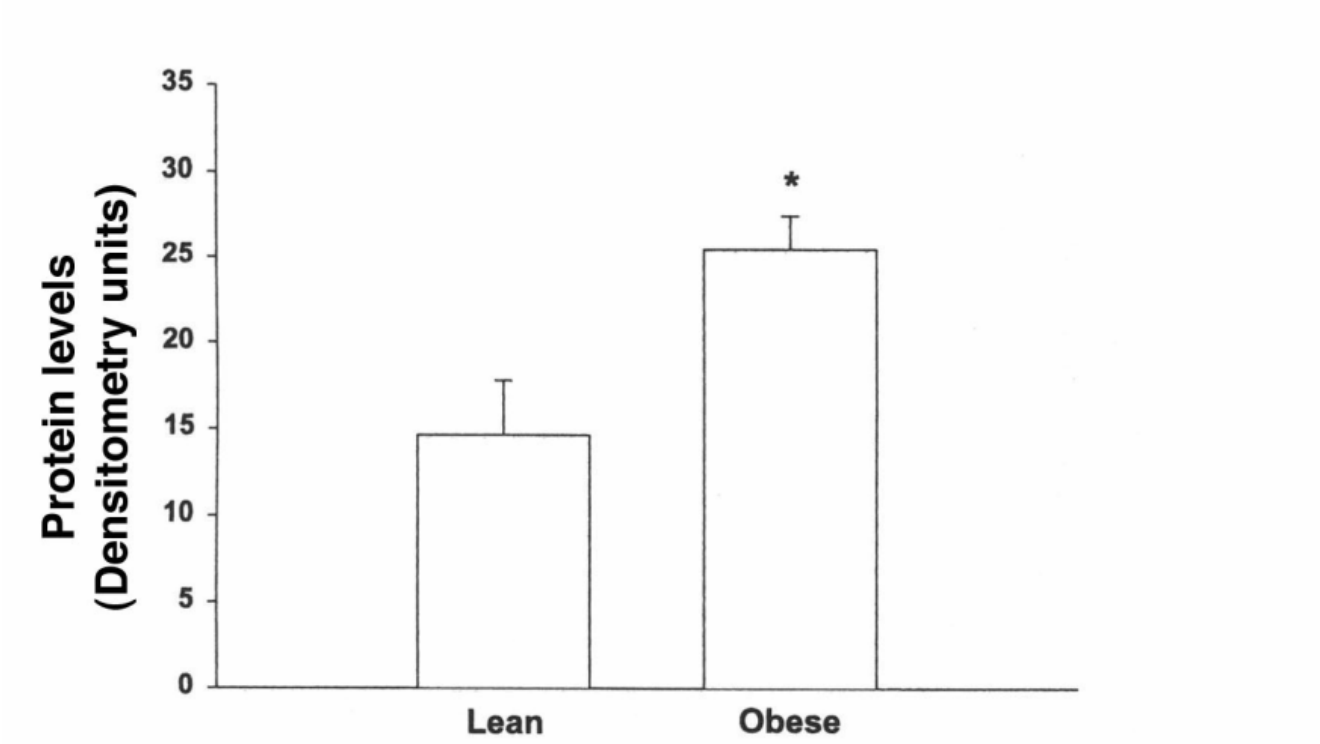


Figure 4. Summary of data obtained from densitometric analysis of gels shown in figure 3. Each column represents the mean $\pm$ SEM of all the lanes for a given group. Results are in arbitrary densitometric units., *$p<0.05$.

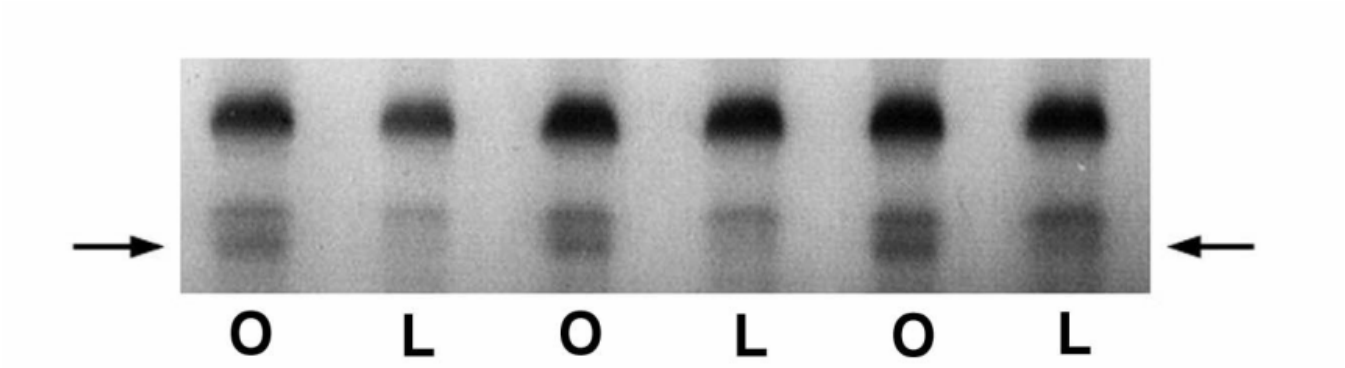


Figure 5. SDS-PAGE analysis of plasma from lean and obese LA/N fa^f rats. Plasma samples were heated in SDS sample buffer prior to being loaded onto the gels. Proteins were separated on 10% polyacrylamide gels and the resultant bands were stained with Coomassie Blue R-250 for visualization. O = lanes containing plasma from obese rats; L = lanes containing plasma from lean rats.

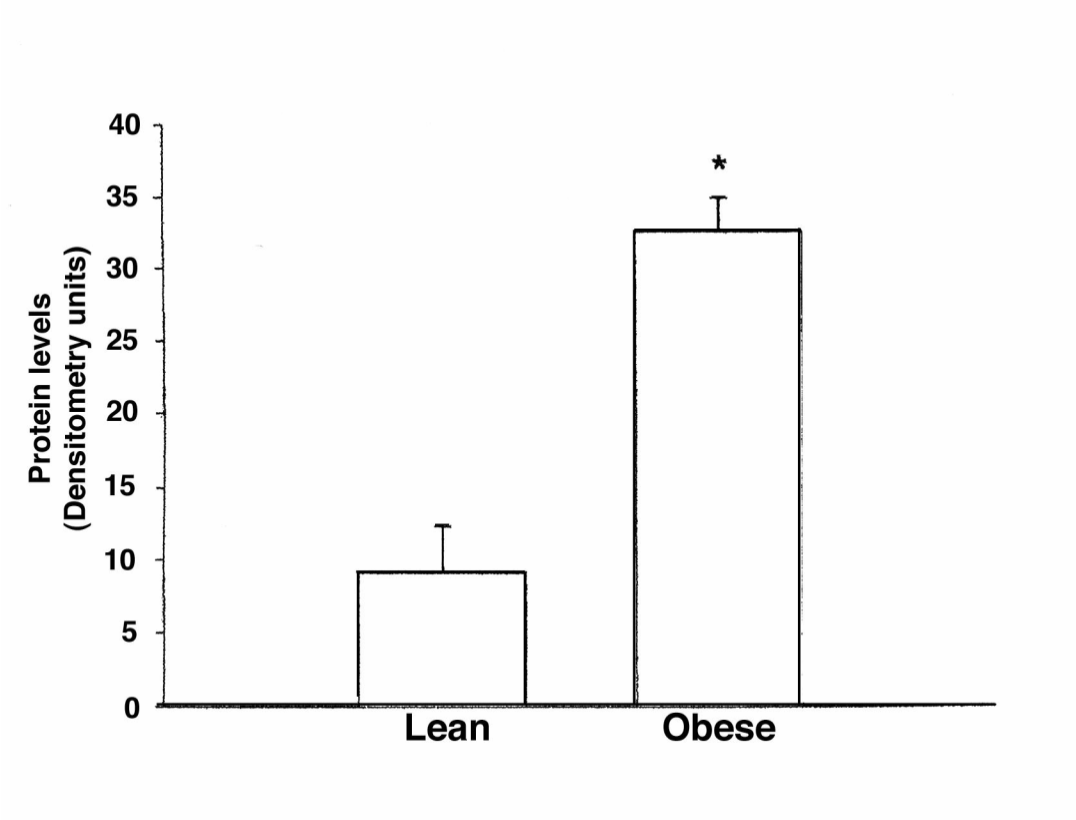


Figure 6. Summary of data obtained from densitometric analysis of the gel shown in figure 5. Each column represents the mean $\pm$ SEM of the 3 scanned lanes in each group. Results are expressed in arbitrary densitometric units, *$p<0.01$.

To identify the differentially expressed protein, plasma samples were fractionated and transferred electrophoretically to Immobilon-P membranes as described in the methods section. The band in question was excised and a partial amino acid sequence was obtained. Using DNASTAR software to compare the resultant sequence with known protein/peptide sequences, it was found that 17 of the first 18 amino acids at the amino terminus of the peptide were identical with the corresponding amino acids in the alpha chain of complement component C3 (Figure 7). Subsequently, western blot analysis was used to confirm the identity of the peptide. Following gel electrophoresis, the band was transferred to a nitrocellulose membrane and probed with polyclonal antibodies directed against rat complement component C3 (Figure 8). Densitometry scans of the resultant western blot indicated that there was three times as much immunoreactive complement component C3 in the plasma of obese Zucker rats as there was in the plasma of lean Zucker rats (Figure 9).

	1 5 9
C3, A Chain	H_2N-ser-val-gln-leu-met-glu-arg-arg-met-
Peptide	H_2N-ser-val-gln-leu-met-glu-ile-arg-met-

	10 14 18
C3, A Chain	asp-lys-ala-gly-gln-try-thr-asp-lys-
Peptide	asp-lys-ala-gly-gln-try-thr-asp-lys-

Figure 7. Sequence of the first 18 amino acids of the amino terminus of the differentially expressed protein compared with the corresponding region of the A chain of complement C3.

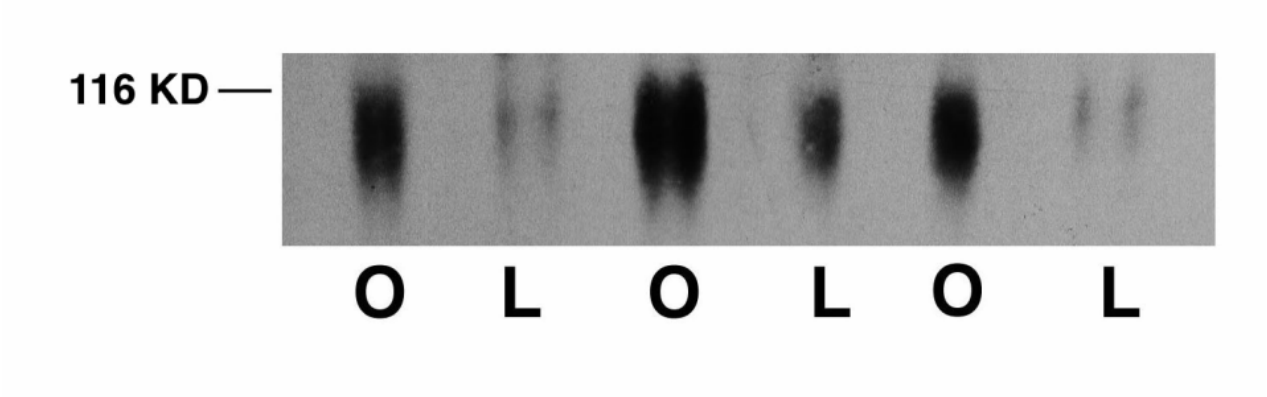


Figure 8. Western blot analysis of plasma from lean and obese Zucker fa rats. Plasma samples were subjected to SDS-PAGE as described in the methods, transferred to a Millipore HA nitrocellulose membrane and probed with a polyclonal antibody to rat Complement C3. Bands were visualized with Amersham's ECL detection kit. O = lanes containing plasma from obese rats; L = lanes containing plasma from lean rats; 116 indicates area of 116 KD MW marker in relation to band of interest.

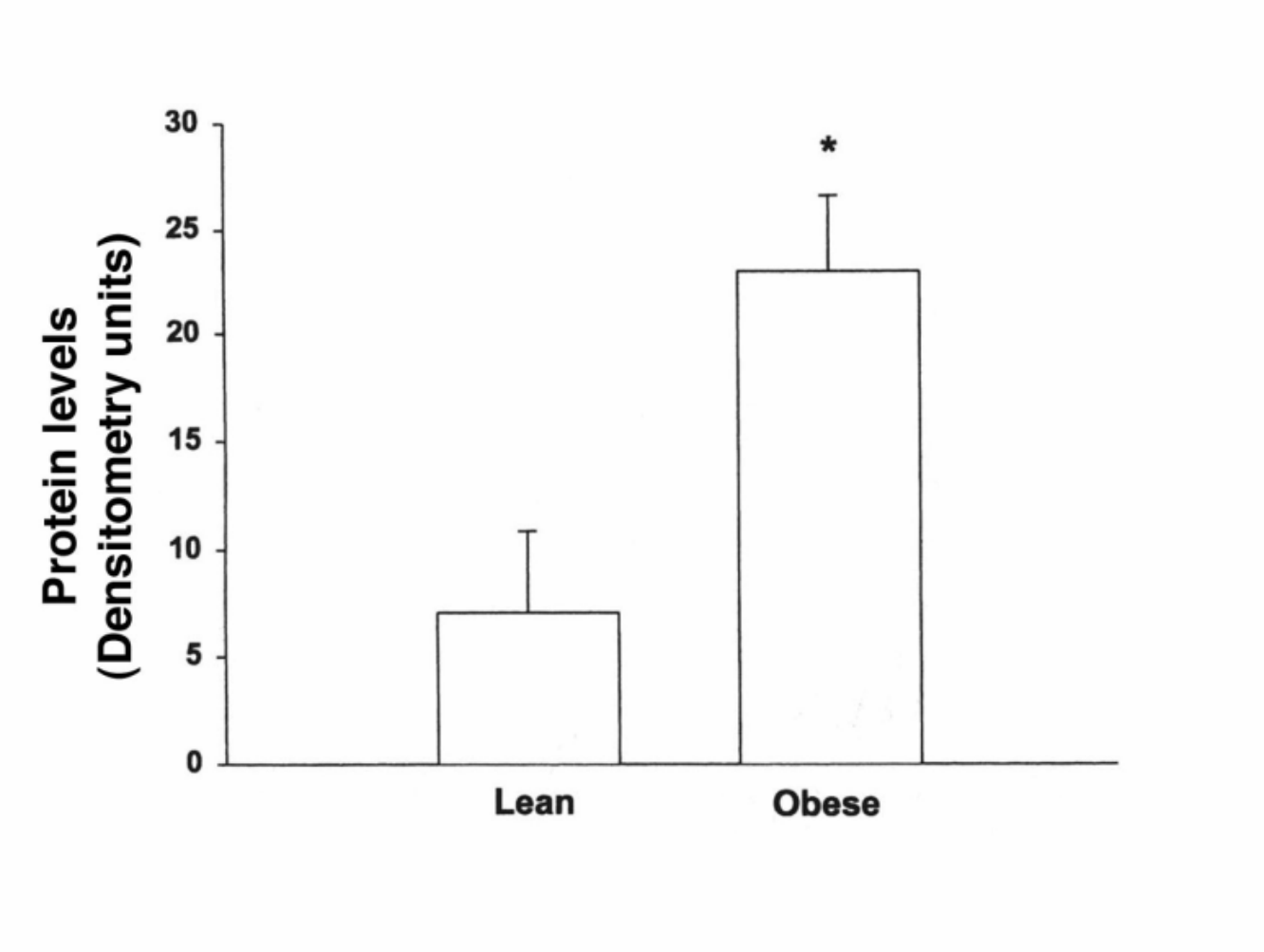


Figure 9. Summary of data obtained from densitometric analysis of the western blot shown in figure 8. Each column represents the mean $\pm$ SEM of the 3 samples in each group. Results are expressed in arbitrary densitometric units, *$p<0.05$.

Because the sequence determination and western blot analysis indicated that the differentially expressed peptide was the alpha chain of complement component C3, sera from lean and obese rats were assayed for complement component C3 activity to determine whether there was a corresponding increase in the activity of this complement component in the serum of obese rats. The assay, which assesses the capacity of the serum sample to promote the lysis of sensitized erythrocytes in a system containing a constant amount of complement component C3-deficient serum, indicated that the serum of obese rats had a hemolytic activity 1.8 times that of lean rats (Figure 10). These results suggest that the elevation in complement component C3 in the blood of obese rats detected by PAGE and western blot analysis represents a corresponding increase in functional capacity.

In an additional experiment, the mobility of the protein running at 116 KD in SDS-PAGE was compared with that of the α chain of purified complement component C3. A 10% polyacrylamide gel with lanes containing purified C3, obese Zucker rat serum, purified C3 plus obese Zucker rat serum, and C3-deficient serum was subjected to electrophoresis as described in the methods section (Figure 11). The alpha-chain of the purified complement component C3 traveled the same distance as the band presumed to contain the alpha-chain of C3 whether alone or in rat serum. Additionally, a band corresponding to complement component C3 a-chain was absent from the lane containing complement component C3-deficient serum.

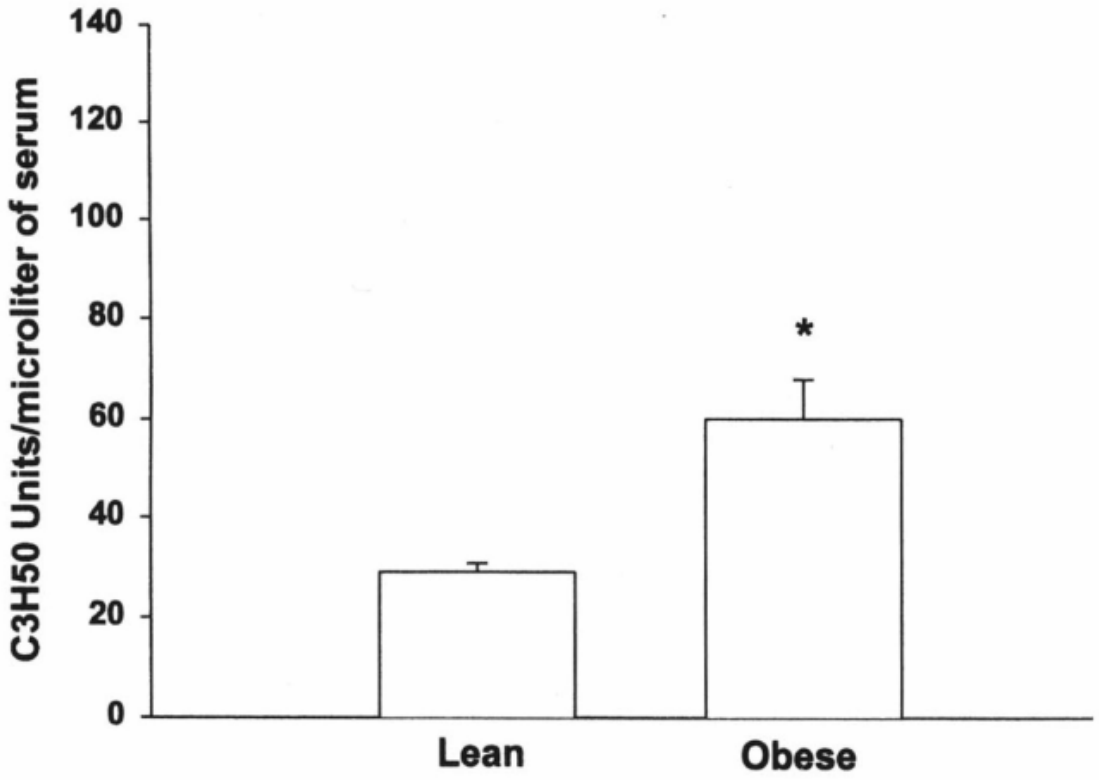


Figure 10. Complement C3 activity of sera from lean and obese Zucker fa rats. Assays were run on fresh samples as described in the methods. Bars represent the means and brackets the SEM of data from 14 obese and 17 lean rats. *$p<0.001$.

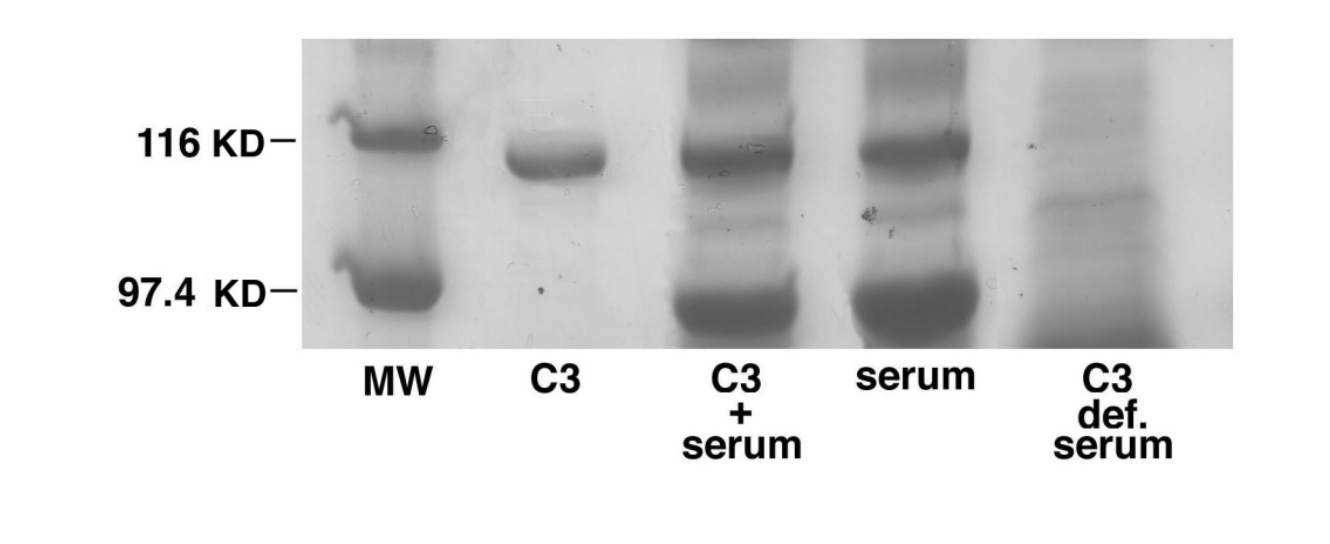


Figure 11. SDS-PAGE analysis demonstrating the relative positions of the 116 KD molecular weight marker, purified complement component C3, purified C3 in obese rat serum, obese rat serum alone, and C3 deficient human serum. Serum and C3 samples were heated in SDS sample buffer before being loaded onto 10% polyacrylamide gels. MW = molecular weight markers, C3 = purified complement component C3, serum = obese Zucker rat serum, C3 def serum = C3 deficient human serum. The numbers to the left of the gel indicate the 116 KD and 97.4 MW markers.

Results II

Analysis of plasma samples from lean and obese female Zucker rats by SDS-PAGE using 15% polyacrylamide gels and shorter electrophoresis times revealed another band which was more heavily expressed in obese rats than in their lean counterparts (Figure 12). The protein in this band appears to have a molecular weight of ~22 KD. Furthermore, the protein/peptide was either expressed at an intermediate level or appeared to be nonexistent in plasma samples from lean Zucker rats.

Since the initial discovery of this 22 KD protein band was made using plasma of female Zucker rats, additional studies were done to determine whether the differential expression was sex linked. To address this issue, samples from age-matched lean and obese male Zucker rats were analyzed by 15% SDS-PAGE electrophoresis as described in the materials and methods section. These plasma samples also demonstrated an increased expression of the 22 KD protein/peptide in obese rats compared to their lean counterparts. (Fig. 13) Densitometry scans of gels comparing lean and obese plasma samples indicate that there is approximately 2.5 times more of this protein in plasma from obese rats than in plasma from detectable lean rats. Samples from lean rats having undetectable levels of this protein are represented in the lean II column. (Fig. 14)

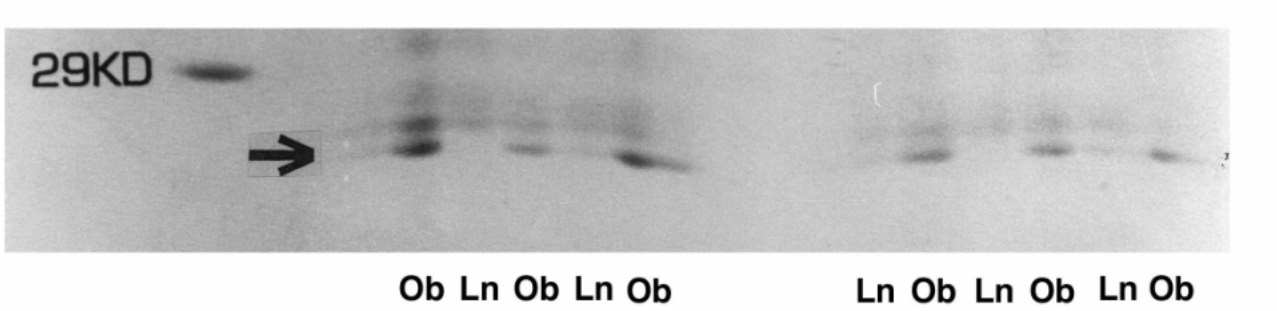


Figure 12. SDS-PAGE analysis of plasma from female lean and obese Zucker fa rats. Plasma samples were heated in SDS sample buffer prior to being loaded onto the gels. Proteins were separated on 15% polyacrylamide gels and the resultant bands stained with Coomassie Blue R-250 for visualization. Ob = lanes containing plasma from obese rats; Ln = lanes containing plasma from lean rats.

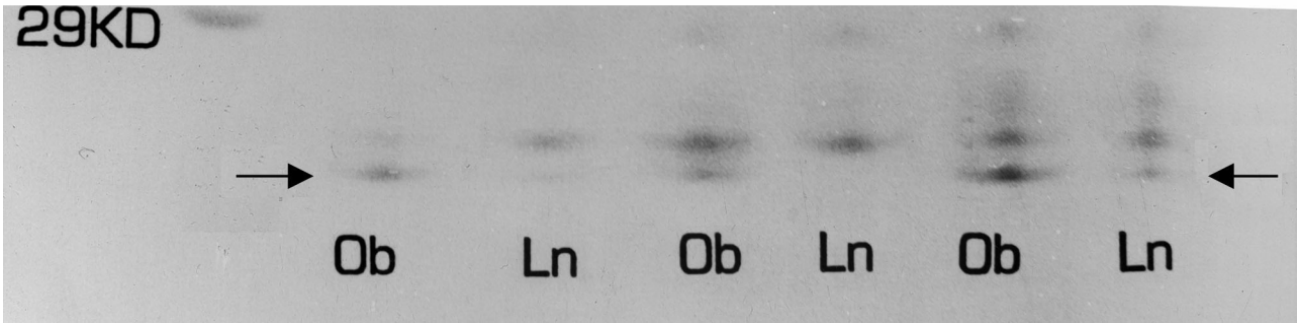


Figure 13. SDS-PAGE analysis of plasma from male lean and obese Zucker fa rats. Plasma samples were heated in SDS sample buffer prior to being loaded onto the gels. Proteins were separated on 15% polyacrylamide gels and the resultant bands stained with Coomassie Blue R-250 for visualization. Ob = lanes containing plasma from obese rats; Ln = lanes containing plasma from lean rats.

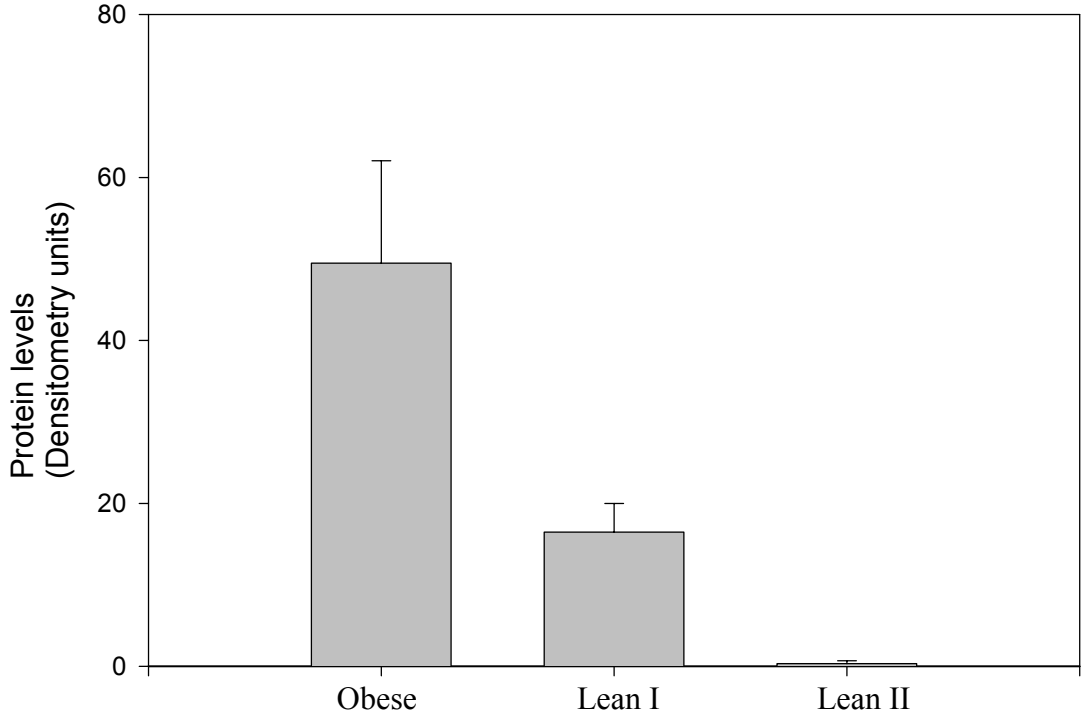


Figure 14. Summary of data obtained from densitometric analysis of 22 KD bands from SDS-PAGE gels. (Gel not shown). Columns 1 and 2 represent 10 samples in each group. Column 3 represents 5 samples. Group 1 contains samples from obese Zucker rats, column 2 represents samples from lean Zucker rats demonstrating reduced expression of this 22 KD protein and column 3 represents samples from lean Zucker rats demonstrating nondetectable expression of the 22 KD protein. Results are expressed in arbitrary densitometry units. $P \leq 0.05$

Since this 22 KD protein was expressed at high levels in obese Zucker rats and demonstrated either faint or non-detectable expression in lean Zucker rats, it was hypothesized that perhaps this was due to partial expression of the protein in heterozygous lean rats and no expression in homozygous lean rats. To determine if the level of the 22 KD protein expression could be used to predict rat genotype and to determine if partial expression were indeed occurring in a gene dosage fashion, backcrossing studies were performed as described previously. The schematic diagram in figure 15 describes the layout of these breedings. Heterozygous lean male and female rats were mated. Plasma samples obtained from the offspring of these crossings (F_1 generation) were analyzed by 15% SDS-PAGE as described in the methods section. Figure 16 shows the results from a litter of Zucker rat pups from one of these crossings. Plasma samples from known lean and obese (adult) rats and molecular weight markers were included for comparison purposes. Offspring from the F_1 generation were backcrossed with known heterozygotes as depicted in figure 15. The genotypic predictions shown in figure 16 were confirmed with backcrossing in the case of lean offspring. The pup predicted to have the obese genotype became overweight/obese after five weeks of age. The pups with partial expression of this protein were proven to be heterozygous lean. This is suggestive of a possible gene dosage effect with this protein, and demonstrates the possible predictive value of the expression level of this protein for determining rat genotype.

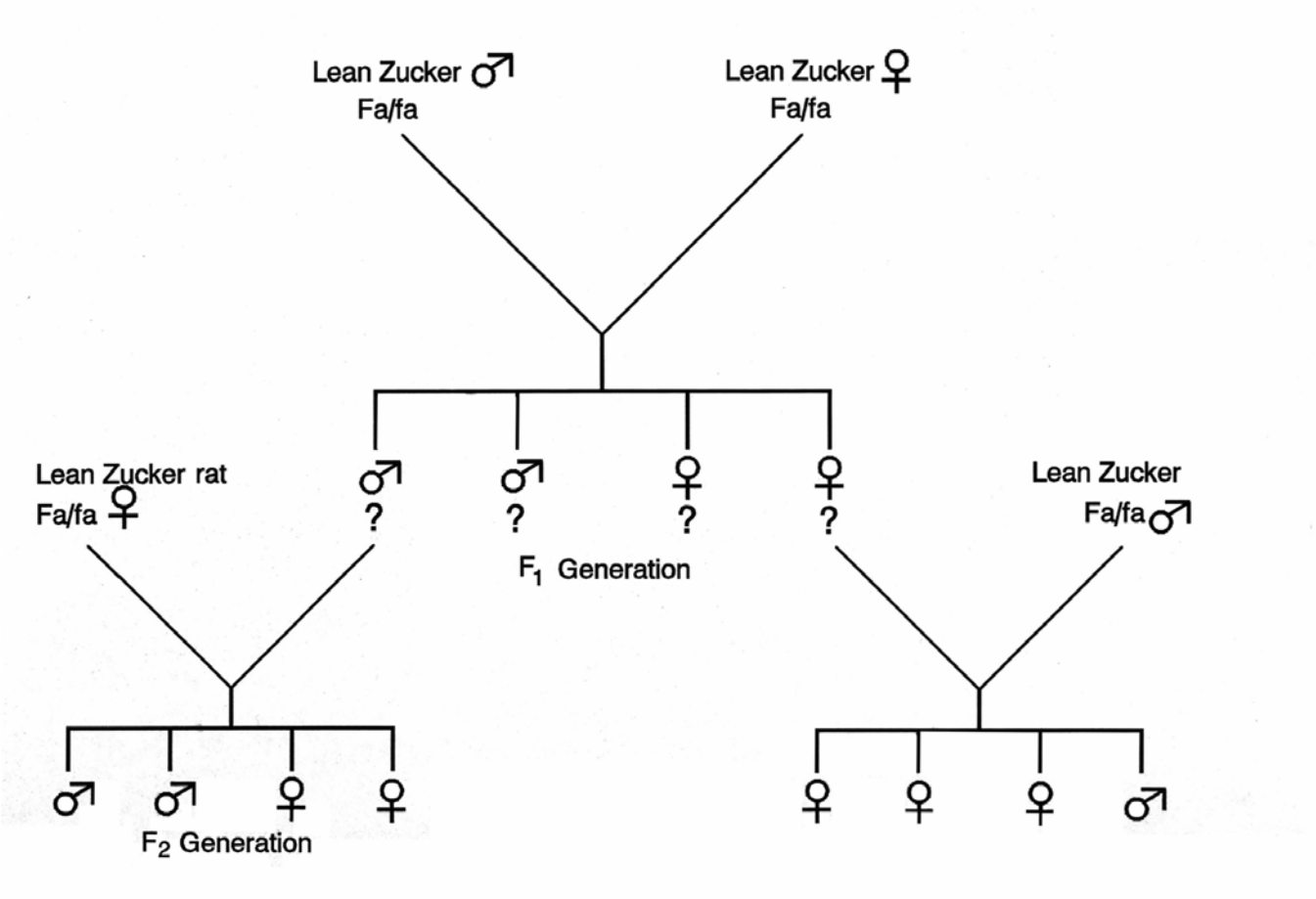


Figure 15. Schematic diagram representing genetic backcrosses to determine if the differentially expressed 22 KD protein band could be used as a predictor of obesity. All backcrosses took place with known heterozygous (Fa/fa) breeders. A backcross resulting in an obese pup and the unknown parent is labeled as Fa/fa, backcrosses not resulting in an obese offspring labeled the unknown parent as Fa/Fa after 3 backcrosses or 25 offspring.

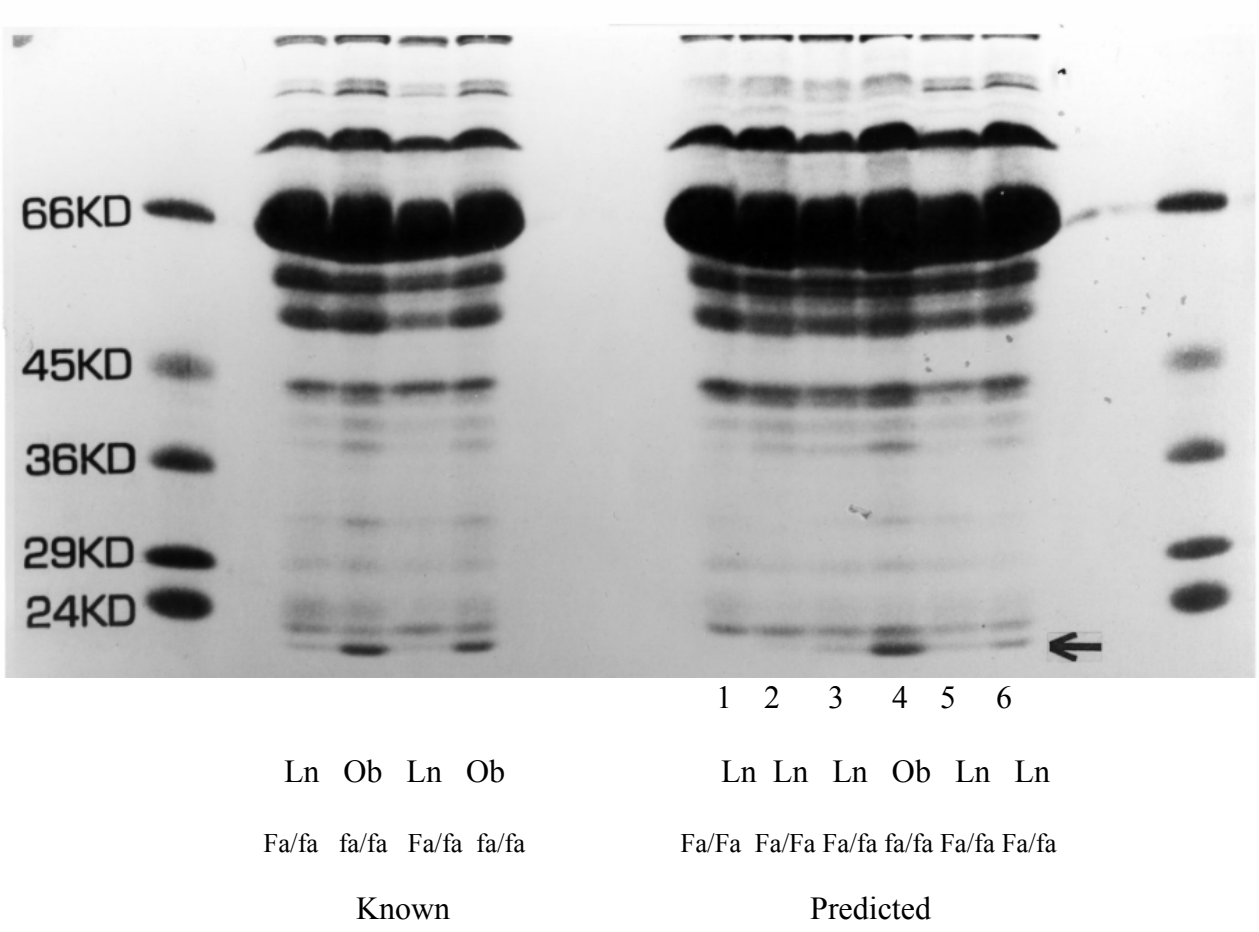


Figure 16. SDS-PAGE analysis of plasma from a litter of Zucker rat pups. All plasma samples from pups were obtained prior to 5 weeks of age. Plasma samples were heated in SDS sample buffer prior to being loaded onto the gels. Proteins were separated on 15% polyacrylamide gels and the resultant bands stained with Coomassie Blue R-250 for visualization. Arrow = band of interest, Ob = lanes containing sample from obese rats or from rats predicted to be obese, Ln = lanes containing sample from lean rats or rats predicted to be lean, Known = adult rat plasma samples, Predicted = sample from pre-five week old rat pups, Fa/Fa = homozygous lean, Fa/fa = heterozygous lean, fa/fa = homozygous obese. Arrow indicates 22 KD protein.

Table IV: Table demonstrating backcrossing data for the litter of Zucker rat pups analyzed in figure 16.

Animal	**Predicted Genotype**	**Crossings**	**Pups**	
			Lean	**Obese**
1	**Fa/Fa**	**3**	**22**	**0**
2	**Fa/Fa**	**2**	**26**	**0**
3	**Fa/fa**	**1**	**8**	**2**
4	**fa/fa**	**0**	**Became obese.**	
5	**Fa/fa**	**1**	**6**	**2**
6	**Fa/fa**	**2**	**8**	**1**

To identify this unknown protein band, plasma proteins from lean and obese Zucker rats were fractionated by SDS-PAGE on 15% gels as described in the methods section. The 22 KD band of interest was excised from lanes containing plasma samples from obese Zucker rats. The excised bands from 8 samples were randomized and a set of three bands was sent to Dr. Carol Beach, Ph.D. at the University of Kentucky Macromolecular Structure Analysis Facility.

The protein in the 3 excised bands was subjected to enzymatic digestion with trypsin and the resulting peptide mixture was analyzed by HPLC. The mixture was loaded onto a reverse phase column in a sodium acetate buffer system at a pH of 4.0 and

eluted with increasing concentrations of acetonitrile/propanol. Analysis of the resulting peaks indicated that each obese band contained approximately 50 picomoles of protein. A chromatogram of the HPLC analysis is shown in figure 17. Two peaks, 58.758 and 79.159 (marked by arrowns on figure 17), were selected for analysis by automated

Edman

Degradation reaction. The sequence analysis of these two peptide fractions is shown in figure 18. By comparing the sequences with the NCBI online national protein database it was determined that both sequences matched rat Apolipoprotein A1, an important protein involved in the body's fat transport system. This sequence was verified by sequence analysis performed by Dr. William Price with the Department of Chemistry, Marshall University College of Science using GC/Mass Spectrometry.

Western Blot analysis was used as a further confirmation of the protein's identity. Plasma samples were analyzed by SDS-PAGE utilizing 15% gels (as described in the methods section). The proteins were then transferred to Millipore Immobilon P^{SQ} membranes and probed with anti-apolipoprotein A1 antibodies as described in the methods section. An anti-apolipoprotein A-I positive band at 22 KD was more heavily expressed in the lanes containing obese rat plasma than in lanes containing lean rat plasma (figure 19). There also appeared to be a second fainter band below the 22 KD band in the lanes containing plasma from obese rats. This could be an immunoreactive degradation product of apolipoprotein A1. Another possibility for this faint band could be the existence of an apolipoprotein A-I isoform which is more heavily expressed in the obese animal. Densitometry analysis of the western blot indicates there is ~ 2 times the amount of apolipoprotein A-I in the obese samples compared to lean (figure 20).

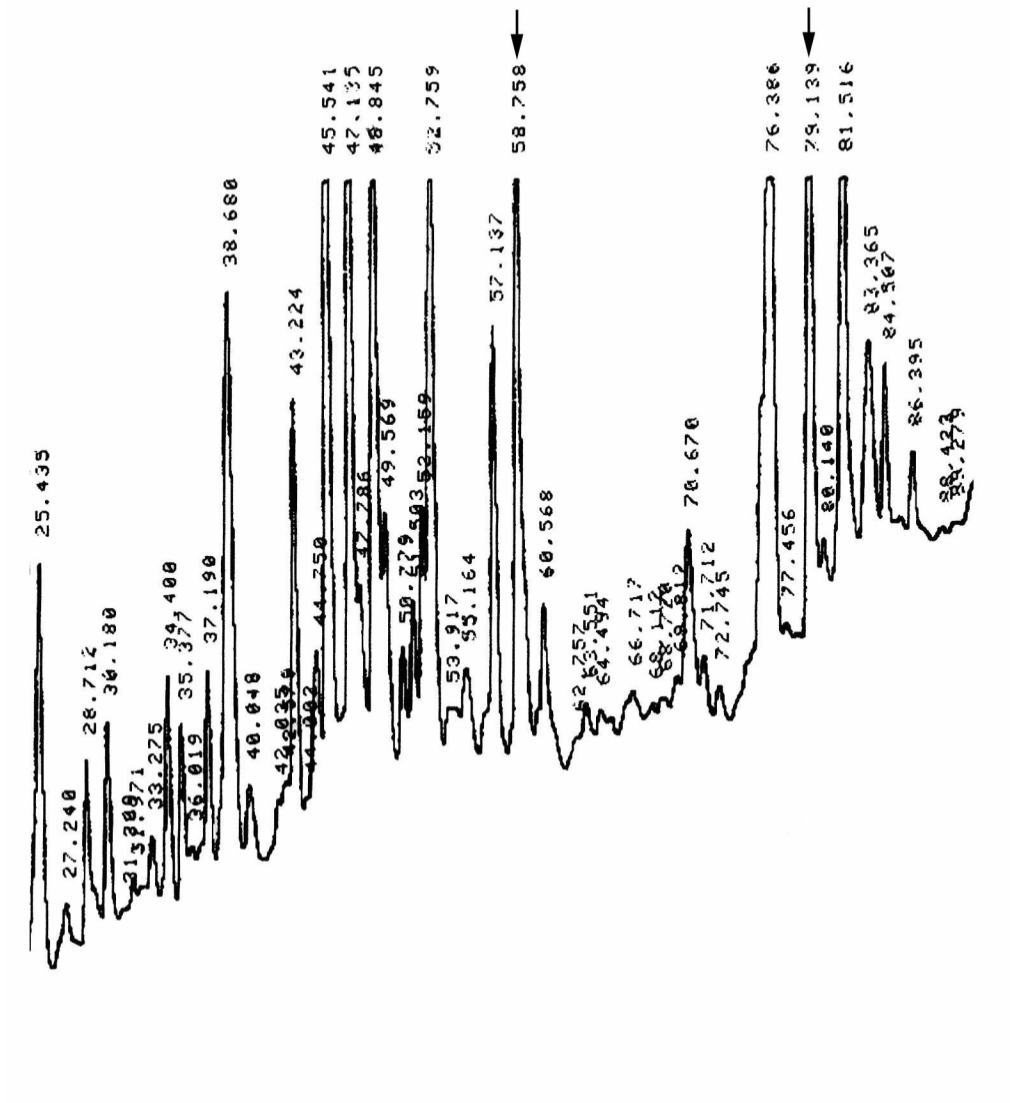


Figure 17. Chromatogram representing peak fractions obtained from the HPLC analysis of a trypsin digest of the 22 kDa protein band. Following digestion the peptides were resolved into fractions via HPLC analysis. The two arrows indicate the two fractions, 58.758 and 79.139, which were chosen for peptide sequencing.

Sequence analysis:

First Peak: Fragment from peak at position 58.758

N-term. Fragment Asp-Tyr-Val-Ser-Gln-Phe-Glu-Ser-Ser-Thr-Leu-Gly-Lys

Known Sequence Asp-Tyr-Val-Ser-Gln-Phe-Glu-Ser-Ser-Thr-Leu-Gly-Lys

51 63

Second peak: Fragment from peak at position 79.139

N-Term. Fragment Gln-Leu-Asn-Leu-Asn-Leu-Leu-Asp-Asn-Trp-Asp-Thr-Leu-Gly-Ser-Thr-Val-Gly

Known Sequence Gln-Leu-Asn-Leu-Asn-Leu-Leu-Asp-Asn-Trp-Asp-Thr-Leu-Gly-Ser-Thr-Val-Gly

64 81

Figure 18. Complete sequences obtained of the amino terminus of the two peptides selected for sequencing by Edman Degradation reaction. Both of these peptide fragments demonstrate a match for apolipoprotein A-I. The first fragment from peak position 58.758 matches the mature apolipoprotein A-I fragment from amino acids #51 to #63. The second fragment from peak position 79.139 matches the mature apolipoprotein A-I fragment from amino acids # 64 to 81. Peptide sequence provided by Dr. Carol Beach, University of Kentucky, using DNAstar software and the NCBI protein sequence database.

Sequences obtained from two independent laboratories and western blot analysis strongly support the conclusion that the protein in the 22 KD band is apolipoprotein A-I. Since apolipoprotein A-I is an important member of the lipid transport system, this could mean there is an alteration in this system in these obese rats.

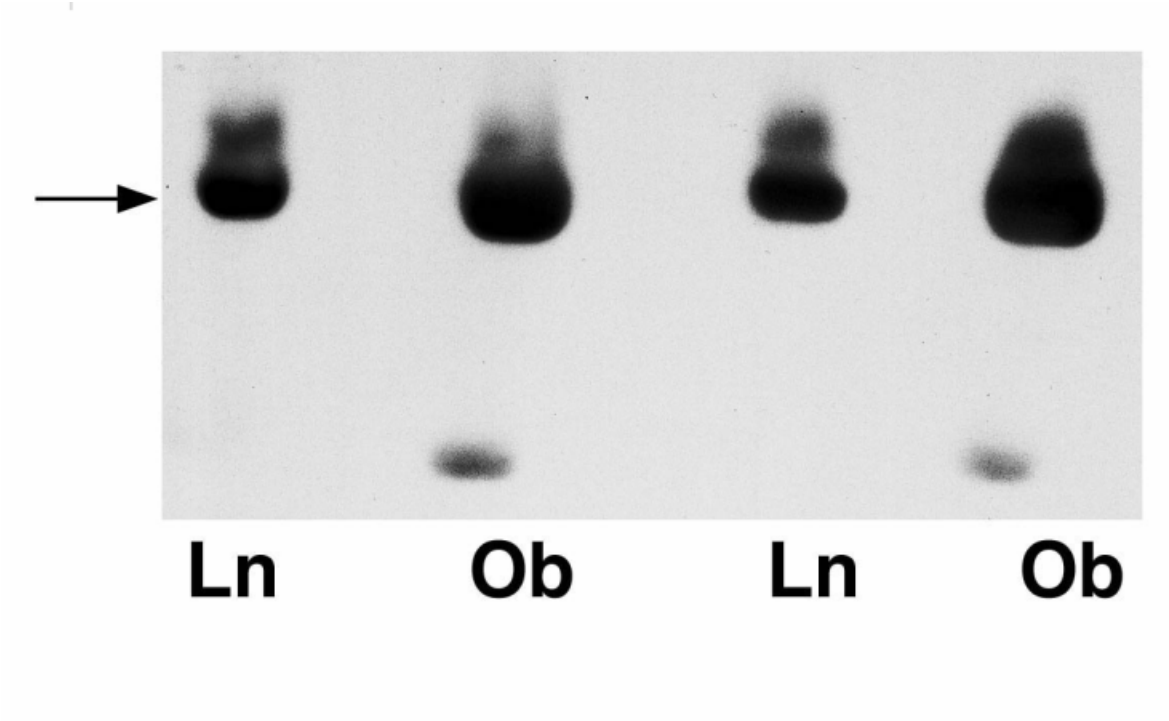


Figure 19. Western blot analysis of plasma from lean and obese Zucker fa rats. Plasma samples were subjected to SDS-PAGE as described in the methods, transferred to a Millipore Immobilon P^{SQ} nitrocellulose membrane and probed with a polyclonal antibody for apolipoprotein A-I. Arrows indicate the 22 KD protein. Note the faint bands below the bands of interest in obese samples.

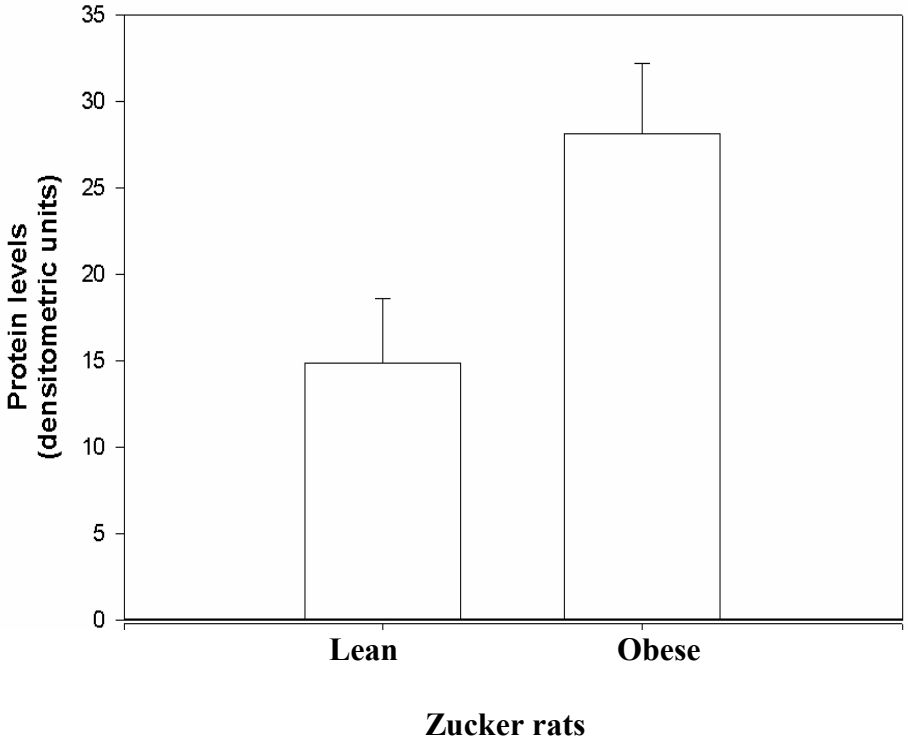


Figure 20: Densitometric scan of western blot in figure 19. Each column represents the mean $\pm$ SEM of the two samples in each group. The lean group includes detectable leans. Column 1 = lean Zucker rats. Column 2 = obese Zucker rats. Units are in arbitraty densitometric units. Densitometry analysis indicates there is an ~ 2 fold increase in apolipoprotein A-I in obese versus lean samples.

Discussion

We have demonstrated the differential expression of 116 KD and 22 KD proteins between obese and lean rats. These bands were subjected to sequence analysis and identified as complement component C3 α-chain and apo-lipoprotein A-I respectively. The protein identities were confirmed through Western blotting with specific antibodies. One protein is important in immune system function, the other in fat and cholesterol transport, both have an important role to play in obesity.

Complement Component C3:

The complement system is part of the body's immune system and is composed of a group of interacting proteins, which upon activation produces a cascade of reactions leading to the attack and destruction of foreign microorganisms. The complement cascade can be initiated in two ways, through the actions of an antigen-antibody complex (classical pathway) or through an antibody independent mechanism (alternative pathway). Complement component C3 is the pivotal member in both pathways (Frank & Fries, 1989).

The link between the immune system and obesity came with the discovery of adipsin, a 34 KD protein which is secreted primarily by adipocytes and which is decreased 58 to 80 percent in obese versus lean ob/ob and db/db mice (Rosen, et al, 1989). While adipsin was first identified as a gene product of differentiating adipocytes in culture with serine protease activities (Cook, et al, 1985), it was later found in the

bloodstream (Flier, et al, 1987). This protein has been demonstrated to be identical to complement factor D (Rosen, et al, 1989; White, et al, 1979).

Adipsin levels are low in several models of obesity, the ob/ob and db/db mouse (Flier, et al, 1987), the fatty Zucker rat (Johnson, et al, 1990) and monosodium glutamate induced obesity in mice. Overfeeding of normal rats has no effect on adipsin (Flier, et al, 1987). In Zucker rats, the reduction in adipsin was observed in both the circulating protein and in the amount of adipsin mRNA (Johnson, et al, 1990). Physiologic states resulting in the conditions of hyperglycemia and hyperinsulinemia, such as the infusion of glucose, suppresses adipsin while conditions resulting in insulin deficiency such as streptozotocin treatment result in increased levels of adipsin (Flier, et al, 1987). Levels of circulating adipsin have been found to be elevated in obese humans (Napolitano, et al, 1991).

Increased levels of circulating complement component C3 have been found in obese versus lean Zucker and LA/N-cp rats (Boggs, et al, 1998). The importance of this finding may lie in one of the cleavage products of complement component C3, C3a-desArg or acylation stimulating protein (ASP). ASP has been shown to stimulate triglyceride synthesis in human adipocytes and fibroblasts (Baldo, et al, 1993; Cianflone, et al, 1987). Like complement factor D, complement components C3 and B are all produced by adipocytes (Choy, et al, 1992) and together can activate the proximal part of the alternative pathway for complement activation.

Together these factors can work to locally activate the proximal part of the alternative pathway for complement activation. Proteolytic cleavage of complement component C3 to C3a and C3b is the initial reaction which activates the alternative

pathway. Complement factor B then forms a complex with C3b and is cleaved by factor D while complexed to form Ba and Bb. Bb remains associated with C3b and the resultant C3bBb acts as a C3 convertase. C3 convertase acts in a positive feedback manner to promote the cleavage of C3 to C3a and C3b (Frank and Fries, 1989). Subsequently, C3a is metabolized in the serum by carboxypeptidase N to form C3a-desArg or acylation stimulating protein. The cytokines TNF-α, IL-1, and IL-6 and interferon- g, increase complement factor expression in many cell types, including liver (Falus, et al. 1990; May, et al. 1988; Ramadori, et al. 1988; Perimutter, et al. 1986), endothelial cells (Ripoche, et al. 1988), skin fibroblasts (Katz, et al. 1989; Katz, Revel, et al. 1989), and monocytes (Lappin, et al. 1990). Adipose tissue also synthesizes both TNF-α and IL-6 which could promote further synthesis of complement component C3.

The data presented here indicate that circulating complement component C3 levels are elevated in two widely utilized models of genetic obesity. The elevated levels of C3 in the obese rat may represent a reservoir of substrate from which increased amounts of ASP could be produced. Also, the decreased levels of complement factor D/adipsin could also play a part in the increased levels of C3 as complement factor D is rate limiting in the formation of the C3 convertase in the alternate pathway. Interestingly, ASP levels have been shown to be elevated in obese women relative to non-obese controls in clinical studies (Sniderman, et al. 1991). These increased levels of ASP may mean that there is the possibility of increased levels of complement component C3 in some human obesities as well.

Apolipoprotein A-I:

Apolipoprotein A-I (apo A-I) is an important component of high-density lipoprotein particles. As such apo A-I plays an important part in the reverse cholesterol transport system, which is responsible for transporting cholesterol from peripheral tissues back to the liver for processing and breakdown. Apo A-I is produced by a variety of tissues in the body including adipose tissue and liver.

High Density Lipoprotein particles (HDL) are made up of apolipoprotein A-I and apolipoprotein A-II. Agarose gel electrophoresis of HDL particles has demonstrated that HDL exists in several subpopulations classified as pre-β- and αHDL (Kunitake, et al, 1992; Ishida, et al, 1987). Further analysis of pre-β-HDL by 2-dimensional polyacrylamide gradient gel electrophoresis separates it into several subclasses, pre-β_1, pre-β_2, and pre-β_3-HDL (Castro, et al, 1988). All of these subclasses of pre-β-HDL are thought to play a role in cholesterol transport with pre-β_1-HDL being the initial receptor of cholesterol from peripheral cells (Castro, et al, 1988; Miida, et al, 1992). By this theory the cholesterol is then transferred to pre-β_2, followed by pre-β_3 and then to α-HDL where it is esterified (Huang, et al, 1993; von Eckardstein, et al, 1995; Huang, et al, 1995; Kawano, et al, 1993; Shige, et al, 2000; Castro & Fielding, 1988; Francone, et al, 1989).

One theory for the formation of HDL suggests that free apolipoproteins, namely apo A-I, apo A-II, and apo E interact with cholesterol and phospholipid to generate small pre-β-HDL like particles (Sasahara, et al, 1997; Hara & Yokohama, 1992). Further, experiments involving short-term incubations with donor cells and human plasma have shown these particles to be the initial recipients of effluxed cellular cholesterol (Castro & Fielding, 1988; Miida, et al, 1992; Sasahara, et al, 1997). Additionally, two pathways

of cholesterol efflux have been identified involving pre-β-HDL, the first is directly proportional to the plasma pre-β-HDL concentration and involves a cell-surface protein. The second is a non-specific efflux mechanism, which is protease insensitive (Sasahara, et al, 1997; Kawano, et al, 1993).

There is quite a bit of controversy regarding the levels of Apolipoprotein A-I in obese versus lean human subjects. Sveger and colleagues determined, in their studies that there were no differences in apo A-I levels between age-matched boys and girls and in obese versus lean children. In both of these cases, almost identical levels of apolipoprotein A-I and apolipoprotein B were found (Sveger, et al, 1989). Previous studies had shown a difference between apo A-I and apo B levels in age-matched boys and girls with boys demonstrating higher levels of apo-A-I and girls demonstrating higher levels of apo B (Srinivasan, et al, 1986).

In 10 to 11 year old obese children, increased levels of apo B and a low ratio of apo A-I:B exist. Both of these conditions happen to correlate with increased incidence of coronary artery disease in adults. However, a positive correlation exists between the physical fitness of obese children and their apo A-I concentrations and apo A-I:B ratio (Schwartz, 1987). A reduction in cholesterol has been found in obese adolescents after exercise plus diet interventions (Becque, et al, 1988). Exercise affects primarily the reverse cholesterol transport system, whereas dietary weight loss has the greatest effect upon the VLDL and LDL delivery system, findings that may explain the normal apo A-I concentrations noted in some groups of children (Schwartz, 1987).

In studying obese subjects put on exercise regimens versus those placed on diets, it becomes clear that the plasma lipoprotein profile in these two groups are affected in

different ways. In the diet group, 25% of total weight loss came from fat free mass. It is also apparent that weight loss affects mainly the transport of cholesterol and triglycerides to cells as very low and low-density lipoproteins. Weight loss has definitely been shown to improve the plasma lipoprotein profile in obese subjects (Wolf, et al, 1983; Dattilo, et al, 1992; Yamashita, et al, 1998). The elevated plasma triglyceride, cholesterol, and low-density lipoprotein (LDL) cholesterol levels in obese subjects generally decrease after weight reduction. Paradoxically, HDL cholesterol commonly decreases with weight reduction in the short term, especially during active weight loss (Wolf, et al, 1983; Dattilo, et al, 1992; Yamashita, et al, 1998).

Aerobic exercise appears to more directly affect the proposed HDL-mediated reverse cholesterol transport system by increasing both HDL-C and apo A-I proportionately (Schwartz, 1987). HDL-C was increased after diet or exercise but apo A-I increased only in the exercise groups (Schwartz, 1987).

High-density lipoprotein (HDL) plays an important role in the process of reverse cholesterol transport, which may become sub-optimal with increasing fatness (Shige, et al, 2000). In many obese subjects there are disorders of plasma lipid transport (Schuller, et al, 2000). While there is disagreement about the levels of apo A-I in obese versus lean humans, there is also disagreement concerning apo A-I levels in obese and lean Zucker rats. Studies comparing the gene expression of apo A-I, apo A-IV, and apo C-III among the lean and obese rats of three genetic strains, both on normal and high fat oil diets have been conducted. These studies have indicated that no difference exists in the level of apo A-I gene expression between the lean and obese Zucker rat (Schuller, et al, 2000). This is contrary to discoveries made in our lab of the circulating levels of apo A-I in obese

versus lean Zucker rats. We demonstrated increased plasma expression in obese versus lean Zucker rats of a 22 kDa peptide (Boggs, et al, 1995). Upon sequencing the protein in this band, it was found to be apo A-I.

It has been theorized that apo A-I turnover is regulated by obesity and/or leptin signaling. In the ob/ob mouse and db/db mouse, there are markedly elevated levels of plasma HDL-C and apo A-I was found to be increased 1.3 fold (Silver, et al, 1999). At the same time, the apo A-I mRNA was found to be markedly decreased, prompting Silver and his colleagues to postulate that perhaps a defect in apo A-I catabolism was to blame and that the decrease in apo A-I mRNA was due to the buildup of apo A-I. This hypothesis was confirmed using radiolabels showing a decrease in catabolism and a 4-fold lower hepatic uptake of HDL. Additionally, leptin treatment restored apo A-I mRNA in ob/ob mice to normal levels and reduced HDL cholesterol (Silver, et al, 1999).

A similar phenomenon may be responsible for the altered apo A-I levels we have observed in the Zucker fatty rat. While the Zucker fatty rat does not suffer from a deficit of leptin, it does have a mutation in the leptin receptor, which may possibly interfere with the signaling necessary to initiate HDL catabolism. Additionally, the confusion resulting from conflicting reports of apo A-I levels in Zucker rats may have more to do with HDL size than expression. HDL in some obese subjects exhibiting hypertriglyceridemia have been shown to be smaller and more susceptible to renal filtration (Horowitz, et al, 1993). Thus, in some Zucker rats, HDL size may vary and though a defect in catabolism is present, increased renal filtration may remove some HDL.

While the Zucker fatty rat has other abnormalities besides the defect in leptin receptors, such as hyperinsulinemia, hyperphagia, hypertriglyceridemia, and obesity;

comparisons among other rodent obesity models such as the LAN-cp model and the OB/OB mouse eliminate some of these other factors from the genetic background and allow a different view of the action of defects in this gene. Several obese diabetic mouse models studied do not have elevated HDL levels proving these observations are not caused by the obesity and diabetes at least in the models studied demonstrating that obesity in and of itself does not always cause elevated HDL or apo-lipoprotein levels (Silver, et al, 1999; Huszar, et al, 1997).

Further studies may hold the key to the question of does obesity usually result in suboptimal resverse cholesterol transport, while in the Zucker fatty rat there appears to be an abundance of HDL, the primary transport vehicle. Several studies have concluded that, at least in humans, there is an altered distribution of isoforms of HDL. These studies suggest that there is increased pre-β_1-HDL in obese human subjects versus lean human subjects. One possibility for this may be a diminished transformation of pre-β_1-HDL to pre-β_2-HDL which could explain this buildup (Sasahara, et al, 1997). This would also explain the suboptimal reverse cholesterol transport and lower levels of HDL-C in some human and rodent obese subjects.

This research demonstrates that Zucker fatty rats have an increased level of apolipoprotein A-I, whether in the form of α-HDL or another subfraction such as pre-β_1-HDL. This might be indicative of a decreased catabolism of HDL due to some defect, possibly involving leptin as there does not appear to be an increased expression of apo A-I mRNA. Also, HDL size differences in obese subjects exhibiting hypertriglyceridemia need to be investigated as this could lead to increased loss of HDL due to renal filtration

and possibly lead to the conclusion that a roughly normal level of apo A-I exists in circulation when in fact higher levels are being maintained.

This highlights the need for further research into the distribution of apo A-I in subfractions of HDL in obese Zucker rats and human subjects as well as effectiveness of reverse cholesterol transport. Further studies are needed to investigate the possible gene dosage effects of the gene for apo A-I in the Zucker rat model of obesity to investigate its potential as a predictor of obesity in pre-obese young. Evidence suggestive of this possibility is demonstrated in the analysis of plasma from pre-obese Zucker rat pups in figure 16, which demonstrates a differential expression of this protein between heterozygous lean and homozygous lean Zucker rat pups. Furthermore, studies need to be conducted regarding the interaction of pre-β subfractions of HDL with adipocytes from obese Zucker rats and humans to determine possible roles in stimulating cell surface receptors and membrane bound enzymes in these cells.

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Objective

A key management position in a goal oriented company with responsibilities in quality, planning, and/or operations management.

Qualifications

Eight years of management experience in controlled laboratory and industrial environments in a Fortune 500 company.
Experience supervising both hourly and salaried professionals.
Experience in quality systems and laboratory operations.
Advanced degree in Biomedical Sciences.
Excellent communication, analytical, and leadership abilities.
Excellent problem solving skills.

Professional Experience

Section Manager, Quality Control 2002 – Present
AK Steel Corp., Ashland, Kentucky
Manage Product Metallurgists and Process Engineers in developing process improvement plans and changes to directly affect plant quality. Track quality numbers (defects, cost of quality, corrective actions, process changes and effectiveness of corrective actions). Lead ISO and QS 9000 audits for the plant.

Section Manager, Chemistry 2001 – 2002
AK Steel Corp., Ashland, Kentucky
Managed a group of 30 employees including chemists, laboratory managers, analytical technicians, and an electrical engineer.
Maintained a budget of 3 million dollars per year.
Led cost control measures resulting in being under budget with more output.
Responsible for laboratory modernization project, in which I evaluated and sourced instrumentation for the labs including new LECO's, Optical Emission Spectrometers, XRAY fluorescence spectrometers, ICAP plasma spectrometers and GC chromatography instruments.
Worked with Environmental Department to attain ISO-14001 certification (the first steel plant in US to obtain).

Laboratories Manager 1995 – 2001
Led ISO 9000/QS 9000 audits for QC department.
Responsible for day to day operations of laboratories.
Worked with IR group in managing a mixed group of salaried and hourly personnel.
Established quality crosscheck programs.
Responsible for tracking instrument and equipment maintenance in QC group.
Reorganized labs for cost containment while increasing output.

Graduate Assistant 1993 – 1995
Marshall University School of Medicine, Huntington, West Virginia
Set up and taught laboratory courses in biochemistry, microbiology and genetics.
Studied receptor chemistry, proteomics, and molecular biology.
Antibody studies including manufacture and isolation of polyclonals in animal models.
Protein purification, sequencing, and identification.

Mill Technician 1990 – 1993
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Education

Bachelor of Science Degree in Biology/Chemistry. 1990
Georgetown College, Georgetown KY.

Doctor of Philosophy Degree in Biomedical Sciences 2003
Marshall University School of Medicine, Huntington, WV.

Publications

Boggs RD, McCumbee WD, Cobbs SL, et al: Increased expression of complement component C3 in the plasma of obese Zucker fa and LA/N fa^f rats compared with their lean counterparts. Obes Res 6(5): 361, 1998.

Boggs RD, McCumbee WD, Reichenbecher VE: Characterization of low molecular weight plasma protein differences in lean and obese Zucker rats. Proc WV Acad Sci 67(1): 24, 1995.

Interests

Foreign cultures, traveling, outdoor sports, history, tech gadgets, hiking, photography, astronomy, boating, entertaining.