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Role of Dietary Carbohydrate Source in the Development of Obesity in Rodent Models of Diet-Induced Obesity

Kristin Lynnet Morris
University of Tennessee, Knoxville

To the Graduate Council:

I am submitting herewith a dissertation written by Kristin Lynnet Morris entitled "Role of Dietary Carbohydrate Source in the Development of Obesity in Rodent Models of Diet-Induced Obesity." I have examined the final electronic copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Human Ecology.

Michael R. Zemel, Major Professor

We have read this dissertation and recommend its acceptance:

Naima Moustaid-Moussa, Jay Whelan, Michael Karlstad

Accepted for the Council:

Carolyn R. Hodges

Vice Provost and Dean of the Graduate School

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

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We have read this dissertation
and recommend its acceptance:

Naima Moustaid-Moussa, Ph.D.

Jay Whelan, Ph.D.

Michael Karlstad, Ph.D.

Accepted for the Council:

Anne Mayhew
Vice Provost and Dean of Graduate Studies

ROLE OF DIETARY CARBOHYDRATE IN THE DEVELOPMENT OF OBESITY IN RODENT MODELS OF DIET-INDUCED OBESITY

A Dissertation
Presented for the
Doctor of Philosophy
Degree
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Kristin Lynnet Morris
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DEDICATION

This dissertation is dedicated to my parents, Frank and Shirley Morris, and my sister, Alissa Morris Wolfe, whose love, patience, and support made this undertaking, and many others, possible.

It is also dedicated to my niece and nephew, Tatum and Wilson Wolfe, who continually boost my spirits.

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ABSTRACT

Dietary macronutrient composition plays a critical role in health and disease. The contribution of dietary carbohydrate source in the development of obesity and related diseases is often given minimal attention. The present studies demonstrate that both quantitative and qualitative changes in dietary carbohydrate influence body composition and adiposity in a rodent model of diet-induced obesity. In heterozygous (*fa*/+) Zucker rats, consumption of a high-fat, carbohydrate-free diet *ad libitum*, attenuated weight gain and adiposity by increasing energy efficiency and blunting expression of fatty acid synthase, a key enzyme in *de novo* lipogenesis. These effects were independent of significant changes in plasma insulin levels. Moreover, the addition of a modest level of sucrose to the high-fat diet completely reversed the effects of carbohydrate restriction, resulting in significant increases in body weight and adiposity, mediated in part by enhanced expression of fatty acid synthase.

In a separate series of experiments, we evaluated the effects of *ad libitum* or energy restricted (70% of *ad libitum*) high-fat diets, varying in carbohydrate source on adiposity in aP2-*Agouti* transgenic mice. In the context of an energy restricted diet, animals consuming diets shown to result in lower postprandial blood glucose levels (ROLL and MUNG) reduced adipose tissue accumulation in the perirenal and retroperitoneal and resulted in smaller adipocytes compared with diets evoking greater postprandial blood glucose excursions.

In *ad libitum* fed animals, the expression of lipogenic enzymes in the liver and selected adipose tissue depots was significantly enhanced by consumption of a high-fat, sucrose-rich diet. Expression of genes of fatty acid oxidation was enhanced in the muscle of

animals consuming the low-glucose response diets. These data suggest that dietary carbohydrate source modulates adipose tissue accumulation and body weight by partitioning substrate utilization between lipid oxidation in skeletal muscle, and reducing lipogenesis in visceral adipose tissue.

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PART 1

INTRODUCTION

I. Introduction

The World Health Organization (WHO) criteria for the classification of overweight and obesity are based primarily on the association between body mass index (BMI, kg/m^2) and mortality. Overweight is classified as having a BMI of 25 - 29.9. A BMI greater than 30 is indicative of obesity (1). Data from the Third National Health and Nutrition Examination Survey (NHANES III, 1988-1991) demonstrate that age-adjusted prevalence of overweight in the United States was 55.9%, while the prevalence of obesity was 22.9% (2,3). However, more recent data indicates the prevalence of overweight increased to 64.5% in 1999-2000, while the prevalence of obesity increased to 30.5% during the same period (4). Specifically, 67% of men and 62% of women were overweight, while 30.5% of men and 33.4% of women are obese (4). Furthermore, the prevalence of overweight among children and adolescents is continuing to increase. In 1999-2000, more than 15% of 6- through 19-year olds were overweight, while more than 10% of 2-through 5-year olds were overweight (5). In addition, childhood obesity is on the rise as documented by prevalence statistics, which identify 25% of children as overweight (6).

Obesity is second only to cigarette smoking as a cause of death in the United States and is cited as a contributing factor in more than 300,000 deaths annually (7-12). Allison, et al. (7) analyzed data from six US studies in conjunction with 1991 national statistics on BMI distribution, population, and overall death rates and consistently estimated approximately 280,000 annual deaths attributable to overweight and obesity, either directly or indirectly through related metabolic abnormalities known to occur concomitantly with excess body weight. The largest proportion of these deaths occurred

in persons with a BMI of at least 30 kg/m^2 (7), which is approximately 30% of adults age 25 and over according to the most recent data from the NHANES continuous survey (4). Data from the Framingham Heart Study indicates that the risk of death increases by 1% for each pound gained during ages 30 and 42 years, and by 2% between ages 50 and 62 years (11). Furthermore, data from the Health Professionals Follow-Up Study demonstrate a positive, linear relationship between BMI and overall and cardiovascular disease mortality among men less than 65 years (12). Among men over 65 years, waist circumference was a stronger predictor of risk of death from cardiovascular disease (12).

Obesity and related co-morbidities have a significant economic impact, accounting for 2-7% of total health care costs in the United States (13,14). Health care costs for overweight individuals are more than four thousand dollars greater than those of lean subjects subjects. Obese individuals spend roughly $10,000 to 40,000 more for lifetime health care than their lean counterparts (13).

Despite numerous studies demonstrating a positive association between dietary fat intake and body fat (15-18), the role of dietary fat in the pathogenesis of obesity remains controversial (19-24). Dietary intervention studies indicate that only 2-25% of the variation in body weight and composition can be explained by dietary fat intake (19-21); a finding variably cited in arguments both for and against limiting dietary fat intake in the prevention and treatment of obesity. While data from the Third National Health and Nutrition Examination Survey (NHANES III, 1988-1991) (25) demonstrated a significant decrease in the mean percentage of total food energy intake (TFEI) and saturated fat since the 1960s, the prevalence of obesity, non-insulin-dependent diabetes mellitus and related disorders increased during the same period (3-6). These findings

suggest that excessive intake of dietary fat is not the only dietary factor influencing body weight regulation.

Despite the controversy regarding dietary fat consumption, major professional organizations almost universally recommend consumption of a diet rich in fruits and vegetables, low-fat or non-fat dairy products, cereals and whole grains, legumes, nuts, fish, poultry and lean meat, while restricting total energy derived from dietary fat to less than 30 energy percent (en%), and saturated fat restricted to less than 10% of total daily energy (26-32). With regards to dietary carbohydrate, the American Heart Association (AHA) recommendations (28,31) encourage the consumption of grain products and foods high in soluble fiber, while stating starchy-foods (eg, bread, pasta, cereal, potatoes) are preferable to sugars (monosaccharides and disaccharides) (32). The American Diabetes Association (ADA) recommends carbohydrate from foods such as whole grains, fruits and vegetables (30). However, with regards to glycemic index, ADA recommendations state that the total amount of carbohydrate in meals and snacks is more important than the source or type of carbohydrate (30).

Diets based on the recommendations of the AHA and ADA generally provide between 20 and 30% of total energy from fat and 55 to 60 % of energy from carbohydrates (26-32). While such diets are typically energy restrictive and may reduce the risk of obesity and related disorders, the appropriateness of this type diet for all individuals is not universally accepted (33-35). Low-fat, high-carbohydrate diets have been shown to elevate plasma triglyceride levels (35), even when the diet is low in simple sugars (36,37). This type diet is also associated with an increase in the number of small, dense LDL particles (38,39) and reductions in HDL levels (40), an atherogenic lipid

profile which is also seen in subjects with visceral obesity, in whom fasting and postprandial hyperinsulinemia and peripheral insulin resistance are also commonly observed (41-43).

Although the metabolic abnormalities associated with low-fat, high carbohydrate diets, as well as those seen in obesity, are hypothesized to be the result of higher insulin levels, evidence in support of this hypothesis is scant. Fatty acid synthesis induced by consumption of a low-fat diet containing a high proportion of simple sugars is increased to a similar extent in lean and obese subjects, despite plasma insulin levels that are 2-fold higher in the latter (44). Furthermore, no significant relationship is found between insulin and fatty acid synthesis when lean and obese subjects are considered separately (44). These findings suggest that stimulation of fatty acid synthesis by dietary carbohydrate is not directly related to body mass index or plasma insulin levels. Consequently, other physiological regulators common to both lean and obese subjects may be more relevant to dietary regulation of fatty acid synthesis *in vivo*.

The macronutrient composition of the diet influences energy metabolism at several levels. High-fat diets are generally more palatable than fat-restricted diets, a factor that may promote passive overconsumption of high-fat foods (45). Long-term studies of fat-reduced diets suggest there is a compensatory increase in energy intake as the duration of fat restriction continues (46). In the Women's Health Trial (47), compliance to a diet which reduced fat intake from approximately 38% to 20% of total energy intake was high for the first six months of dietary intervention and was accompanied by a significant loss of body weight. Compliance waned as the duration of intervention was extended to 24 months and consequently, weight loss was similar to subjects consuming

their habitual diet (47). Similarly, in overweight subjects, reducing energy intake derived from dietary fat to 17.6% of total energy for one year did not significantly increase fat loss compared with weight-matched control subjects (47,48). These findings suggest that weight loss induced by low-fat diets is transient and difficult to maintain.

The failure of conventional fat-reduced diets to promote and maintain weight loss may partly explain the increasing popularity of low-carbohydrate diets. In a comprehensive review of carbohydrate-restricted diets, lower-carbohydrate diets were found to produce greater weight loss than diets containing greater quantities of carbohydrate (49). This affect is hypothesized to result from a reduction in energy intake rather carbohydrate restriction. However, there are few long-term studies of carbohydrate-restricted diets and consequently, there is a notable lack of information regarding the efficacy of low-carbohydrate diets in maintaining reduced body weight or the possibility of adverse consequences.

The first long-term (one year), multicenter, randomized, controlled trial of a low-carbohydrate, high-protein, high-fat, Atkins'-type (50) diet was conducted by Foster, et al. (51). The central feature of this diet involves limiting carbohydrate intake without restricting consumption of fat and protein. For the first two weeks of the diet, carbohydrate intake is limited to 20 grams per day and is then gradually increased until a stable and desirable weight is achieved and maintained (50). The Atkins-type diet was compared to a high-carbohydrate, low-fat, reduced-calorie (1200-1500 and 1500-1800 kcal/d for women and men, respectively) diet for the same duration. Subjects consuming the low-carbohydrate diet had lost significantly more weight than the low-fat group at 3 months ($p<0.01$) and 6 months ($p=0.02$); however, the difference in weight

loss was not statistically different between the two groups at 12 months. Both diets were associated with improvements in insulin sensitivity, as determined by an oral glucose tolerance test. However, the data failed to demonstrate an effect of macronutrient composition, independent of weight loss, in obese subjects without diabetes. Compared with the low-fat diet, the low-carbohydrate diet was associated with greater decreases in serum triglycerides and greater increases in HDL-cholesterol (51), the levels of which are recognized as important risk factors for cardiovascular disease (52,53).

In a similar, albeit shorter (6 months) study, Samaha, et al. (54) reported that severely obese subjects (mean BMI: 43) lost more than twice as much weight ($p<0.01$) when consuming a hypocaloric (by 500 kcal/d) low-carbohydrate (< 30 g carbohydrate per day) diet compared with weight-matched subjects consuming an isocaloric, low-fat (~30 en% fat) diet. In nondiabetic subjects, the low-carbohydrate diet induced a greater increase in insulin sensitivity ($p = 0.01$) than did the low-fat diet. Furthermore, assignment to the low-carbohydrate diet and the amount of weight lost were each independent predictors of improved insulin sensitivity. Compared to the low-fat diet, the low-carbohydrate diet induced a greater reduction in serum triglyceride levels, which could not be solely attributed to greater weight loss in the low-carbohydrate group (54). A study of the same duration was conducting in mildly obese subjects consuming a carbohydrate-restricted (<25 g/d) diet, with no limit in total energy intake (55). After six months, body weight was approximately 10% lower than baseline ($p<0.001$), leading to a significant reduction in BMI ($p<0.01$). The authors of this study noted that the reduction in body weight achieved by adherence to a very-low-carbohydrate diet for six

months was similar to that achieved in mildly obese subjects treated with Sibutramine® (56).

A 12-week study comparing the effects of a low-fat versus a low-carbohydrate diet was conducted in overweight adolescents (57). This study is one of few in which energy intake was unrestricted, whereas other studies of low-carbohydrate diets are generally hypocaloric (58,59). Subjects assigned to the low-carbohydrate diet were permitted *ad libitum* intake of dietary protein and fat throughout the 12 week study. During the first two weeks, carbohydrate intake was restricted to less than 20 grams per day. Carbohydrate intake during weeks 3-12 was increased to 40 grams per day, to be derived from nuts, fruits, and whole grain foods. Subjects assigned to the low-fat diet were permitted *ad libitum* intake of fat-free dairy products, fruits and vegetables, while daily intake of carbohydrate and fat was restricted to 75 and 40 grams, respectively. Despite consuming significantly more energy, particularly from dietary fat ($p<0.001$), subjects in the low-carbohydrate group lost 141% ($p<0.04$) more weight, than did subjects in the low-fat group. Consequently, there was a significantly greater improvement in BMI in the low-carbohydrate versus the low-fat group ($p<0.05$). Simply stated, subjects assigned to the low-carbohydrate diet were able to lose a significant amount of weight while consuming between 1500-2500 kilocalories per day (kcal/d) (57).

Conventional wisdom suggests that the main factor in body weight regulation is total daily energy intake. However, the partitioning of calories between carbohydrate and fat may also influence energy balance. Postprandial fuel utilization is influenced by a variety of nutritional factors including circulating levels of glucose, free fatty acids, insulin, leptin and gut hormones. The carbohydrate content of a meal is a critical determinant of

insulin secretion, which influences postprandial hormonal and metabolite levels, and consequently, may alter the pattern of substrate utilization.

Because of the controversy surrounding the optimal diet for weight loss or prevention of obesity, we sought to design studies to address the influence of dietary carbohydrate on body weight and composition and to relate these changes to circulating metabolites and the expression pattern of several genes implicated in the regulation of carbohydrate and lipid metabolism. In our first studies, we evaluated the effects of a high-fat diet devoid of carbohydrate on body weight, plasma metabolites, adiposity, and expression of fatty acid synthase, a key enzyme of *de novo* lipogenesis, in both the liver and adipose tissue. Furthermore, we examined the effects of adding a minimal amount of sucrose to the high-fat diet as means to evaluate the metabolic response to small quantities of refined sugars in the context of a high-fat diet.

Secondly, we designed studies to evaluate the effects of a qualitative change in diet, accomplished by varying the source of dietary carbohydrate, on adiposity, plasma metabolites and substrate utilization. This approach is quite different from other studies which manipulate dietary lipid content or induce elevations in plasma free fatty acids through lipid infusion. Our aim was to determine if dietary carbohydrate source *per se* could alter substrate partitioning between adipose tissue, skeletal muscle, and liver and ultimately, influence body composition. To this end, we evaluated the expression of UCP-2, PPAR-α, PPAR-γ, and FAS in white adipose tissue; UCP-3 and PPAR-α in gastrocnemius and soleus muscle; and FAS, and PPAR-α in the liver. We sought to identify relationships plasma leptin, insulin, and glucose levels and alterations in gene expression in response to dietary carbohydrate source.

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PART 2

LITERATURE REVIEW

I. Components of the Insulin Resistance Syndrome

Obesity represents a compensated state of insulin resistance in that hyperinsulinemia is observed in obese subjects with normal glucose tolerance and this is believed to represent beta-cell compensation for peripheral insulin resistance (1-4). Chronic hyperinsulinemia may be the earliest detectable metabolic abnormality attributable to obesity. The transition to impaired glucose tolerance occurs with the progressive loss of compensatory metabolic changes (hyperinsulinemia and post-prandial hyperglycemia) and corresponds with the development of defects in both oxidative and non-oxidative glucose metabolism (5). Insulin resistance manifests as reduced insulin-stimulated glucose uptake and metabolism in adipocytes and skeletal muscle and impaired insulin-mediated suppression of hepatic glucose uptake (3,6). Thus, there are significant impairments in the three main targets of insulin action: liver, skeletal muscle and adipose tissue.

The Insulin Secretory Response

In normal persons, glucose-induced insulin secretion follows a bi-phasic pattern and is initiated when circulating glucose levels exceed 5.5 mmol/L (2,7,8). The first phase of insulin secretion lasts for 5-10 minutes and is followed by a second, more sustained phase which persists for the duration of hyperglycemia (3,8). Chronic hyperglycemia (>6.4 mM) dramatically reduces or abolishes the first phase response (9). Insulin secretion is very sensitive to changes in plasma glucose levels. The glucose ED_{50} for insulin secretion is 10-15 mmol/L glucose, which is in the lower range of the insulin-glucose dose response curve (10-13).

The rapidity of the early phase response is believed to result from release of pre-formed insulin from secretory granules docked on or near the plasma membrane of the beta cell (14,15). As the early phase response fades, there is a second, more prolonged period of insulin release, which persists for as long as plasma glucose levels remain elevated (3,4). Fasting and 2-hour oral glucose tolerance test (OGTT) insulin concentrations are inversely related to insulin action and consequently, can be interpreted as indices of insulin resistance (8,12).

Normal insulin secretory dynamics, and the acute response in particular, are critical to the maintenance of normal glucose tolerance. This has been demonstrated experimentally in healthy subjects during hyperglycemic clamps by co-infusion of somatostatin to selectively abolish the acute phase of insulin secretion (16,17). In subjects with normal first and second phase insulin secretory responses, infusion of glucose alone results in the near complete suppression of hepatic glucose output within 20 minutes. Abolishing the first phase response by co-infusion of somatostatin, severely blunts insulin-mediated suppression of hepatic glucose output, such that only 50% suppression is achieved within 20 minutes. The infusion of insulin 10 minutes following the simultaneous infusion of somatostatin and glucose, mimics the selective loss of the first phase of insulin secretion while the late phase of insulin release is preserved. Furthermore, hepatic glucose production remains incompletely suppressed at 150 minutes, when the normal late phase of insulin secretion is preserved by initiation of insulin infusion 10 minutes following the simultaneous infusion of glucose and somatostatin, demonstrating the essential role of early insulin secretion in the maintenance of glucose homeostasis (17).

Marked impairments in the first phase insulin response are observed early in the transition from normal to impaired glucose tolerance (2,4,7). β-cell compensation for the insulin resistance associated with obesity is necessary to maintain normal glucose tolerance (18). In order to overcome the loss of insulin responsiveness in insulin resistance states such as obesity, the higher insulin concentration needed must overcome the degree of insulin resistance at any given plasma insulin concentration as well as normalizing any kinetic defects in insulin action.

Insulin Hypersecretion

Insulin hypersecretion in the basal state and in response to an oral or intravenous glucose load is a consequence of obesity (1,3). In obese subjects with normal β-cell function, insulin resistance is accompanied by an enhancement in the early phase of insulin secretion (2). Bonadonna, et al. (19) have shown fasting plasma insulin levels to be roughly two-fold higher in obese subjects compared to age-matched, lean controls ($p<0.001$). Hyperinsulinemia in obese subjects was the result of enhanced insulin secretion in both the early (0 – 10 min) and late (10 – 120 min) phases of glucose-induced insulin release, culminating in a total insulin response which was 75% greater in obese compared to lean subjects ($p<0.01$). This is consistent with the findings of Hollenbeck, et al. (20), who have demonstrated greater prehepatic insulin production in obese subjects compared to lean subjects at all times during an oral glucose tolerance test (OGTT).

In lean and obese subjects insulin sensitivity decreases linearly with BMI, however, insulin resistance is 2-3 times more prevalent in obese subjects (21). According to one

report, as many as 41% of obese subjects exhibit fasting hyperinsulinemia. The frequency of subjects characterized by enhanced insulin secretion rises in parallel with BMI, with nearly half of all subjects with a BMI between 30.1 and 34.9 kg/m^2 classified as hypersecretors, a figure which rises to 80% in subjects whose BMI exceeded 35 kg/m^2 (21,22).

First-Pass Hepatic Insulin Extraction

Following synthesis in the β cell, proinsulin is cleaved to yield insulin and c-peptide, which are secreted in equimolar quantities (23,24). Once secreted, insulin must first traverse the liver. In normal subjects, roughly 50% of total secreted insulin is extracted and removed in a receptor mediated process during the initial portal passage (25). Normal hepatic extraction is necessary to prevent hyperinsulinemia, and is acutely regulated by both glucose and free fatty acids, suggesting this function is linked to peripheral indicators of insulin sensitivity (25). As insulin resistance worsens, reduced hepatic extraction is necessary to compensate for reduced peripheral sensitivity to insulin (28). Thus, the liver plays an important role in regulating the amount of insulin reaching systemic circulation.

Genuth, et al. (25) have demonstrated similar rates of insulin clearance in normal and diabetic subjects, while obese subjects were found to more rapidly catabolized insulin. Despite having fasting plasma insulin levels which were 1.5-fold higher ($p<0.001$) than those observed in normal weight subjects, obese subjects were found to have significantly elevated insulin clearance rates ($p<0.05$). This supports the role of

compensatory hyperinsulinemia as an adaptive mechanism for peripheral insulin resistance associated with obesity (25).

Influence of Excess Adiposity

Overnutrition facilitates the development of abnormalities in insulin kinetics and insulin action. Mott, et al. (29) placed normal weight subjects on a weight maintaining diet for 14 days, after which time the energy content of the diet was increased progressively over 3 days to induce weight gain. Following 13 days of overnutrition, plasma insulin and glucose responses during an OGTT were not significantly different from baseline. However, a significant increase in the insulin response to a meal tolerance test (47 en% fat, 29 en% carbohydrate, 24 en% protein) was observed and as a consequence, plasma insulin levels were significantly higher over three hours compared to subjects consuming a weight maintaining diet (29).

In vivo insulin action is characterized by a physiological delay between observed increases in plasma insulin levels and onset of insulin action with respect to glucose metabolism (30-32). This "rate of activation" for insulin may be altered in insulin resistant states. In subjects undergoing euglycemic clamps, the rate of glucose metabolism can be computed to detect temporal differences in insulin action. This method was employed by Biolo, et al. (30) to determine the effects of overall adiposity and body fat distribution on insulin-stimulated glucose metabolism. Despite similar BMI values in subjects with lower body (40 ± 2 kg/m^2) and upper body obesity (41 ± 3 kg/m^2), body fat distribution was found to influence several aspects of insulin action in vivo. The rate of glucose metabolism over 120 minutes was greatest in normal weight subjects

and was reduced in lower body obesity ($p<0.05$). Upper body obesity further worsened insulin action, as indicated by rates of glucose metabolism significantly lower ($p<0.05$) than normal weight subjects and subjects with lower body obesity but similar BMI. Rates of glucose infusion required to maintain euglycemia were similar for obese subjects regardless of body fat distribution, and significantly lower ($p<0.05$) than that required in normal weight subjects.

The rate of onset of insulin action ($T_{1/2}$), expressed as the time necessary to achieve half maximal rates of glucose infusion, was also influenced by body fat distribution. Normal weight control subjects and those with lower body obesity had similar $T_{1/2}$ values, whereas subjects with upper body obesity demonstrated significant delays in the onset of insulin action ($p<0.05$). The results of this study demonstrate a selective association between body fat distribution and the kinetics of insulin action; upper body obesity is associated with a slower rate of onset in insulin action as indicated by $GIR_{20\text{-}120\ min}$ and $T_{1/2}$ values compared to lower body obesity, despite similar BMIs (30).

Abnormalities Associated with Abdominal Adiposity

The dynamics of insulin secretion, clearance and physiological actions are subject to metabolic influences (4,6,12,29,32-36). Abdominal obesity is strongly associated with peripheral insulin resistance and appears to cause several defects in the dynamics of insulin secretion and normal hepatic clearance (6,31,34). Steady state insulin secretion and clearance dynamics have been studied during a period of overnight rest in normal weight and abdominally obese subjects (33). During this period, abdominally obese subjects had significantly higher plasma glucose levels compared to normal weight

controls ($p<0.001$). Insulin secretion rates were 2.3-fold higher in obese compared to normal weight control ($p<0.001$) subjects, whereas hepatic insulin extraction was similar in both groups. Consequently, average plasma insulin levels were also significantly elevated (3.4-fold) in abdominally obese subjects ($p<0.001$). Subjects were then observed over a 24-hour period during which three mixed meals were consumed. Despite similar plasma glucose levels, insulin secretion rates were 2.3-fold higher in abdominally obese compared to non-obese subjects ($p<0.001$), resulting in significantly greater insulin output over 24 hours in the abdominally obese subjects ($p<0.001$), accompanied by a reduction in hepatic insulin extraction. As a result, average plasma insulin levels were more than 4-fold higher in abdominally obese subjects ($p<0.001$) (6).

Polonsky, et al. (3) observed a significant, negative relationship between basal hepatic insulin extraction and fasting insulin levels in lean and obese subjects ($r=-0.63$. $p<0.02$). Although average basal and 24-hour glucose concentrations were similar in obese and lean men, obesity was accompanied by significantly higher (2-fold, $p<0.05$) basal insulin levels and higher rates of insulin secretion in the basal state, over a 24-hour period and during a euglycemic hyperinsulinemic clamp. Furthermore BMI was positively correlated with basal and 24-hour insulin secretion rates ($r=0.801$ and $r=0.895$, respectively, $p<0.001$ for both), indicating that the amount of insulin secreted increases with increasing body mass. In both obese and lean subjects, fasting insulin levels were positively associated with basal rates of insulin secretion ($r=0.79$-0.82, $p<0.003$). In obese subjects, there was a significant negative correlation between hepatic extraction and basal and 24-hour insulin secretion ($p<0.002$ for both), indicating that impaired

hepatic extraction contributes to elevated plasma insulin levels observed in obese subjects (3,4).

The influence of total adiposity and body fat distribution on prehepatic insulin production has been estimated by measuring peripheral c-peptide turnover in premenopausal women by Peiris, et al. (34). Following an overnight fast, basal c-peptide levels were more than 3-fold higher ($p<0.05$) in obese women compared to age-matched non-obese control subjects; an effect largely attributable to a nearly 4-fold greater ($p<0.05$) rate of prehepatic insulin production in obese subjects. These abnormalities in insulin secretion were also present when stimulatory concentrations of glucose were administered, when obesity was associated with elevated c-peptide levels ($p<0.05$) and greater insulin secretion ($p<0.05$). Consequently, the cumulative insulin response was 2-fold higher ($p<0.01$) in obese subjects compared to their lean counterparts. In fasting, nonobese women, approximately 70% of secreted insulin was extracted during the first portal passage. Hyperglycemia reduced hepatic extraction to approximately 50% of secreted insulin in lean subjects. There was a wide range of fractional extraction in obese women, ranging from 53-94% in the fasting state and 5-87% during hyperglycemia. In all subjects, plasma insulin levels were correlated with post-hepatic insulin delivery in the basal state ($r=0.85$, $p<0.01$), as well as during intravenous glucose administration ($r=0.93$, $p<0.01$). A positive relationship was observed between percent body fat and prehepatic insulin production in the basal state ($r=0.52$, $p<0.05$) and following intravenous glucose administration ($r=0.5$, $p<0.05$) (34).

Using the same subjects, Peiris, et al. (34) determined the influence of body fat distribution on plasma insulin levels and pre-hepatic insulin clearance. Upper body

obesity (WHR > 0.85) was associated with increased post-hepatic insulin delivery ($p<0.05$) and plasma insulin levels ($p<0.05$) compared with lower body obesity (WHR < 0.78). Furthermore, it was noted that as WHR increased, there was a progressive decrease in hepatic insulin extraction. Upper body obese women also had greater impairments in insulin-mediated glucose disposal, which was a function of both the decrease in hepatic extraction ($r=-0.72$, $p<0.01$) and the rise in systemic insulin delivery following glucose stimulation ($r=0.59$, $p<0.01$). Thus, the degree of adiposity and site of fat deposition influence insulin metabolism by two distinct mechanisms (34).

Polonsky, et al. (3,4) have demonstrated a significant elevation in total insulin released over 24-hours of mixed meal feeding in obese compared to lean subjects ($p<0.001$). Glucose tolerance exerts an effect independent of obesity and persons with impaired glucose tolerance (IGT) secrete almost twice as much insulin ($p<0.0001$) as persons with normal glucose tolerance (NGT).

Proposed Mechanisms of Insulin Resistance in Human Obesity

Early studies assessing the relationship between obesity and impaired insulin action yielded conflicting results. In nonobese males, a statistically significant, negative relationship ($r=-0.70$, $p<0.01$) has been demonstrated between percent body fat, estimated by skinfold thickness and *in vivo* insulin action measured by the euglycemic clamp technique (37). A similar negative relationship ($r=-0.70$, $p<0.01$) has been reported between obesity defined by BMI and insulin action (assessed by steady state plasma glucose levels) in lean and obese subjects (21). Insulin mediated glucose disposal

progressively declines as percent body fat approaches 28-30%; no further decrements in insulin action are observed above this level of adiposity (21).

In lean subjects, insulin stimulates a dose-dependent increase in the rate of glucose disposal, with an EC_{50} of ~130 μU/ml (32). A decrease in the biological effects of insulin at any given level of circulating insulin may be due to reduced peripheral sensitivity to insulin and/or decreased maximal responsiveness to the hormone (13,38,39). The dose response curve for insulin-stimulated glucose disposal is shifted to the right in obesity (32). In obese subjects, reductions in insulin sensitivity may result solely from a decrease in the number of insulin receptors (5). Consequently, there is a reduction in the level of cellular insulin binding with a parallel reduction in insulin-stimulated glucose disposal at physiological levels of insulin. While higher plasma insulin levels are needed to achieve half-maximal effects on glucose metabolism (EC_{50}: 210 μU/ml), the response to maximally-stimulating levels of insulin is preserved, suggesting the signaling cascade distal to insulin binding its receptor remains intact. A postreceptor defect manifests as a rightward shift in the insulin dose response curve (EC_{50}:370 μU/ml), and loss of responsiveness (32,40). Consequently, there is a blunting of all responses to insulin action at maximally stimulating levels of insulin. Compared with subjects with a reduced number of insulin receptors, subjects with post-receptor defects are more hyperinsulinemic and appear to be more insulin resistant, as indicated by greater decrements in glucose disposal at maximally-stimulating concentrations of insulin (13,32,40).

Rizza, et al. (32) used the euglycemic, hyperinsulinemic clamp technique to characterize the dose-dependent effects of insulin on glucose production and utilization

in lean subjects. To determine the effect of insulin on whole body glucose metabolism, the steady-state glucose infusion rate (SSGIR) at each sequential step of the insulin infusion was plotted against the corresponding plasma insulin concentration. This generated a sigmoidal curve, in which the SSGIR rose steeply up to a plasma insulin level of ~100 μU/ml. The ED_{50} for insulin stimulated glucose utilization was determined to be ~60 μU/ml, with maximum rates of glucose disposal achieved when plasma insulin levels reached ~675 μU/ml.

Whole body glucose homeostasis also relies on the ability of insulin to suppress hepatic glucose production (41). Hepatic glucose production is more sensitive to changes in circulating insulin levels than is peripheral tissues glucose utilization; while a 10-20 μU/ml increase in circulating insulin is sufficient for half-maximal suppression of endogenous glucose production, half-maximal stimulation of glucose utilization requires an increment in plasma insulin of 40-50 μU/ml (41,42). Complete suppression of endogenous glucose production occurred when plasma insulin levels reached ~60μU/ml, which is much lower than the concentration required to achieve maximum stimulation of glucose utilization (32,42).

Experimentally induced hyperinsulinemia (25-35 μU/ml) induces insulin resistance in lean subjects within 40 hours (43). This level of hyperinsulinemia significantly reduced insulin sensitivity, such that a lower rate of glucose infusion was required to maintain euglycemia at submaximally effective plasma insulin levels ($p<0.01$ vs. saline infusion). The requirement for lower rates of glucose infusion was maintained at maximally stimulating levels of insulin, indicating an impairment in insulin responsiveness. Despite plasma insulin concentrations that were significantly greater following insulin versus

saline infusion ($p<0.05$), adipocyte insulin binding following 40 hours of hyperinsulinemia was similar to that observed following saline infusion. This study clearly demonstrates that hyperinsulinemia results in small but significant reductions in insulin-stimulated glucose utilization, at both submaximal and maximally effective plasma insulin levels (43). Adipocytes and monocytes isolated from subjects in whom hyperinsulinemia had been experimental induced and compared to cells isolated from the same subjects prior to hyperinsulinemia. Despite significantly higher insulin levels during experimental hyperinsulinemia, insulin binding to adipocytes and monocytes was unchanged, which suggests that hyperinsulinemia impairs insulin action at a step distal to insulin binding its receptor (32,43).

The hyperinsulinemia and insulin resistance of obesity are subject to improvement with weight loss. Jones, et al. (44) have demonstrated that a 10% reduction in body weight significantly improves hyperinsulinemia and insulin resistance in obese subjects. Nonetheless, insulin-stimulated glucose utilization remains lower ($p<0.05$) and the total integrated insulin response to a test meal higher ($p<0.05$), than in lean subjects, suggesting greater reductions in body weight are necessary for normalization of these aspects of the metabolic syndrome.

In summary, the pathophysiology leading to the exaggerated hyperinsulinemia observed in obesity is the result of the additive effects of peripheral insulin resistance (38), reduced hepatic extraction (25,26), and β-cell hypersecretion (4). Chronic hyperinsulinemia is the price the obese must pay to preserve glucose homeostasis. Ultimately, compensatory hyperinsulinemia will worsen peripheral insulin resistance and cause further deterioration of whole-body glucose metabolism. The transition from

normal to impaired glucose tolerance corresponds to the appearance of compensatory mechanisms which maintain normal glucose homeostasis including hyperinsulinemia and postprandial hyperglycemia which prevent defective peripheral glucose utilization and storage. Hyperinsulinemia is believed to signal β-cell compensation for peripheral insulin resistance and may be the first clinically detectable abnormality in the transition to impaired glucose tolerance in obese subjects (45).

Resistance to glucose storage is also a basic feature of obese subjects still maintaining normal glucose tolerance (46,47). A key difference between the obese subject with impaired glucose tolerance and the obese subject with overt NIDDM is the inability of the latter to maintain plasma glucose levels within the normal range in both the basal state and post-glucose challenge (46-48). However, as insulin resistance worsens, such compensatory mechanisms will fail leading to overt glucose intolerance and non-insulin dependent diabetes mellitus.

II. Physiology of Glucose Homeostasis

Glucose Homeostasis

The postabsorptive phase occurs following a 14-16 hour overnight fast. During this time, plasma glucose levels average 5mM and plasma insulin levels are low (60 -75 pM) (41). The liver is responsible for approximately 80% of glucose released into circulation during this period (49,50). Postabsorptively, there is no net storage of glucose and glycogenolysis results in the rapid depletion of hepatic glycogen stores in an effort to maintain normal circulating glucose levels (49). In this state, the rate at which glucose is released into circulation by both the liver and kidney must closely approximate the rate at which glucose is removed from circulation, both of which are roughly 10 μmol/kg body weight/min (49-51). Initially, 50% of glucose released by the liver is derived from glycogenolysis with the remainder arising from gluconeogenesis (49). The fraction of glucose produced from gluconeogenic substrates (lactate, alanine, glutamine) will increase as fasting continues (52,53). Studies by Landau, et al. (49) demonstrate that gluconeogenesis accounts for roughly 65% of glucose produced during the first 22 hours of fasting. This figure rises to ~82% with an additional 24 hours of fasting and 96% when fasting is extended for 46-64 hours.

In the basal state, glucose taken up by tissues is either completely oxidized to carbon dioxide or is released back into circulation, primarily as alanine and lactate, from which glucose can be re-derived via the glucose-alanine and Cori cycles, respectively (53,54). Postabsorptive energy needs are largely met by oxidation of free fatty acids, which compete with glucose as the fuel substrate of choice for tissues such as the heart and

skeletal muscle (55-57). This concept will become even more important in the postprandial state.

Following ingestion of a 75 gram oral glucose load, roughly one-third of ingested glucose will be extracted by splanchnic tissues, presumably for direct conversion to glycogen (41,58,59). The appearance of ingested glucose in systemic circulation is apparent within 15 minutes, but peaks between 60 and 120 minutes following glucose ingestion. Overall systemic glucose disappearance follows a similar pattern, although the rate of glucose clearance peaks around 90 minutes and remains slightly above the postabsorptive rate three hours after glucose ingestion (41,58,59).

The muscle is the major tissue responsible for disposal of an oral glucose load (41). Muscle glucose uptake is greatest in the first two hours following glucose ingestion, during which time the rate at which glucose is disposed in this tissue is five-fold greater than in the postabsorptive period (41). As much as 20% of an oral glucose load is extracted from circulation by both the liver and kidney (41). The liver may extract glucose as glucose *per se* or as lactate, alanine, and/or glutamine, whose carbon backbones originated from previously ingested glucose (41,50). The rate at which the brain disposes of glucose is estimated to be ~80 mg/min (58). Because the brain has an absolute requirement for glucose, this rate should remain unchanged following a glucose challenge. Based on the data presented above, four tissues are responsible for disposal of at least 90% of an oral glucose load: skeletal muscle (40%), liver (25-35%), kidney (15-20%), and brain (15-20%).

The complete digestion and absorption of the components of a mixed meal and restoration of the postabsorptive state takes roughly 6 hours (41). Therefore, persons

adhering to the typical 3-meal per day pattern spend most of the day in the postprandial state. The rate at which ingested glucose appears in circulation is influenced by many factors including the rate of gastric emptying (60), rate of nutrient absorption from the gut (61), splanchnic glucose uptake (59), glucose transport dynamics (62), and first- pass hepatic glucose extraction (25). The pattern of substrate exchange has been examined in normal men by Capaldo, et al (63). Subjects were studied basally and for 5 hours following ingestion of a commonly consumed meal (pizza) which contained 75 grams of starch, 37 grams of protein, and 17 grams of fat. Consistent with previous studies, blood glucose levels peaked ($p<0.001$ vs. basal) 90 minutes after meal consumption. Plasma insulin levels, which were low in the basal state, followed similar, albeit delayed pattern of increase as observed with plasma glucose levels, and peaked ($p<0.01$) roughly 120 minutes following meal ingestion. Net splanchnic glucose balance was negative in the basal state, indicating net export of glucose. During the 30-60 minutes following meal intake, net splanchnic glucose balance rose by 270% ($p<0.001$) before gradually declining towards basal levels. Ingested glucose appeared in portal circulation within 15 minutes and peaked between 30 and 60 minutes. However, meal-derived glucose continued to appear in the portal system at the end of the 5-hour period, although at a slower rate than that observed in the immediate postprandial period. In total, the gut absorbed approximately 60% of ingested starch. Thus, in contrast to an oral glucose load, digestion and absorption of the components of a mixed meal is incomplete over 5 hours. Interestingly, meal ingestion was not associated with rapid suppression of hepatic glucose production (HGP). The basal rate of HGP was maintained throughout the first 90 minutes of the postprandial period. At the end of the 5-hour period, maximum

suppression of HGP was only 30% lower than the basal rate. This is at odds with the findings of Taylor, et al. (64), who have reported a dramatic decrease in hepatic glucose production within 10 minutes of ingestion of a mixed liquid meal (milkshake), which reached nadir of 67% suppression ($p<0.01$) within 30 minutes. This apparent discrepancy is likely due to the form of the test meal. Taylor, et al. (64) supplied all carbohydrate as glucose in a readily digestible form. Thus, a liquid meal containing glucose as the sole carbohydrate is much more readily digested and absorbed than is a solid meal containing more structurally complex carbohydrates and requiring extensive mechanical and enzymatic processing prior to absorption.

Returning to the findings of Capaldo, et al. (63), splanchnic glucose uptake rose from the basal level to peak around 120 minutes. Splanchnic glucose clearance followed a similar pattern, rising 20-40% above the postabsorptive rate, with peak rates observed between 120 and 180 minutes. Leg glucose uptake rose from the basal rate to peak within 30 minutes. If one assumes that leg muscle represents 25% of whole body skeletal muscle mass, the total amount of glucose taken up by skeletal muscle would be roughly 30% of meal-derived glucose. Splanchnic lactate balance switched from net uptake to net output at 60 minutes, after which lactate balance was near zero. The rate of leg lactate production fell to near zero within 90 minutes following meal consumption and remained suppressed by 50% at the end of the five hour period. Plasma free fatty acids fell rapidly following meal ingestion and remained low throughout the five hour period of the study (63).

Altered Hepatic Glucose Metabolism in Obesity

Obesity is characterized by numerous abnormalities in carbohydrate metabolism (65,66). The liver is particularly sensitive to insulin-mediated suppression of endogenous glucose production (ED_{50}: ~25-50 μU/ml) (32). Endogenously produced glucose arises from gluconeogenesis and glycogenolysis, and the regulation of these processes may be altered in obesity (49,67-69). When presented with variations in substrate availability, the liver is responsible for adjusting the rates of glycolysis, glycogenolysis, and gluconeogenesis in order to maintain constant hepatic glucose output (70).

In normal humans, hepatic glycogen content varies considerably during the day. Following an overnight fast, the liver contains roughly 75 grams of glycogen. Peak glycogen levels of 115-120 grams are observed in the hours immediately following the last meal consumed on any given day (69,70). Glycogen synthesis is the critical determinant of non-oxidative glucose metabolism and consequently, it has been suggested that expansion of hepatic glycogen stores in obesity may contribute to enhanced hepatic glucose production and consequently, glucose intolerance (71,82).

In order to understand the role of hepatic glycogen stores on glucose homeostasis, Clore, et al. (73) overfed normal subjects in excess of 1000 calories per day for 5 days in order to greatly expand hepatic glycogen. Overfeeding was associated with significant increases in plasma metabolites, including glucose ($p<0.01$), insulin ($p<0.01$), and alanine ($p<0.01$). In response to overfeeding, hepatic glucose output increased significantly ($p<0.01$) and was accompanied by a 42% decrease ($p<0.01$) in alanine gluconeogenesis, as well as a reduction in overall gluconeogenesis.

In a study of obese non-diabetic subjects, Muller, et al. (74) found similar rates of endogenous glucose production in lean and obese subjects. However, fractional gluconeogenesis was 2-fold higher ($p<0.05$) in obese subjects compared to their lean counterparts. In obese subjects, elevated gluconeogenesis was compensated for by blunted (by ~50%) glycogenolysis ($p<0.05$) and thus, normal regulation of endogenous glucose production was preserved. Hepatic glycogen stores were increased in obese compared to lean ($p<0.05$) subjects, presumably the result of impaired glycogenolysis.

In contrast to Muller, et al. (74), Felig, et al. (66) demonstrated dysregulation of hepatic metabolism in obese subjects. In the basal state, splanchnic glucose release was similar in obese and normal weight subjects. Nonetheless, splanchnic uptake of glucose precursors (lactate, alanine and glycerol) was 50-130% higher in obese compared with lean subjects (66). This suggests that gluconeogenesis makes a greater contribution to total hepatic glucose output in obesity. Support for this hypothesis came from balance data for glucose and glucose precursors across the splanchnic bed, which demonstrated that gluconeogenesis accounts for 19% of endogenous glucose output in normal weight controls compared to 30% in obese subjects. A factor contributing to enhanced gluconeogenesis in obesity is the greater fractional extraction of gluconeogenic precursors. In lean subjects, 38% of alanine and 26% of lactate is extracted by the liver, whereas, 55% of alanine and 39% of lactate, is extracted in obese subjects ($p<0.05$).

When plasma insulin levels are increased 60% by glucose infusion, splanchnic glucose output is suppressed by 75% in normal subjects. Under the same conditions, HGP in obese subjects is suppressed by only 38% ($p<0.05$ vs. lean), and significant suppression

of glucose output in obese subjects is achieved only by increasing plasma insulin levels 200% above basal (66).

Severe obesity is accompanied by several noteworthy morphological and physiological changes in hepatocytes (75). Hepatocytes from morbidly obese subjects, although morphologically intact, are significantly larger in terms of both volume ($\mu l/10^6$ cells) and surface area (μm^2). Fatty infiltration contributes to a 2-fold increase in hepatocyte volume and a doubling of surface area compared to non-obese control subjects ($p<0.01$ for both). Insulin responsiveness is maintained only with maximally stimulating concentrations of insulin in hepatocytes isolated from obese subjects. Although receptor affinity and number of binding sites in hepatocytes from obese subjects are comparable to controls, these effects will be diluted by the excess surface area and volume (75).

Cellular Mechanisms Responsible for Abnormal Hepatic Metabolism in Obesity

Obesity is associated with impairments in insulin-mediated suppression of hepatic glucose output as evidenced by a rightward shift in the dose response curve. The maximum response to insulin is maintained in obesity, however the ED_{50} is increased (32). Furthermore, alterations in the kinetics of insulin action on hepatic glucose production are also observed in obese persons (31). These changes manifest as slower onset of suppression, and premature loss of insulin-mediated suppression (76).

Because glucose transport across the hepatocyte membrane occurs via the non-insulin dependent glucose transporter, GLUT2 (62), the first regulated step of hepatic glucose metabolism is phosphorylation of glucose to glucose-6-phosphate (G6P) by

glucokinase (GK) (77). In the basal state, GK is sequestered within the nucleus in association with its regulatory protein (78). Glucose concentrations greater than 5mM, as encountered in the postprandial state, trigger the dissociation of GK from its nuclear regulatory protein and translocation of GK from its intracellular site to the cytoplasm of the hepatocyte (78). The movement of GK within the hepatocyte is associated with an increase in enzyme activity and concomitant rise in intracellular glucose-6-phosphate ($[G6P]_i$) levels (78).

In normal subjects, an acute rise in intracellular concentrations of G6P after a carbohydrate-containing meal or oral glucose load acts in concert with insulin to stimulate glycogen synthase activity, thus promoting glycogen synthesis (79,80). The stimulatory effect of G6P on glycogen synthase activity occurs simultaneously with inhibition of glycogen phosphorylase (81,82). These events correspond to the duration of the rise in plasma glucose and insulin levels above basal levels. The return of blood glucose levels to basal levels, removes the constraint placed on glycogen phosphorylase activity by the meal-associated increase in intracellular G6P levels, and therefore, promotes glycogen breakdown (42). The ability of the liver to alternate between glycogen synthesis and degradation represents an important physiological mechanism by which glucose can be stored or oxidized depending on the energy needs of the organism.

III. Insulin Stimulated Glucose Transport

Insulin Receptor Structure and Activation by Ligand Binding

The insulin receptor, a member of the tyrosine kinase receptor family, is composed of two extracellular α-subunits and two β-subunits linked by disulfide bonds (83,84). The extracellular ligand binding domain of the α-subunit is coupled to the tyrosine kinase activity of the β-subunit by a single transmembrane domain (85-87). Within the α-subunits, the insulin binding sites are contained in a cysteine-rich region (88,89). The β-subunits are membrane spanning proteins with an external domain composed of 194 amino acids, a single 23 amino acid transmembrane domain, and a large intracellular domain containing the receptor tyrosine kinase (85-87).

In the basal state, the α-subunits are unoccupied and the insulin receptor is phosphorylated on serine and threonine residues in the β-subunit, which exerts an autoinhibitory effect on the receptor kinase activity (90). Binding of insulin to the α-subunit monomers alters the nature of the interaction between the α-β heterodimers, relieving the inhibitory constraint and permitting the activation loop of the tyrosine kinase domain to contact the other β-subunit (91-93). This initiates trans-autophosphorylation by which the tyrosine kinase activity of one β-subunit phosphorylates specific tyrosine residues on the other β-subunit (93,94).

Following stimulation and activation of the insulin receptor, the insulin:insulin receptor complex undergoes rapid internalization into endosomal structures (95,96). Receptor internalization is a multi-step process initiated by insulin-induced

autophosphorylation of specific residues (Tyr^{1146}, Tyr^{1150}, and Tyr^{1151}) of the β-subunit (95). Subsequently, the insulin-receptor complexes are redistributed from cell membrane microvilli, where the receptor preferentially localizes in the basal state, along the cell surface (97) and internalized into clatherin-coated pits for uptake into intracellular endosomal structures (98).

Signaling Events Following Insulin Receptor Activation

Several clusters of autophosphorylation sites have been identified in the β-subunit of the insulin receptor and are critical to normal insulin signaling (99-101). In particular, Cys^{860} in the extracellular domain of the insulin receptor β-subunit appears to be critical for the biological actions of insulin (100). Mutation of this residue to serine (C860S) does not alter normal autophosphorylation of the receptor itself (101). However, insulin-mediated phosphorylation of its major downstream substrate, IRS-1, is severely impaired in cells expressing the C860S mutation and internalization of the activated receptor is prevented. Consequently, there is a major reduction in the metabolic actions of insulin in these cells, which supports the importance of this domain in propagation of the insulin signaling cascade (100,101).

The juxtamembrane domain is critical for the interaction of the insulin receptor with IRS-1 (102). Within the juxtamembrane domain, phosphorylation of Tyr^{960}, which lies just inside the cell membrane, creates a recognition motif for the phosphotyrosine binding (PTB) domain of the IRS proteins (103). While mutation of this residue interferes with the phosphorylation of endogenous substrates, including IRS-1, and thus,

impedes propagation of the insulin signal, autophosphorylation of the receptor itself is unaffected (103,104).

The C-terminal region of the β-subunit contains several amino acid clusters, which either propagate or restrain the insulin signal (105,106). Phosphorylation of three specific residues (Tyr^{1146}, Tyr^{1150}, Tyr^{1151}) within the kinase regulatory domain increases the activity of the receptor kinase towards intracellular substrates (107,108). Also in this region are specific residues (Tyr^{1316}, Tyr^{1322}), which serve to restrain the level of kinase activation achieved upon phosphorylation of Tyr^{1146}, Tyr^{1150}, and Tyr^{1151} within the kinase activation loop (106,109,110). Point mutations or truncations of the β-subunit which delete these residues increase both the autophosphorylation capacity and the activity of the β-subunit tyrosine kinase towards intracellular substrates and consequently, leads to excessive activation of the insulin signal (111,112). In addition, specific serine and threonine (Ser^{1293}, Ser^{1294} and Thr^{1336}) in the c-terminal region are phosphorylated in the basal state and appear to prevent unnecessary commencement of the signaling cascade in the absence of insulin (113).

The major substrate for the activated insulin receptor is IRS-1 (114-116). Tyr^{960} is one of the first residues phosphorylated in the insulin receptor following ligand binding and is essential for coupling the insulin receptor to IRS-1 (103,114). Two domains within IRS-1 interact with the phosphorylated insulin receptor, primarily through the region encompassing Tyr^{960} (117-119). The amino terminal region of IRS-1 contains a pleckstrin homology (PH) domain and an adjacent phosphotyrosine binding (PTB) domain, both of which are necessary for interaction with the insulin receptor under physiological conditions (118-120). The PH domain provides the most sensitive

interaction for coupling the insulin receptor with IRS-1, while the PTB domain contributes an additional site of interaction, thereby increasing the sensitivity of the association between the receptor and IRS-1 (121). The cooperative nature of these domains may provide a means by which the cell adjusts the rigidity of the interaction with the insulin receptor and thus, regulates amplification of the insulin signal.

At the molecular level there is evidence for induction of insulin resistance by enhanced serine (ser)/threonine (thr) phosphorylation of IRS-1, which impedes its interaction with the juxtamembrane region of the insulin receptor and consequently, makes IRS-1 a poor substrate for insulin-mediated tyrosine phosphorylation (122,123). Consequently, IRS-1 is unable to recruit downstream effector molecules of the insulin signaling cascade leading to loss of the biological actions of insulin.

Several tyrosine residues (Tyr^{608}, Tyr^{628}, Tyr^{939}, Tyr^{987}) of IRS-1 are phosphorylated in response to activation of insulin receptor kinase activity (124). These residues are found in the C-terminal region of IRS-1 and, upon phosphorylation, serve as docking sites for src homology 2 (SH2) domain of phosphotidylinositol-3-kinase (PI3-K) (125). PI3-K is a heterodimeric, dual function lipid kinase composed of a regulatory subunit (p85) and a catalytic subunit (p110) (126). The p85 subunit serves as an adaptor protein by virtue of its tandem SH2 domains, which must interact simultaneously with specific residues in IRS-1 and the p110 subunit to achieve direct coupling and full activation of the catalytic activity (124-127).

Activation of PI3-K activity occurs by one of two mechanisms (128,129). First, the interaction of the SH2 domains of the p85 subunit with specific phosphotyrosine residues on IRS-1 may increase the existing catalytic activity PI3-K (130,131).

Alternatively, PI3-K may be recruited to its natural substrates in close proximity to the plasma membrane (132). The major in vivo catalytic action of PI3-K is phosphorylation of the D-3 position of the inositol ring of phosphatidyl-inositol(4,5)bis phosphate to yield phosphatidyl-inositol-(3,4,5)-phosphate (PIP3) (133). The lipid by-products generated via PI3-K are essential for the major metabolic actions of insulin, including glucose uptake (134).

Glucose Transport Proteins and Cellular Glut4 Trafficking

The hydrophobic nature of the cell membrane lipid bilayer is impermeable to glucose and thus, a transport system is necessary for glucose uptake by cells. To date, five homologous transmembrane proteins (GLUT-1, -2, -3, -4, and -5) have been identified, which transport glucose by facilitated diffusion down concentration gradients (135). GLUT-1 is a constitutively active glucose transporter with a broad expression pattern. Expression of GLUT-1 is highest in the brain, erythrocytes, and endothelial cells and glucose transport via GLUT-1 is independent of insulin stimulation (62). The low affinity (high K_m) glucose transporter, GLUT-2, is responsible for glucose sensing and uptake in the kidney, small intestine epithelial cells, liver, and pancreatic β-cells. GLUT-3 expression is limited to neurons and the placenta, where it facilitates glucose transport across the blood brain barrier. Fructose transport is accomplished by GLUT-5, which has very low affinity for glucose. GLUT-5 is expressed in the small intestine, kidney, brain, adipose tissue, and skeletal muscle (136).

GLUT-4 is the primary insulin-responsive glucose transporter and is predominantly expressed in skeletal muscle and adipose tissue (137-139). Unlike the

other facilitative glucose transporters, 90% of GLUT-4 protein is sequestered in intracellular compartments in the basal state (140,141). As postprandial blood glucose levels rise, the resulting increase in circulating insulin levels activates signaling cascades which ultimately result in the translocation of GLUT-4 storage vesicles to the plasma membrane (142,143). Importantly, this process is readily reversed as insulin levels fall.

In the absence of insulin, GLUT-4 is slowly recycled from intracellular storage vesicles to the plasma membrane and T-tubules in skeletal muscle (139,141,142). To date, three such compartments have been identified. The majority of GLUT-4 protein (~60%) is found in the tubulo-vesicular elements beneath the plasma membrane (144,145). The remaining protein is localized to the trans-Golgi network, clathrin-coated vesicles, and endosomal structures (141,142).

Insulin-stimulated trafficking of GLUT-4-containing vesicles depends on complex intracellular machinery. Evidence suggests there is a distinct subpopulation of GLUT-4 containing vesicles which is responsible for the majority of insulin-stimulated glucose translocation (142,146,147). These vesicles are enriched in soluble N-ethylmaleimide-sensitive factor attachment protein receptor (v-SNARE) designated vesicle-associated membrane protein (VAMP2) (148,149). At the plasma membrane, docking sites for VAMP2 are created by the SNARE proteins syntaxin-4 (Syn4) and syntaxin-associated protein-23 (SNAP23) (149,150).

A protein identified as Synip has been found to associate with Syn4 in the basal state in adipose tissue and skeletal muscle (151). The interaction of Synip and Syn4 is mutually exclusive of VAMP2 and thus precludes the interaction of the VAMP2-enriched vesicles with the t-SNARE complex, effectively preventing fusion of Glut4-containing vesicles

with the plasma membrane (151,152). Insulin stimulation prompts the dissociation of Synip, facilitating the VAMP2-Syn4 interaction necessary for GLUT4 vesicle fusion and presentation of GLUT4 proteins on the membrane surface (152,153).

IV. Adipose Tissue Metabolism and Alterations in the Metabolic Syndrome

Major Functions of Adipose Tissue

The primary function of adipose tissue in humans is regulation of energy balance by coordinating the storage of triglycerides during times of energy excess and release of free fatty acids (FFA) and glycerol for use as fuel substrates by other tissues during times of energy deprivation. Adipocytes store lipid, primarily as triacylglycerol (TAG), within large lipid droplets. Lipolysis of these lipid stores yields non-esterified fatty acids (NEFAs) and glycerol. Once believed to be metabolically inactive, adipose tissue is now recognized as a tissue with endocrine, neurocrine and paracrine actions by virtue of its many secretory products and expression of various receptors.

Adipose tissue accumulation is governed by the balance between triacylglycerol synthesis (lipogenesis) and breakdown (lipolysis). As its name suggests, hormone-sensitive lipase (HSL), the chief regulator of lipolysis, responds to various hormonal stimuli, including those signals arising from nutritional and dietary factors. Opposing the action of HSL are several lipogenic enzymes including lipoprotein lipase (LPL), the primary enzyme governing lipid deposition, and fatty acid synthase (FAS), a key enzyme of de novo lipogenesis.

Hormone-Sensitive Lipase

Insulin and catecholamines are the only hormones which have significant acute effects on human adipose tissue lipolysis (154,155). Lipid mobilization and release of free fatty acids are mediated by adipocyte adrenoceptors, which regulate lipolysis via

control of hormone sensitive lipase (AT-HSL) (156). Unlike adipocytes from other mammalian species, catecholamines have acute stimulatory and inhibitory effects on lipolysis in humans, which are mediated by β- and α_2-adrenergic receptors (156,157). Catecholamines, such as norepinepherine, initiate the lipolytic cascade by binding to plasma membrane β_1- and β_2-adrenergic receptors (ARs), which are positively coupled to adenylate cyclase via the stimulatory G_s protein (158). The activation of adenylate cyclase results in elevations in intracellular cAMP ($[cAMP]_i$) levels. Once formed, cAMP interacts with the regulatory subunits of protein kinase A (PKA) resulting in the release of the catalytic subunits, and the subsequent phosphorylation and thus, activation of AT-HSL (156). Following its activation, AT-HSL translocates from the cytosol to the surface of the lipid droplet (159). In vivo, AT-HSL catalyzes the rate-limiting step in hydrolysis of stored triacylglycerols to diacylglycerol. The net result of increased AT-HSL activity is an increase in the release of NEFAs and glycerol from adipocytes (160). Binding of catecholamines to the adipocyte α_2-AR, which is negatively coupled to adenylate cyclase via the inhibitory G_i protein, counters the stimulatory effects of β-AR activation (156,157).

Hyperglycemia and hyperinsulinemia are common findings in obesity and NIDDM. These conditions are also associated with elevated rates of lipolysis (161,162). *In vitro*, neither glucose nor insulin significantly increase isoproterenol-stimulated lipolysis. However, the combination of glucose and insulin significantly increases both basal and stimulated lipolysis, possibly linking obesity-associated hyperinsulinemia to elevated rates of lipolysis (163).

Reynisdottir, et al. (163) suggest that catecholamine resistance in subjects with the metabolic syndrome is entirely attributable to defects in the β_2-AR system. In studies of abdominal adipocytes isolated from subjects with clinical features of the metabolic syndrome (BMI>26 kg/m^2, WHR >0.93, insulin resistance, impaired glucose tolerance), significant correlations were observed between glucose intolerance and basal lipolytic rate, maximum stimulated lipolysis, and β_2-AR density. Furthermore, insulin sensitivity, as determined by euglycemic-hyperinsulinemic clamp, was found to be strongly associated with β_2-AR number, fat cell volume, WHR, fasting insulin levels, and glucose tolerance. A marked reduction in the maximum lipolytic rate in response to the β_2-AR selective agonist, terbutaline, was observed in insulin-resistant subjects compared to healthy subjects ($p<0.001$), and was associated with an approximate 50% reduction in β_2-AR density in subjects with metabolic syndrome. Regardless of the stimulatory agent used, lipolytic rates were significantly increased over basal level in all subjects, yet these responses were blunted by 44-75% in adipocytes from subjects with the metabolic syndrome ($p<0.01$).

Lipoprotein Lipase

Lipoprotein lipase is expressed predominantly in skeletal muscle and adipose tissue and is regulated in a tissue-specific manner (164-166). Lipoprotein lipase is the rate-limiting step for triglyceride catabolism by peripheral tissues. The activity of AT-LPL is critical for hydrolysis and exchange of lipoprotein-associated triglycerides among extrahepatic tissues and circulating lipoproteins, which provide the substrates for an expanding adipose tissue mass (165). Greater than 95% of the body's triglycerides are

stored in adipose tissue, having been derived from both dietary and endogenous triglycerides (160).

While triglycerides are a very dense source of fuel, they, unlike glucose, are insoluble in plasma and must be transported associated with lipoproteins. In addition, triglyceride-rich lipoproteins are too large to cross the capillary endothelium of most tissues; consequently, they must be modified in a way that permits their uptake by peripheral tissues. This is accomplished through the actions of AT-LPL, which following synthesis within parenchymal cells of adipose tissue migrates to the interstitial space and ultimately resides on the luminal side of the capillary endothelium of most tissues (165). Triglyceride-rich lipoproteins passing through the capillary endothelium are subject to hydrolysis by AT-LPL, resulting in the release of glycerol and free fatty acids which can be taken up by parenchymal cells of adipose tissue (160).

The fate of absorbed fatty acids is largely determined by the activity of AT-LPL relative to the expression and activity of LPL in peripheral tissues, particularly skeletal and cardiac muscle, and is ultimately determined by nutritional and hormonal influences (161,164,166). Animal studies have demonstrated that the activity of AT-LPL is sensitive to energy and hormonal balance, particularly the availability of glucose and insulin (166,167). Insulin upregulates AT-LPL in concert with acylation-stimulating protein (ASIP), which is produced locally by adipocytes in response to circulating chylomicrons (168). The coordinated actions of insulin and ASIP ensure that the rate of triglyceride synthesis in adipocytes is sufficiently rapid so that circulating fatty acid concentrations do not exceed a threshold level at which rapid clearance of circulating triglycerides would be initiated (167,168).

Adipose tissue LPL is increased in genetic and diet-induced obesity, while its activity is decreased in insulin-resistant states, including NIDDM (161,166,169). Furthermore, the degree to which AT-LPL expression is stimulated in response to weight gain suggests that the enzyme acts to preserve the expanded fat mass (170). This is consistent with heightened activity of the enzyme in the postprandial state, during which time fatty acids are directed to adipose tissue for storage and re-esterification (167,168). The post-prandial increase in AT-LPL activity is the result of post-transcriptional regulation (171,172). Opposing the actions of AT-LPL is the skeletal muscle isoform (SM-LPL), the activity of which is one-eighth that of the adipose tissue isoform (164).

Availability of fuel substrates, specifically fatty acids and carbohydrates, influence the activity and expression of lipogenic enzymes (173,174). An inverse relationship exists between fatty acids and carbohydrates, such that an increase in the provision of fatty acids ensures inhibition of glucose utilization (56,167,175). A significant negative correlation exists between AT-LPL mRNA and steady state glucose levels ($p<0.001$). The activity of plasma LPL is weakly correlated with plasma insulin levels ($p<0.06$), suggesting that further reductions in LPL activity can be anticipated as insulin resistance worsens (167).

Steady state AT-LPL mRNA levels have been found to be significantly lower in insulin-resistant compared to insulin-sensitive ($p<0.001$) subjects. The degree to which peripheral tissues are refractory to insulin is also negatively correlated with both plasma LPL activity ($p<0.03$) and AT-LPL mRNA ($p<0.001$). However, insulin resistance does not adversely influence the activity of AT-LPL. When steady state plasma glucose levels

are used as an indicator of insulin resistance, there is a significant inverse relationship between plasma glucose levels and AT-LPL mRNA ($p<0.001$) (167).

Fatty Acid Synthase

Fatty acids in adipocytes can be derived from circulating lipoproteins via the action of lipoprotein lipase or they can be synthesized *de novo* from carbohydrate-derived precursors. Excess glucose promotes lipogenesis by increasing glycolytic flux and consequently, increasing the availability of acetyl CoA. Acetyl CoA is the chief substrate for the synthesis of long-chain saturated fatty acids which can be subsequently esterified and stored as triacylglycerols in adipose tissue . Fatty acid synthase (FAS) catalyzes all reactions involved in the synthesis of long-chain saturated fatty acids (palmitate) from acetyl CoA, malonyl CoA and NADPH (176).

Human FAS is expressed in several tissues (177). Semenkovich, et al. (178) report that FAS expression is induced by glucose in a time- and concentration-dependent manner, independently of insulin, in HepG2 cells. The level of FAS mRNA increases with glucose in the range of 100-4500 mg/L, with much of the increase occurring within the physiological range (500-2000 mg/L) for humans with fasting and refeeding and maximal effects observed between 11-15 hours of exposure to glucose. The effects of glucose on FAS activity are pretranslational as evidenced by parallel increases in FAS mRNA and activity (178).

Insulin stimulates FAS expression and activity similarly in lean and obese subjects (179). Furthermore, the insulin-induced increase in FAS expression is readily reversed upon removal of insulin. Addition of insulin to previously deprived cells increases FAS

mRNA content, whereas withdrawal of insulin from insulin-treated cells decreases the level of FAS mRNA to levels observed in cells which were never exposed to insulin. Thus, adipocytes from both lean and obese subjects increase FAS expression when continuously exposed to insulin (179).

Normal mice fasted for 48 hours have low circulating insulin levels and low hepatic FAS activity, while refeeding a high-carbohydrate, fat-free diet significantly increases FAS activity in the liver and adipose tissue, concomitant with increases in circulating insulin levels (180). The increase in FAS expression in response to refeeding is less vigorous in diabetic animals, suggesting insulin is required for induction of FAS expression in vivo (181).

Antilipolytic Actions of Insulin

In nonobese, normoglycemic individuals, adipose tissue is exquisitely sensitive to the anti-lipolytic effects of insulin. In fact, insulin-mediated suppression of lipid mobilization occurs at concentrations of insulin (ED_{50} = 12-20 pmol/L) much less than those required to half-maximally stimulate glucose uptake by skeletal muscle (155,175). Insulin-mediated suppression of free fatty acid release is markedly blunted in obese subjects as evidenced by a rightward shift in the dose response curve compared to normal subjects (182). Consequently, plasma free fatty acid levels are often elevated in obesity (183). In addition, rates of lipid oxidation are often elevated in obese subjects, despite significantly higher plasma insulin levels relative to normal subjects (184).

While adipose tissue insulin sensitivity is critical to euglycemia, the exquisite sensitivity of the intramuscular compartment to insulin-mediated suppression of lipolysis

also has important implications in postprandial metabolism (185). In the postabsorptive state, free fatty acids (derived from adipose tissue and intramuscular triglycerides) are the preferred fuel substrate for resting muscle. During the transition to the postprandial state when there is greater glucose availability, the heightened sensitivity of skeletal muscle lipolysis to insulin, effectively prevents the continued hydrolysis of intramuscular triglycerides and subsequent release of free fatty acids, which would be expected to compete with glucose for oxidation. Thus, the acute sensitivity of skeletal muscle to insulin-mediated suppression of lipolysis ensures that the switch from the preferential use of free fatty acids to oxidation of glucose by skeletal muscle occurs in the postprandial state (185).

The rapid uptake of glucose by adipose tissue following insulin stimulation is dependent on GLUT4. While insulin resistance *per se* does not negatively impact the expression of GLUT4 in skeletal muscle of humans or rodents, adipocyte GLUT4 expression is reduced up to 50% in obese compared with lean subjects (186,187). The reduction in adipose tissue GLUT4 expression in obesity may represent a physiological attempt to limit further expansion of adipose tissue stores.

Regional Adipose Tissue Deposition

The significance of regional fat distribution was first alluded to more than 50 years ago by Vague (188,189), who noted a clustering of metabolic abnormalities including NIDDM, atherosclerosis, hypertension, biliary calculus and gout in individuals with excessive adiposity in the truncal region. It is now clear that the anatomical site of

excessive adipose tissue accumulation significantly impacts the risk of developing chronic disease (190-194).

The two general types of fat distribution are android (apple shape), in which fat accumulates in the truncal region, and gynoid (pear shape), in which fat is distributed around the hips and buttocks (191,195). Android obesity is the typical male-pattern obesity and is associated with increased risk of cardiovascular disease, cerebrovascular disease, non-insulin-dependent diabetes mellitus (NIDDM) and hypertension in obese men (196,197). Gynoid obesity appears most often in women, however, accumulation of fat in the truncal region in women results in metabolic abnormalities similar to those seen in abdominally obese men (196,197).

Waist circumference (WC) and waist:hip circumference ratio (WHR) are the most widely used anthropometric measures for assessing regional adiposity (198). WC is an aggregate measure of total and abdominal fat mass and is most sensitive when used with overweight adults (BMI=25.1-29.9) (199). Men and women with WC values less than 94 cm and 80 cm, respectively, are less likely to develop obesity-related chronic diseases than are individuals whose WC values exceed 102 cm (men) and 88 cm (women) (199).

Several studies have demonstrated increases in fasting insulin and glucose levels with increasing waist girth. Analysis of data from the Nurse's Health Study (197) has revealed WC to be more strongly correlated with weight ($r=0.82$) and BMI ($r=0.81$) than WHR ($r=0.31$ and 0.34, respectively). In addition, information gleaned from the Nurse's Health Study and others, indicate WC may be a more sensitive predictor of chronic disease than WHR (197-201). The relative risk for developing cardiovascular disease in women with a waist girth of 96.5 cm or greater is 3.24 compared to women whose WC is

71.1 cm or less. Furthermore, the Nurse's Health Study determined that for every 2.54 cm (1 inch) increase above 71.1 cm, relative risk for coronary heart disease is increased 1.07 fold (197).

WHR or waist circumference alone has been consistently correlated with intra-abdominal fat as assessed by computerized tomography (CT) or magnetic resonance imaging (MRI) (198,200). The clinical usefulness of the waist-to-hip ratio in predicting cardiovascular events in men and women has been established. Megnien, et al. (202) found WHR to be significantly positively associated with systolic blood pressure ($p < 0.05$), total-to-HDL-cholesterol ratio ($p < 0.01$ for men and $p<0.001$ for women), fibrinogen ($p < 0.001$ for men and $p<0.02$ for women), and diabetes ($p<0.001$ for men and $0<0.01$ for women). Furthermore, WHR was strongly associated with estimated 10-year mortality and morbidity from cardiovascular events. The estimated percentage of coronary heart disease ($p < 0.001$) and death ($p<0.01$), myocardial infarction ($p<0.01$), stroke ($p<0.01$) and total cardiovascular disease ($p<0.001$) and death ($p<0.01$) increased in parallel with WHR in both men and women (202).

Site Differences in Adipose Tissue Metabolism and Insulin Sensitivity

The preferential deposition of adipose tissue in one anatomical region over another is related to the balance between triglyceride uptake and fatty acid release. There have been numerous reports of depot-related differences in adipose tissue metabolism (191-200). Compared to subcutaneous adipocytes isolated from the gluteal-femoral region, visceral abdominal adipocytes exhibit higher rates of basal lipolysis, extensive sympathetic innervation, acute sensitivity to the stimulatory actions of catecholamines,

yet remains relatively resistant to the antilipolytic actions of insulin (182,203). Visceral adipose tissue deposits are also responsible for the bulk of free fatty acids released into circulation and the proximity of this depot to the liver may be an important factor in the pathology associated with central obesity. In fact, visceral adipose tissue drains directly to the liver via the portal venous system (191). Thus, the liver will be fully exposed to the range of fatty acids and cytokines released from the abdominal adipose tissue depot (75). Increased substrate flux from the liver, particularly free fatty acids, may induce adipocyte hypertrophy, which perpetuates insulin resistance (204-206). Enlarged, insulin resistant abdominal adipocytes are slow to respond to the effects of insulin and may demonstrate reduced rates of esterification of dietary-derived fatty acids, contributing to post-prandial hypertriglyceridemia (207-210). Consequently, circulating free fatty acid levels would remain elevated at the most metabolically inopportune time: when glucose and insulin levels are elevated and fatty acid oxidation by peripheral tissues is suppressed.

The metabolic profile of subcutaneous adipocytes is much different (211,212). Subcutaneous adipocytes derived from the gluteal depot exhibit low rates of basal lipolysis, are acutely sensitive to the anti-lipolytic actions of insulin and demonstrate reduced responsiveness to catecholamine stimulation (213-215). In addition there is significant expression of the inhibitory α_2-AR in adipocytes from this depot (215). Thus, adrenergic receptor profile of subcutaneous adipocytes makes them both antilipolytic and relatively resistant to pro-lipolytic catecholamines.

Hormonal responsiveness is significantly impaired in enlarged adipocytes (208). Although basal and catecholamine-induced lipolysis are positively correlated with cell size, the antilipolytic effects of insulin are more pronounced in smaller adipocytes. In

addition, the capacity to increase glucose conversion to carbon dioxide and triglycerides in response to insulin stimulation is significantly blunted in enlarged adipocytes (205,206,208). Weight loss as a consequence of dietary intervention or surgical treatment is associated with a decrease in adipocyte size as well as reversal of impaired insulin responsiveness (204). Improved insulin sensitivity has also been demonstrated in animals by surgical removal of visceral fat. Removal of visceral fat reduced plasma insulin levels, enhance hepatic insulin sensitivity, and improved insulin-stimulated glucose utilization relative to animals in whom visceral fat remained intact. Animals retaining visceral fat were less sensitive to the suppressive actions of insulin on hepatic glucose output, which supports a causative role for visceral fat in manifestation of at least some aspects of the insulin resistance syndrome (216).

Contribution of De Novo Lipogenesis in Adipose Tissue

The contribution of *de novo* lipogenesis in human adipose tissue is the subject of much debate. Recent evidence suggests *de novo* lipogenesis in adipose tissue significantly contributes to increased adiposity in humans (217,218). Body weight gain of normal subjects overfed with carbohydrate is not explained by the increase in liver lipogenesis alone (217). Lean, obese and obese, weight-reduced subjects have been studied to assess changes in lipogenic capacity of liver and adipose tissue which may contribute to obesity. Compared with control subjects, obese subjects had higher plasma glucose ($p<0.01$), cholesterol ($p<0.05$), triglycerides ($p<0.05$), insulin ($p<0.01$) and leptin ($p<0.05$) levels. In the obese subjects studied after weight loss induced by a hypocaloric diet, plasma triglycerides, cholesterol, insulin and leptin all decreased ($p<0.05$ for all) compared with

the values observed prior to weight reduction (217). The contribution of hepatic lipogenesis to total de novo lipid synthesis was enhanced in obese compared to lean ($p<0.01$) subjects. The reduction in energy intake induced by the hypocaloric diet, reduced hepatic lipogenesis ($p<0.05$) to values comparable to lean subjects (218).

The Ob Gene Product, Leptin

The *ob* gene product, leptin, is 167 amino acid protein produced and secreted predominately by adipose tissue which plays an important role in regulation of energy intake, body weight, and adiposity (219-221). Serum leptin levels strongly correlate with adiposity ($r=0.85$, $p<0.001$), BMI ($r=0.69$, $p<0.05$), and fasting insulin levels ($r=0.57$, $p<0.0001$) in both men and women, although women generally have higher circulating leptin levels than men (222) In the fed state, leptin is secreted in proportion to adipose tissue triglyceride stores and circulates as free leptin or associated with a soluble form of the leptin receptor (222-224).

Leptin Deficient ob/ob Mice

A mutation in the *ob* gene results in the leptin-deficient *ob/ob* mouse, whose phenotype is characterized by early onset obesity, hyperphagia, reduced energy expenditure, and severe insulin resistance (221,225). *ob/ob* mice with complete leptin deficiency fail to undergo sexual maturation and demonstrate structural neuronal abnormalities and impaired myelination in the brain (226-228). Leptin treatment can accelerate puberty in normal prepubescent mice and leptin levels are known to increase before puberty in humans (229,230). Despite stable plasma insulin levels in newborn

mice, plasma leptin levels increase 5-10-fold ($p<0.05$) within 10 days in C57BL/6J mice prior to decreasing to adult levels (231,232). The rise in circulating leptin levels coincide with a 6-10-fold increase in *ob* mRNA in white adipose tissue (232). This surge in leptin secretion and expression occurs by a mechanism independent of body fat stores, which are stable during the neonatal period during which the increase in leptin levels is observed (232).

In neonatal mice, 12-hours of food deprivation has no effect on plasma leptin levels (231,232). In contrast, leptin decreases by 40% in adult mice deprived of food for 12-hours (233). In normal adult mice housed under 12-hour light and dark cycles and fed ad libitum, maximum food intake occurs during the dark cycle, and plasma leptin levels exhibit pulsatile, circadian rhythm (233,234). Plasma leptin levels follow the same diurnal pattern in humans, falling to their lowest levels humans just prior to rising (235). The circadian rhythm is absent in obese Zucker (*fa/fa*) rats (236).

Administration of recombinant leptin to *ob/ob* mice results in significant weight loss by suppression of food intake and increased energy expenditure as well as normalizing blood glucose and insulin levels (237-240). The rise in energy expenditure is a result of both increased physical activity and thermogenesis, primarily involving brown adipose tissue (237,238,241). The improvements in glycemia and insulin sensitivity in leptin-treated *ob/ob* mice were initially believed attributable to reduced adiposity. However, in pair-feeding studies, leptin infusion resulted in more pronounced reductions in adiposity and plasma insulin levels than was produced by equivalent caloric restriction in pair-fed animals, suggesting leptin influences both central and peripheral tissues (242).

Leptin Resistant db/db Mice

Leptin relays peripherally-derived information regarding fat reserves to the central nervous system by binding a specific receptor (OB-R) encoded by the *db* gene in the choroids plexus of the brain (243). Alternative splicing of the *db* gene results in truncation of the carboxy terminal cytoplasmic domains (244). Ob-Ra, a shorter isoform of OB-R, is expressed in the microvasculature constituting the blood brain barrier and is the vehicle by which peripherally secreted leptin gains access to target neurons in the hypothalamus (245). The long isoform, OB-Rb, mediates the actions of leptin in the hypothalamus (246). Mutation of the *db* gene results in the *db/db* mouse, which lacks a functional leptin receptor (244,245). The phenotype of the *db/db* mouse is characterized by early-onset obesity, fasting hyperglycemia, and hyperinsulinemia (225). In addition, despite overexpression of the *ob* gene in adipose tissue, *db/db* mice are unable to respond to circulating leptin (247).

Leptin Levels in Normal Weight and Obese Humans

Considine, et al. (222) found that serum leptin levels in obese subjects were more than 4-fold higher than those of their age-matched lean ($p<0.001$) counterparts. Leptin levels increase in a linear fashion with increasing adiposity, such that an obese subject with a serum leptin level of 30 ng/ml will have approximately three-fold more fat mass than a lean subject with a serum leptin level of 10 ng/ml (222). Serum leptin levels strongly correlate with adiposity ($r=0.85$, $p<0.001$), BMI ($r=0.69$, $p<0.05$) and fasting insulin levels ($r=0.57$, $p<0.0001$) (248,249). However, the demonstration that serum leptin levels are elevated in obesity appear to conflict with studies documenting a reduction in

food intake in *ob/ob* mice given exogenous leptin (240). Thus, it seems likely that obesity, in addition to being an insulin resistant state, is also characterized by leptin resistance.

The relationships between circulating leptin levels and adiposity have been examined in normal weight and overweight/obese subjects by Havel, et al. (249). Although plasma insulin levels were increased two-fold in overweight/obese compared to lean ($p<0.01$) subjects, and similar increases ($p<0.01$) in plasma leptin levels are observed in obese subjects. In normal weight and obese subjects, there is a significant correlation between fasting plasma insulin and leptin levels ($r=0.61$, $p<0.0001$). Consistent with the notion that circulating leptin levels are reflective of total body fat reserves, plasma leptin was highly correlated with BMI ($r=0.81$, $p<0.0001$) in all subjects, and this relationship was persisted when normal weight ($r=0.58$, $p<0.001$) and overweight/obese ($r=0.59$, $p<0.001$) were considered separately. Percent body fat was similarly correlated with plasma leptin levels overall ($r=0.80$, $p<0.0001$), in normal weight ($r=0.64$, $p<0.005$) and overweight/obese ($r=0.69$, $p<0.002$) subjects (249).

A strong positive association has also been observed between cerebrospinal fluid (CSF) leptin levels and BMI ($r=0.40$, $p<0.05$). Despite serum leptin levels that were more than 3-fold higher in obesity, CSF leptin is only marginally higher (by 30%, $p>0.05$) in obese than in lean subjects. Consequently, the CSF/serum leptin ratio was 4.3-fold higher in lean compared to obese ($p<0.05$) subjects and was inversely associated with BMI in all subjects ($r=-0.58$, $p<0.05$). The finding of significantly higher CSF/serum leptin ratio in lean versus obese individuals suggests that leptin enters the brain by a saturable transport mechanism and that this may be the rate-limiting step in

leptin action. Obese subjects are often hyperleptinemic, yet fail to reduce their energy intake accordingly. The four-fold higher CSF/serum leptin ratio may partially account for this effect (250). In this study, an increase in leptin levels above 25 ng/ml as observed in obese subjects fails to compensate for leptin resistance. Furthermore, Schwartz, et al. (248) have demonstrated that the efficiency of leptin uptake, as measured by the CSF/serum leptin ratio, is significantly lower in obese compared to lean subjects.

Leptin binding sites have been identified in many regions of the brain including the choroid plexus, which is responsible for secretion of cerebrospinal fluid (251). Banks, et al. (252) have shown that the transport rate of leptin across the blood brain barrier is impaired in obese mice, which may be due to an inability to upregulate the leptin transporter in the face of increasing serum leptin levels. Subsequently, Banks, et al. (253) demonstrated that the leptin transport system is partially saturated at physiological leptin levels (~10 ng/ml).

Leptin circulates in serum as free hormone and bound to several proteins, including the soluble leptin receptor isoform. The soluble leptin receptor is the shortest isoform and lacks the hydrophobic transmembrane domain. Levels of free leptin selectively reflect body fat mass while bound leptin serves as a marker of resting energy expenditure (254). In both lean and obese women, free leptin levels are closely correlated with percent body fat ($r=0.73$, $p<0.0001$) (254).

Regulation of Leptin Synthesis and Secretion and Influence of Insulin

The pattern of *obese* gene expression has been examined in human adipose tissue. A single *ob* mRNA species 4.5 kb in size is abundantly expressed in adipose tissue from the

subcutaneous, omental, retroperitoneal, perilymphatic, and mesenteric fat pads. There are significant variations in leptin expression between depots in the same individual. The highest level of *ob* expression was detected in the subcutaneous adipose tissue depot. No significant amount of *ob* mRNA has been observed in brain, heart, lung, liver, stomach, pancreas, small intestine or skeletal muscle. Furthermore, the lack of an appreciable amount of *ob* mRNA in preadipocytes suggests that *ob* gene expression is induced during the process of adipocytes differentiation and maturation (255).

Williams, et al. (256) examined the level of *ob* mRNA and the response to weight loss. Serum leptin levels were shown to be four-fold higher in obese compared with lean subjects. However, the *ob* mRNA content of abdominal subcutaneous adipocytes is only two-fold higher in obese compared to lean ($p=0.005$) subjects, suggesting that adipocyte hypertrophy contributes to higher leptin levels in obesity. To determine if leptin expression is responsive to changes in body weight, obese subjects were placed on a hypocaloric diet for a period of 8 – 12 weeks, which led to a 10% reduction in body weight and lowered plasma insulin and leptin levels by 25 and 53%, respectively. Serum leptin levels were reduced as a result of a 38% reduction in *ob* mRNA content of abdominal subcutaneous adipocytes. Following a four-week period of weight maintenance, serum leptin levels and *ob* mRNA levels rose slightly but remained significantly lower than those observed prior to weight reduction. Thus, an increase in adipose tissue mass results in an increase in serum leptin levels at the level of *ob* gene transcription.

Leptin expression and secretion vary by adipose tissue depot. The levels of mRNA and leptin secreted into culture media are about two-fold higher in subcutaneous

compared to omental adipocytes. Consequently, leptin content is roughly 1.5-fold higher in subcutaneous compared to omental adipose tissue ($p<0.005$). To determine whether leptin secretion or content of adipose tissue is dependent on the synthesis of new protein, tissue samples were incubated for 3 hours in the presence of 10 μg/ml cycloheximide. Compared to control samples, cycloheximide treatment significantly decreased leptin content by 46% and 36% in the omental and subcutaneous adipose tissue samples, respectively ($p<0.01$ for both) without affecting the rate of leptin secretion. In the presence of cyclohexamide, leptin secretion is maintained at 80% of untreated levels for the first three hours, but subsequently falls to levels ~28% of control by the fifth hour. This suggests that adipose tissue secretes preformed leptin for approximately three hours, after which time new protein synthesis is required to continually secrete leptin (257).

To investigate the mechanisms underlying increased circulating leptin levels in obesity, Lonnqvist, et al. (258) measured secretion rates in vitro and mRNA levels of leptin in subcutaneous adipose tissue biopsies from lean and obese women. Plasma leptin levels were elevated five-fold in obese compared to lean women ($p<0.05$). Rates of leptin secretion were two- to seven-fold more rapid in obese compared to non-obese women, when expressed per lipid weight ($p<0.05$) or per cell number ($p<0.05$). Obese subjects had larger adipocytes which contained significantly more lipid compared to adipocytes from control cells, implying that leptin secretion is enhanced in cells with greater lipid volume. Leptin mRNA was elevated two-fold in the obese group ($p<0.05$). Strong positive relationships were found between leptin mRNA and leptin secretion, when expressed per lipid weight ($r=0.66$, $p<0.001$) or per cell number ($r=0.60$, $p<0.001$).

Furthermore, approximately 60% of the variation in plasma leptin levels was attributable to variations in the secretion rate, suggesting this is a critical factor contributing to elevated plasma leptin levels in obesity. In support of this, positive correlations were noted between leptin secretion rate and BMI ($r=0.71$-0.76, $p<0.001$) and fat cell volume ($r=0.85$-0.89, $p<0.001$). Fat cell volume, which was also positively correlated with leptin mRNA, plasma leptin, and leptin secretion ($r=0.7$-0.9, $p<0.001$), explained 50-80% of the variation in plasma leptin levels. This study demonstrates that leptin secretion is subject to regulation by several factors. Chief among these is the steady state leptin mRNA level, which presumably controls the production rate of leptin in adipocytes and was elevated ~two-fold in obese women. Leptin mRNA accounted for ~40% of the variations in circulating and secreted leptin. Chronic hyperinsulinemia may be an additional factor, since plasma insulin levels were strongly correlated with the rate of leptin secretion, when expressed per gram of lipid weight ($r=0.60$, $p<0.01$) or per cell number ($r=0.67$, $p<0.001$) (258).

Uusitainen, et al. (259) have shown that following a 6-hour period supraphysiological hyperinsulinemia, plasma leptin levels were increased by 88% compared increases observed following saline infusion for the same duration. During euglycemic, hyperinsulinemic clamp studies, plasma leptin levels increased ~62% compared with saline infusion. Insulin-induced increases in plasma leptin levels are more pronounced in women than in men. Plasma leptin levels were significantly correlated with percentage of body fat in men ($r=0.52$, $p<0.05$) and women ($r=0.92$, $p<0.001$) (259).

Barr, et al. (260) evaluated leptin secretion following incubation of adipose tissue in the presence or absence of insulin. Insulin increased the amount of leptin secreted by

80% compared with adipocytes incubated in the absence of insulin. Furthermore, insulin changed the subcellular localization of leptin within the cell and decreased immunochemical staining of cellular leptin deposits, which is consistent with secretion of leptin in response to insulin (260).

The role of leptin in long-term regulation of adiposity and energy expenditure has been examined in female Fischer 344 rats raised on either a low-fat complex-carbohydrate (LFCC) or a high-fat sucrose (HFS) diet for 20 months. Leptin levels were significantly elevated in the HFS compared with the LFCC group after 2 weeks of dietary treatment and continued to rise throughout the remainder of the study. Consequently, leptin levels were significantly elevated in HFS animals compared to LFCC animals at all points during the study. Adipocyte size gradually increased in HFS-fed animals during the feeding period ($p<0.01$), being greater at all points in time than adipocytes from LFCC-fed animals. The difference in adipocyte size between the two groups was not significantly different at 2 weeks. However, by 2 months adipocytes from HFS-fed animals twice as large ($p<0.01$) than those from LFCC-fed animals. Adipocyte size continued to increase in HFS-fed animals and reached maximum size at 20 months. In contrast, adipocyte size remained relatively unchanged in LFCC-fed animals at 6 and 20-months. In all animals, there was a strong positive correlation between fat cell size and plasma leptin levels ($r=0.96$, $p<0.001$) (261).

Body Fat Distribution, Insulin Action and Serum Leptin Levels in Humans

Leptin appears to play a role in the regulation of body fat distribution and insulin action in vivo. Sprague-Dawley rats given *ad-libitum* access to standard rodent diet for 8

days were compared to rats with free access to the same diet while receiving continuous exogenous insulin infusion. A pair-fed group that did not receive exogenous leptin were also included. Administration of exogenous leptin elevated plasma leptin levels and reduced food intake by 50% compared with untreated animals fed *ad libitum*. During the 8-day study, body weight increased by 5.7% in *ad libitum* fed animals while it decreased by 8.1% in the leptin-treated animals and by 9% in pair-fed animals. The decreases in body weight observed in leptin-treated and pair-fed animals were attributable to decreased fat mass. Pair-fed animals and leptin-treated animals experienced similar reductions in total body fat (by 24%) and visceral fat (by 21%). However, when visceral adiposity was expressed as the sum of omental, epididymal, and perinephric fat depots), a marked and specific reduction was observed in leptin-treated animals (by 62%, $p<0.001$ vs. pair-fed). Thus, in addition to the well recognized effects of leptin on food intake and total body adiposity, leptin appears to specifically target visceral and intraabdominal adiposity, suggesting the hormone may contribute to the regulation of body fat distribution.

Postabsorptive plasma insulin levels were significantly lower in leptin-treated and pair fed animals compared to untreated animals fed *ad libitum* ($p<0.001$). During physiological hyperinsulinemia, the rate of glucose uptake in leptin treated rats was 52 and 33% higher than in control ($p<0.001$) and pair-fed animals ($p<0.001$), respectively. Thus, leptin administration improved peripheral insulin sensitivity to a greater extent than can be attributed to weight reduction by moderate caloric restriction alone. The observed improvements in the peripheral actions of insulin were partially accounted for by a nearly 2-fold increase in the rate of glycogen synthesis in leptin-treated animals compared to pair-fed animals ($p<0.001$). Leptin also enhanced insulin actions in the

liver, by enhancing insulin-mediated suppression of hepatic glucose output, resulting in a 4-fold reduction in total glucose output in leptin-treated animals ($p<0.001$) compared to control and pair-fed animals. Glucose cycling, which represents the flux through glucose-6-phosphatase back into circulation, was also reduced in leptin treated animals ($p<0.001$). Complete suppression of hepatic glycogenolysis was observed in leptin-treated rats, whereas this process was still active in control and pair-fed animals (262). Thus, leptin plays important roles in regulation of adiposity and glucose homeostasis by several mechanisms which are independent of its effects on food intake. Specifically, this study demonstrates that administration of exogenous leptin selectively decreases visceral adiposity, augments insulin-stimulated glucose disposal, and enhances insulin-mediated suppression of hepatic glucose output.

To examine the relationships between body fat distribution, insulin sensitivity, and leptin levels, these variables have been quantified in healthy individuals classified into three groups: lean insulin-sensitive (LIS), lean insulin-resistant (LIR), and obese insulin-resistant (OIR). Subjects were classified as insulin resistant based on the insulin sensitivity index (S_I) calculated during a frequently sampled intravenous glucose tolerance test (FSIVGTT). Insulin-resistant subjects were relatively hyperinsulinemic as indicated by two-fold higher fasting insulin levels in OIR subjects compared with the insulin-sensitive group ($p<0.001$), with an intermediate level in the LIR group ($p<0.001$ vs. both OIR and LIS). Insulin resistance appeared related to central fat deposition as suggested by higher WHR in both insulin resistant groups compared with lean, insulin sensitive subjects. CT scanning revealed greater fat deposition in the abdominal region of insulin-resistant subjects ($p<0.001$). Despite having similar BMIs, LIR subjects had 45% more

abdominal subcutaneous fat and 70% more visceral abdominal fat compared to the LIS group ($p<0.01$). The increases in abdominal fat were even more pronounced in obese subjects, who had 2.5 and 3-fold greater amounts of subcutaneous and visceral abdominal fat ($p<0.01$ for both) compared to lean subjects, regardless of insulin sensitivity. The increased abdominal fat area in obese subjects was associated with a threefold elevation in fasting leptin levels relative to both sets of lean subjects ($p<0.001$ for both). Leptin levels were also twice as high in LIR subjects compared with the LIS group ($p<0.001$). Insulin sensitivity was inversely correlated with BMI ($r=-0.634$, $p<0.001$), WHR ($r=-0.498$, $p<0.001$), SCAT ($r=-0.57$, $p<0.001$) and IAAT ($r=-0.688$, $p<0.001$). Using multiple regression analysis, the associations between measures of body fat distribution and insulin sensitivity were simultaneously analyzed. In all subjects, visceral abdominal adipose tissue deposition was the strongest predictor of insulin sensitivity. Leptin levels were most strongly correlated with subcutaneous abdominal fat, which explained 54-66% of the variance in leptin levels in men and women (263).

Short-Term Leptin Regulation and Association with Insulin Action

In rodents subjected to a short term fast, plasma leptin levels decrease much more than the amount of adipose tissue lost (240,242). Similarly, lean and obese adults fasted for 52 to 96 hours lost less than 4% of body weight while leptin levels decreased 54 to 72%. In non-obese subjects, seven days of energy restriction mimics fasting, as evidenced by minimal weight loss (4% of initial body weight) in the face of a 36% reduction in serum leptin levels. These findings suggests that serum leptin levels may be more dependent on on-going triglyceride synthesis or glucose uptake by adipocytes

rather than by adipose tissue mass which was negligibly affected by short fast. Refeeding normal subjects after a 3 day fast returns plasma leptin levels to baseline within 12 hours indicating leptin is acutely sensitive to changes in energy availability (264).

Dietary Influences on Serum Leptin Levels

To investigate the effect of diet on plasma leptin fasting and postprandial leptin levels were measured before and after 14 days' ad libitum intake of a fat rich (FAT), starch-rich (STARCH) or sucrose-rich (SUCROSE) diet in normal weight, post-obese women and matched controls. Both the STARCH and SUCROSE diets contained 28 en% fat, 13 en% protein and 59 en% carbohydrate, the difference being in the sucrose content which was 2 en% in the STARCH and 23 en% in the SUCROSE diet. The FAT diet contained 46 en% fat, 13 en% protein and 41 en% carbohydrate (2 en% sucrose). Energy intake over the 14 day ad libitum feeding period was lowest in the STARCH group ($p<0.05$). In both post-obese and control groups, fasting leptin levels on day 15 and the change in fasting leptin (day 15 – day 1) were higher after SUCROSE compared to other groups ($p<0.01$). Total AUC were higher after SUCROSE than STARCH, although these values were not statistically significant. These findings can not be explained by fasting blood glucose levels which did not differ by diet. The increase in fasting leptin may therefore be caused by greater initial postprandial glucose excursions as would be anticipated in response to the SUCROSE compared to STARCH diet (265). These findings are consistent with studies which suggest that dietary carbohydrate per se influences leptin secretion. In a study of non-obese men fed isoenergetic high-fat or high-carbohydrate meals, significant differences were observed in leptin levels at eight

hours following meal consumption (266). Coppack, et al. (267) suggest a specific effect of dietary carbohydrate on leptin levels in vivo. Five hours after consumption of a high carbohydrate (70 en%) meal, a significant increase in tissue leptin was observed demonstrating a slow yet specific effect of dietary carbohydrate on leptin secretion.

To determine the effects of dietary fat content independent of changes in body mass on plasma leptin levels, normal weight and overweight/obese subjects underwent a four month experimental period during which time they consumed a prescribed weight-maintaining diet of 31 en% fat for four weeks, 23 en% fat for six weeks and 14 en% fat for the final six weeks. To examine the effects of sustained weight loss on plasma leptin levels, the four month experimental period was followed by a self-selected ad libitum very low fat (< 15 en%) diet for 8 months. Plasma leptin levels were more than 2-fold higher in overweight/obese subjects compared to their normal weight counterparts but were unaffected by varying the fat content of the diet. At stable body weight, plasma leptin levels correlated with fasting plasma insulin levels ($r=0.61$, $p<0.0001$). The average weight loss after 8 months of the self-selected diet was approximately 7%. Plasma leptin levels were decreased in both normal weight women and overweight/obese women who sustained weight loss of greater than 7% by consumption of a self-selected, low-fat diet ad libitum for 6 months. In all subjects, this level of weight loss was associated with reductions of ~35% in both plasma leptin and insulin levels. The decrement in plasma leptin after weight loss was greatest in overweight/obese women compared to their normal weight counterparts ($p<0.005$), although overweight/obese women continued to maintain higher plasma leptin levels ($p<0.01$). Maintenance of reduced body weight at 8 months was not associated with

additional reductions in leptin levels. The change in plasma leptin on the low fat diet in all subjects was significantly correlated with both the change in BMI ($r=0.42$, $p<0.02$) and the change in percent body fat ($r=0.49$, $p<0.005$). In contrast, changes in plasma insulin were not significantly correlated with the change in BMI or percent body fat. However, the change in plasma leptin on the self-selected low-fat diet was significantly correlated with the change in fasting insulin ($r=0.52$, $p<0.002$). Partial regression analysis demonstrated that the change in plasma leptin was significantly correlated with the change in plasma insulin, independent of changes in BMI ($r=0.45$, $p<0.01$) or percent body fat ($r=0.44$, $p<0.02$). Further analysis was possible by calculating the leptin/percent body fat ratio, which corrects for adiposity. This ratio revealed that overweight/obese women have higher plasma leptin levels per unit of adiposity than normal weight women. However, after 8 months of the self-selected low-fat diet, only overweight/obese women had a significant decrease (~30%) in their leptin/adiposity ratio in whom both the absolute changes of leptin per unit of adiposity and the proportional changes of leptin per proportional change of adiposity were 2.5-3.5 times greater than in normal weight women. Thus, a 10% reduction in total adiposity in an overweight/obese women resulted in a 34% decrease in plasma leptin levels, whereas a similar change in adiposity decreases plasma leptin by only 13% in normal weight women (267).

In energy-restricted obese humans, improvements in insulin sensitivity and glucose tolerance are apparent before any major loss of body fat is detected. The mechanism responsible for the early benefits of caloric restriction are not well understood, but circulating leptin levels may be one factor. The response of serum leptin to energy

restriction has been examined in obese subjects with normal and impaired glucose tolerance. Subjects consumed prescribed diets containing 50% of their usual ad libitum energy intake for a period of 28 days. At the conclusion of this period, weight loss was attributable to comparable loss of fat and lean tissue. Serum leptin levels were significantly correlated with measures of total body adiposity prior to (day 0) and following (day 28) energy restriction. Leptin levels on day 28 were expressed as a percentage of day 0 values in order to test for the influence of specific dietary measures. Serum leptin fell 33% by day 4 ($p<0.001$) with a further 21% reduction seen at day 28 ($p<0.01$). Serum leptin at day 4 (expressed as a percentage of day 0 value) was significantly related to the percentage change in grams of dietary carbohydrate intake per day ($r=0.74$, $p=0.0005$), but no association was noted with changes in dietary fat intake or protein. Similarly, there was no correlation between day 4 serum leptin levels (as a percentage of day 0) and the percentage change in total energy intake. In multiple correlation analysis, which included the three macronutrients, the change in carbohydrate intake was a significantly better independent predictor ($r=0.68$, $p=0.027$) of the change in serum leptin than were dietary protein and fat. Fasting insulin fell significantly by day 4 ($p<0.0001$), with no further change noted on day 28. The early change in serum insulin was significantly correlated with the change in serum leptin ($r=0.57$, $p=0.013$) during the same period, but was not independent of the change in carbohydrate intake. These data suggest that serum leptin levels during the early stages of energy restriction are not reflective of dietary fat intake or reduced adipose tissue mass, but may be more indicative of changes in dietary carbohydrate intake (265).

Dietary carbohydrates may differentially influence 24-hour leptin profiles. For seven days, lean subjects consumed one of four diets which varied in glycemic index of the dietary carbohydrate and level of dietary fat: high GI carbohydrate, 30% fat (highGI); low GI carbohydrate, 30% fat (lowGI); high GI carbohydrate, reduced (20%) fat (highGI, lowfat); and low GI carbohydrate reduced fat (lowGI, lowfat). No significant differences in serum glucose and insulin profiles were observed between diets and there were no statistically significant differences in peaks, troughs, or AUCs for glucose or insulin. Basal leptin levels were not significantly different between diets. The normal 24-hour leptin profile was observed with the two lowGI diets: serum leptin levels remained low throughout the day, rose above the baseline (0800 hr) values after 2300 hr, peaked at 0200 hr, and returned to baseline by 0800 hr. In contrast, the 24-hour leptin profile was altered by the high GI diets following which serum leptin levels rose above baseline (0800 hr) as early as 1300 hr. The leptin AUC from 1230 hr to 2400 hr and the 24-hour AUCs with the high GI diets were significantly higher than for the low GI diets ($p<0.0001$) (268).

Agouti

The *agouti* gene encodes a 131-amino acid protein which is subject to temporal and spatial regulation in rodents(269,270). The *agouti* gene is normally expressed exclusively within the hair follicle, regulating the synthesis of eumelanin (black) and phaeomelanin (yellow) pigment to produce the wild-type agouti pattern of coat coloration: black hairs with a subapical yellow band (269,271). Upon binding of α-melanocyte stimulating hormone (α-MSH) hormone to the melanocortin-receptor-1 (MC1-R), activation of

adenylate cyclase causes an increase in intracellular cAMP levels and the production of eumelanin. *Agouti* is expressed only transiently during the middle of the hair growth cycle during which time it exerts a paracrine effect on melanocytes, shifting pigment production from eumelanin to phaeomelanin mediated by antagonism of melanocortin-receptor-1 (MC1-R) (272). Dominant mutations at the *agouti* locus result in the constitutive and ubiquitous expression of agouti-signaling protein (ASP) and leads to a pleiotropic obesity syndrome. The best characterized dominant mutations are the lethal yellow (A^{y}) and viable yellow (A^{vy}) mutants. In contrast to *ob/ob* and *db/db* mice which are characterized by early-onset obesity, A^{y} and A^{vy} mice develop an obesity syndrome that peaks between 8 and 17 months of age (273). Furthermore, *ob/ob* and *db/db* mice demonstrate significant adipocyte hyperplasia while the increased adiposity of obese yellow mice is primarily due to adipocyte hypertrophy, much like the human obesity syndrome (274). Significant pancreatic β-cell hyperplasia is evident in A^{vy} males by 21 days of age and thus, precedes the development of hyperinsulinemia and obesity (275). Hyperinsulinemia develops in A^{vy} mice at approximately 6 weeks of age, while *db/db* and *ob/ob* mice are hyperinsulinemic at 10 and 15 days, respectively (276). The development of hyperinsulinemia in A^{vy} mice is accompanied by the appearance of other metabolic abnormalities including hyperphagia, hyperglycemia in males, increased rates of hepatic lipogenesis and decreased rates of adipocyte lipolysis (277-279). Obese yellow mice are characterized by a slight increase in linear growth, making them unique from other rodent obesity models, which are somewhat shorter than their wild-type littermates (280).

Dominant mutations at the agouti locus that result in obesity are characterized by the constitutive synthesis of normal (wild-type) *agouti* protein within the hair follicle, accounting for the yellow coat color, and the ectopic expression of agouti in most all tissues of the animal, which is believed responsible for the development of the pleiotropic obesity syndrome as these animals age. Transgenic mice ectopically expressing *agouti* from ubiquitous promoters (e.g. β-actin) recapitulate the obese hyperinsulinemic phenotype, indicating the pleiotropic obesity syndrome of the yellow mouse is a direct result of ectopic *agouti* expression. Disruption of MC1-R function results in obese mice with black fur but lacking the subapical yellow band, indicating the obesity phenotype is not dependent on the synthesis of yellow pigment. The working hypothesis for the induction of obesity by ectopic overexpression of normal *agouti* protein involves antagonism of melanocortin receptors, specifically MC4-R, in regions of the brain known to be critical for regulation of feeding behaviors and body weight. The significance of MC4-R signaling in body weight regulation is supported by MC4-R deficient mice which develop an obesity syndrome which mimics the *agouti* syndrome (281).

Murine Obesity Resulting from Ectopic Agouti Expression

Wild-type *agouti* cDNA was placed under the transcriptional control of the human β-actin promoter to generate transgenic mice that ectopically express normal (wild-type) *agouti* in all tissues. To accentuate the effect of ectopic *agouti* expression on weight gain, animals were fed a high-fat diet throughout their lifespan. The average weight of *agouti* transgenes became greater significantly ($p<0.01$) greater than their wild-type littermates

at 4 and 6 weeks of age in females and males, respectively. Ultimately, *agouti* transgenes were 1.3- to 1.7-fold heavier than their age- and sex-matched wild-type littermates. By 20 weeks of age, *agouti* transgenes have significantly higher insulin levels compared to controls, with the most pronounced hyperinsulinemia occurring in males compared to females. Consistent with a more severe state of hyperinsulinemia, male transgenes had plasma glucose levels that were approximately 70% higher than in control animals at 20 weeks of age (280).

Relationships Between Agouti and Leptin

Both diet-induced obesity and genetic obesity due to the yellow *agouti* mutation are associated with significantly elevated *ob* mRNA levels. In normal C57BL/6J mice fed a high-fat, high-carbohydrate diet for 14 weeks, there was a significant elevation in *ob* mRNA per μg of total RNA compared to the level observed in control mice on standard rodent chow. To produce an equivalent state of acute nutritional stimulation in mice on each diet, food was removed 7 hours prior to the beginning of the dark cycle and mice sacrificed 30 minutes after an intraperitoneal injection of glucose just before lights out. In lean mice, *ob* mRNA was significantly elevated in 7-hour fasted, glucose injected mice compared to mice fasted for 48 hours ($p<0.05$). This acute effect of nutritional status was not observed in mice fed the high-fat diet for 14 weeks. Body weight and serum insulin were also significantly elevated in diet-induced obese mice ($p<0.05$). When lean and obese mice were analyzed together, linear regression indicated that *ob* mRNA was significantly correlated with body weight ($r=0.79$, $p<0.05$) and serum insulin levels ($r=0.54$, $p<0.05$) (282).

Compared to lean wild-type C57BL/6J mice, *ob* mRNA was significantly elevated in genetically obese yellow (C57BL/6J-A^y) mice ($p<0.05$). Administration of glucose to lean mice was associated with a significant induction in *ob* mRNA ($p<0.05$), an effect that was absent in obese *agouti* mice. Because obese yellow mice also have an expanded adipose tissue mass, the amount of total leptin secreted by these mice is even greater (282).

The human homologue of *agouti* is expressed in both adipose tissue and pancreas. The relationship between *agouti* expression and adipocyte fatty acid synthase expression and activity has been investigated in human subcutaneous adipocytes. *Agouti* appears to be up-regulated during the process of human pre-adipocyte differentiation as suggested by the greater abundance (4-fold) of *agouti* protein in human mature adipocytes compared to preadipocytes ($p<0.005$). Using human adipose tissue biopsies obtained during elective surgery, adipose tissue *agouti* content was significantly correlated with both FAS mRNA level ($r=0.846$, $p<0.002$) and FAS activity ($r=0.782$, $p<0.005$) (283).

V. Skeletal Muscle Metabolism and Alterations in the Metabolic Syndrome

Skeletal Muscle Characteristics

Human and rodent skeletal muscle is a mixture of two main muscle fiber types (284-286). In humans, muscle fiber types may be distinguished based on their different metabolic and histochemical characteristics (287,288). Type I fibers are appropriately called "slow-twitch" fibers, based on their slower contraction velocity compared to Type II ("fast-twitch") fibers (289). Type II fibers are subdivided into two classes, Type IIa (fast twitch oxidative fibers) and Type IIb fibers (fast twitch glycolytic fibers) (289). Because there is substantial overlap in the oxidative capacities of Type I and Type II fibers, fiber type does not provide a direct measure of the oxidative capacity of skeletal muscle. Oxidative capacity is known to be related to mitochondrial density, which can be assessed by NADPH staining (290). Studies comparing muscle fibers from lean subjects have shown that type I fibers have the greatest oxidative capacity, as indicated by higher activity of enzymes which serve as markers for oxidative metabolism including succinate dehydrogenase, 3-oxoacid CoA transferase, citrate synthase, 3-hydroxyacyl CoA dehydrogenase, and malate dehydrogenase (291). There is a strong correlation between the proportion of type I fibers and oxidative capacity as determined by mitochondrial NADPH staining ($r=0.95$, $p=0.0001$) and citrate synthase (CS) activity ($r=0.35$, $p=0.03$) (285). Type IIb fibers are adapted for glucose oxidation and demonstrate increased activity of glycolytic enzymes such as phosphofructokinase, hexokinase, lactate dehydrogenase and pyruvate dehydrogenase (287).

Fiber Type Influences Insulin Binding

In vitro studies using rodent cell lines have revealed differences in insulin binding and glucose uptake based on muscle fiber type. Whole muscle sections were incubated with ^{125}I-insulin and 2-deoxy-D-glucose for 4 hours to permit steady state conditions after which time insulin binding and glucose uptake were measured. Greater insulin binding and greater basal and insulin-stimulated 2-deoxy-D-glucose occurred in soleus (primarily Type I fibers) compared to gastrocnemius (predominately Type II fibers) muscles (292,293). A possible explanation for the observed associations between insulin resistance and muscle fiber type can be found in the pattern of glucose transporters expressed by fiber types. Type I fibers have increased levels of GLUT-4, which would be transported to the membrane more readily when the supply of capillaries to the fiber is more dense (293).

Muscle Fiber Type in Obese Subjects

Tanner, et al. (294) have demonstrated a clear relationship between muscle fiber type and obesity. Obese women were shown to have a lower percentage of type I and a higher percentage of type IIb muscle fibers compared to lean subjects ($p<0.001$ for both). Furthermore, BMI was positively related to the relative percentage of type IIb fibers ($r=0.49$, $p<0.001$). This study also found that muscle from obese African-American women contained a lower percentage of type I fibers than comparably obese Caucasian women ($p<0.05$). Consequently, African-American subjects had more type IIb fibers than their Caucasian counterparts ($p<0.01$). This is consistent with epidemiological studies which have documented a higher prevalence of obesity and two

fold greater incidence of NIDDM in obese African-American women compared to obese Caucasian women (295-297). Obese African-American women are also more insulin resistant and have an impaired capacity for fat oxidation compared with Caucasian women (298). These findings are also consistent with a reduction in the percentage of type I fibers in muscle biopsies from obese African-American women compared to weight-matched Caucasian women (296,299).

Obese subjects often fail to lose a significant amount of weight with intervention and are prone to weight regain (300). A reduction in the percentage of type I fibers would be expected to impair the ability of skeletal muscle to oxidize lipids and predispose one to weight regain (294). To determine if a relationship exists between muscle fiber composition and the capacity for weight reduction, biopsies from the rectus abdominus muscle were obtained from women (BMI ~52 kg/m^2) undergoing gastric bypass surgery to induce weight loss (301). Prior to surgery, the percentage of type I and type IIa fibers were roughly equal, and three-fold greater than the percentage of type IIb fibers in abdominal muscle preparations. BMI was significantly reduced following the surgery. The relative percentage of type I fibers at the time of surgery was positively associated with the change in BMI ($r=0.55$, $p<0.05$) and the percentage of body weight lost ($r=0.56$, $p<0.05$). Although this study does not permit the identification of the precise mechanism(s) by which alterations in fiber type contribute to obesity, the data suggests that the histochemical characteristics of muscle fibers may be partially predictive of obesity (301).

Glucose Transport and Uptake by Skeletal Muscle

The importance of skeletal muscle in glucose homeostasis is apparent during an oral glucose tolerance test, when glucose uptake by skeletal muscle increases 5-10 fold (302). Skeletal muscle may contribute to insulin resistance and glucose tolerance that is associated with obesity as suggested by studies demonstrating decreased glucose uptake by skeletal muscle in obese subjects (302,303).

Insulin influences both the uptake and intracellular fate of glucose in skeletal muscle at steps distal to its vasodilatory actions. During moderate insulin stimulation, Kelley, et al. (304) suggests that the phosphorylation of glucose, rather than transmembrane transport, becomes the key step controlling further glucose metabolism by cells. Thus, it seems necessary to define the respective contributions of glucose transport and phosphorylation in normal physiology as well as alterations that occur in insulin resistant states.

Basal glucose uptake by muscle occurs in a process mediated by a specific glucose transport protein, GLUT-1, which appears to be bound to the plasma membrane, without regard to plasma insulin levels (62). Basal glucose transport may also be facilitated by GLUT-3. Insulin-stimulated glucose uptake is facilitated by the insulin-sensitive glucose transport protein, GLUT-4. In the postabsorptive state, the bulk of GLUT-4 proteins are sequestered in intracellular vesicles associated with low-density microsomes. In vastus lateralis muscle biopsies taken from normal fasting human subjects, the ratio of the three principal glucose transporters was determined to be 2:1:3 for GLUT-1, GLUT-3 and GLUT-4. In the basal state, the plasma membrane glucose

transporter pool in the basal state is approximately 25% GLUT-1, 45% GLUT-3, and 30% GLUT-4 (305).

Transport of glucose across the plasma membrane is the first rate-limiting step in glucose metabolism under physiological conditions (306). Once glucose enters the skeletal muscle cell, it is immediately phosphorylated to glucose-6-phosphate (G6P) via the enzyme hexokinase (HK) (307). The conversion of glucose to G6P traps glucose within cells, preventing its efflux from cells, and thus, maintains a concentration gradient for the movement of free glucose across the sarcolemma (307). In the basal state, intracellular free glucose levels are negligible (308). However, when hyperinsulinemia or hyperglycemia are present, free unphosphorylated glucose accumulates within the myocytes and steps distal to transmembrane transport become rate limiting (308,309).

Skeletal muscle expresses two isoforms of hexokinase: hexokinase I (HK I) and hexokinase II (HK II) (307). Although there is significant sequence conservation between the two isoforms, they are regulated in distinct manners in skeletal muscle. HK II appears to be the insulin-responsive isoform, as insulin induces HKII transcription in vitro (310).

In subjects undergoing euglycemic, hyperinsulinemic clamps, insulin rapidly stimulates glucose uptake, while there is a delay of 2-4 hours before changes in HKII expression are detected (311). The acute increase in glucose transport and phosphorylation is mediated by an insulin-mediated shift in the intracellular distribution of HKII from the cytosol, where it is localized in the basal state, to the mitochondria, where the enzyme will be in close proximity to elevated levels of ATP, which will further increase the activity of the enzyme (312).

In the basal state, human skeletal muscle concentrations of G6P are normally ~100 μM (313). Skeletal muscle HK II activity is positively correlated with GLUT-4 activity, which safeguards against excessive accumulation of intracellular G-6P ($[G6P]_i$. Consequently, insulin stimulation of skeletal muscle in vivo increases the expression/translocation of GLUT-4 and causes a simultaneous, parallel increase in HK II expression. G-6P is a critical metabolic intermediate whose levels largely determine the metabolic fate of glucose within the muscle cell (313).

Multiple defects in glucose metabolism occur in insulin resistant conditions, including obesity and NIDDM (314-316). Non-invasive methods for the in vivo investigation of skeletal glucose utilization have facilitated a greater understanding of the proximal steps of glucose metabolism and their contribution to glucose homeostasis. One of these methods, nuclear magnetic resonance (NMR) spectroscopy, has yielded evidence to suggest that the transmembrane transport of glucose is the primary site of regulation of glucose metabolism during euglycemic states. Quantitation of intracellular levels of free glucose and G6P demonstrated that about 90% of glucose flux is controlled by regulating the transmembrane transport of glucose during euglycemia as well as during conditions of mild hyperinsulinemia with hyperglycemia (317). However, this remains controversial since it is unclear if this relationship is maintained over a range of insulin levels.

In lean subjects with normal glucose tolerance, moderate insulin infusion stimulates leg glucose uptake to levels 3-4-fold higher than in obese subjects and subjects with NIDDM. Insulin stimulates an 8-10-fold increase (above basal) in fractional glucose clearance in lean subjects, which is a significantly greater effect than is observed in either

obese or NIDDM subjects, which experienced similar decrements versus lean subjects. Moderate insulin infusion increases glucose phosphorylation 5-fold above basal in lean subjects, while simultaneously reducing the level of free glucose in the interstitial space. These responses are absent in obese and NIDDM subjects, indicating major impairments in both glucose transport and phosphorylation (318).

At supraphysiological levels of insulin, rates of glucose clearance significantly increase above basal levels in lean, obese and NIDDM subjects. This suggests that obese subjects and those with NIDDM are experiencing decrements in insulin sensitivity but maintain the capacity to increase glucose uptake in response to maximally-stimulating levels of insulin. However, hyperinsulinemia reduced free glucose levels to below basal levels and resulted in a more pronounced increase in glucose transport and phosphorylation compared with subjects with NIDDM. Thus, in obese subjects with normal glucose tolerance, hyperinsulinemia can alleviate the impairment in both glucose transport and phosphorylation that were observed with moderate insulin levels. In contrast, hyperinsulinemia failed to significantly reduce free glucose levels, suggesting a more profound defect in glucose transport and phosphorylation in these subjects (318).

Skeletal Muscle Lipid Metabolism and Lipoprotein Lipase

Skeletal muscle demonstrates significant metabolic flexibility, readily altering its pattern of substrate utilization based on availability. Skeletal muscle is a major contributor to whole-body lipid oxidation. Fatty acids oxidized by skeletal muscle may originate from several sources. Nonesterified fatty acids (NEFAs), derived from triacylglycerols released from adipose tissue by the action of hormone sensitive lipase

(HSL), account for the majority (80-90%) of the muscle lipid fuel needs during mild exercise or fasting conditions (319). Alternatively, free fatty acids may be liberated from circulating triglyceride-rich lipoproteins by the muscle isoform of lipoprotein lipase (mLPL). Following feeding, as much as 30% of circulating triglycerides may be utilized by skeletal muscle as an energy substrate (319,320). Finally, skeletal muscle may oxidize free fatty acids derived from endogenously stored intramuscular triglycerides following the action of the isoform of HSL found in skeletal muscle (321).

The muscle isoform of lipoprotein lipase (mLPL) hydrolyzes the bonds between fatty acyl residues and glycerol in triglycerides (321,322). However, the adipose tissue and muscle isoforms are subject to reciprocal regulation such that when LPL activity is increased in one tissue, it will be decreased in the other. In contrast to adipose tissue LPL, insulin decreases the activity of mLPL (323,324). In addition to tissue specificity, the activity of mLPL is five-fold greater in oxidative, slow twitch red muscle compared to glycolytic fast twitch muscles. In addition, mLPL has a phospholipase A_2 activity which facilitates the hydrolysis of the lipid layer surrounding the core triglycerides within lipoproteins (325). This makes the triglyceride rich core of the lipoprotein particle accessible to the lipase activity of mLPL. Roughly half of the fatty acids generated by the action of mLPL on circulating lipoproteins will be extracted and stored within muscles for use as an oxidative fuel source when needed (326).

In resting, fed humans, lipolysis of circulating triglyceride-rich lipoproteins (chylomicrons and very-low-density lipoproteins, VLDL) provide 50-80% of fatty acids taken up by skeletal muscle (326). This is accomplished by the muscle isoform of lipoprotein lipase (mLPL). Isoforms of LPL are found at the capillary endothelium of

various tissues, including adipose tissue, skeletal muscle and cardiac muscle. The highest levels of LPL are found in adipose tissue and skeletal muscle (327). FFAs liberated by the action of LPL are either metabolized immediately to meet prevailing energy needs or are re-esterified and stored in endogenous pools from which they can be mobilized and oxidized on demand as energy needs dictate. In general, muscles with a predominance of slow-twitch oxidative fibers contain the highest level of mLPL, consistent with a greater reliance on lipid oxidation for meeting energy needs (325). In contrast, muscles composed mainly of fast-twitch white fibers which have higher glycolytic capacity, have much less mLPL activity (327).

While insulin stimulates the activity of AT-LPL in a dose-dependent manner, elevations in plasma insulin down-regulate the muscle isoform of LPL (164,166). Physiological elevations in circulating insulin during a euglycemic clamp has been shown to decrease thigh muscle LPL activity ($p<0.05$), whereas thigh glucose uptake is significantly increased ($p<0.05$). The increase in glucose uptake was positively associated with the decrement in mLPL activity ($r=0.93$, $p<0.05$) (328).

The role of mLPL is to provide fatty acids to meet energy needs. The overexpression of LPL has been shown to reduce circulating triglyceride levels, however, this is associated with higher blood glucose levels. Thus, it seems that overexpression of mLPL may induce insulin resistance by forcing skeletal muscle to oxidize lipids at the expense of available glucose. Transgenic mice overexpressing human LPL (MCKhLPL) in skeletal muscle have been shown to have higher blood glucose levels than wild type control mice. This suggests a role for mLPL in skeletal muscle insulin resistance. Following consumption of a high carbohydrate diet, fasting MCKhLPL transgenic mice

demonstrate a decrease in RQ, suggesting a shift in substrate utilization in favor of lipids that is not observed in wild type mice. Consistent with this, fasting levels of citrate, a potent inhibitor of phosphofructokinase, were observed in the quadriceps muscle of MCKhLPL mice compared to wild type mice ($p=0.013$). Higher muscle glucose-6-phosphate levels in MCKhLPL compared to wild type mice ($p=0.013$) may have also contributed to the development of insulin resistance, as suggested by the need for lower rates of glucose infusion to maintain euglycemia during euglycemic hyperinsulinemic clamps in MCKhLPL compared with nontransgenic mice ($p=0.015$) (329).

Obese subjects often have increased triglyceride-rich VLDL levels in circulation (330). This may be the result of overproduction by the liver or decreased removal from circulation, presumably due to defective action of LPL (330,331). Negative correlations have been found between body weight, insulin and mLPL have been found in normal weight and obese subjects (332,333). In a group of obese women, a strong, negative correlation between basal mLPL activity and total body fat as assessed by BMI ($r=-0.67$, $p<0.01$). Moreover, mLPL was inversely related to the insulin response during an oral glucose tolerance test ($r=-0.50$, $p<0.05$) (334). These findings suggest that hyperinsulinemia may be the pathological factor for altered mLPL activity since obese subjects are often hyperinsulinemic and insulin has an acute inhibitory effect on the activity of mLPL (328). A significant negative association was found between fasting triglycerides and mLPL activity ($r=-0.48$, $p<0.05$), suggesting that the lower LPL activity in muscle of obese subjects is at least partly responsible for higher triglyceride levels (334).

Regulation of Fatty Acid Oxidation in Skeletal Muscle

Several plasma membrane proteins that bind free fatty acids have been identified in various tissues including skeletal muscle. These proteins include plasma membrane binding protein ($FABP_{pm}$), fatty acid translocase (FAT/CD36), and fatty acid transport protein (FATP) (334-337). Red oxidative skeletal muscle contains more $FABP_{pm}$ than white glycolytic muscle (335). Fatty acid translocase and fatty acid transport protein are also more abundant in red compared to white muscle (337). Maximal transport of fatty acids into red vesicles is 1.8 fold-greater than in white vesicles (337). Once inside the myocytes, fatty acids are transported by a cytosolic fatty acid binding protein ($FABP_c$) (337).

Following uptake by the myocytes, fatty acids must first be activated to long chain acyl CoAs (LCACoAs) before transport across the mitochondrial membrane by carnitine palmitoyl transferase-1 (CPT-1) (338). Malonyl CoA inhibits the transport of LCACoAs into the mitochondria by directly inhibiting CPT-1 (339-341). Excessive malonyl CoA levels thus leads to accumulation of LCACoAs in the cytosol (342). While intramuscular lipids are unlikely to serve as a source of free fatty acids for use by distant tissues, the accumulation of lipids within myocytes may lead to excessive concentrations of intracellular fatty acyl CoAs or other fatty acid metabolites which can modulate local glucose utilization (343,344). Within muscle, free fatty acids (FFA), primarily palmitate, oleate, and linoleate, are activated to long-chain acyl CoAs before transport across the mitochondrial membranes by CPT-I, for subsequent β-oxidation. LCACoAs are also substrates for the esterification of FFAs for synthesis of triglycerides and phospholipids (345,346).

Triglycerides *per se* are unlikely to be the primary cause of skeletal muscle insulin resistance since they are an inert storage form of lipid. The balance between oxidation and re-esterification of plasma derived free fatty acids within muscle is the key determinant of fatty acid storage within the tissue (348). Nonesterified fatty acids are transported through the cytosol by fatty acid binding proteins (FABPs) (335). Prior to their oxidation, long chain fatty acids must be activated to long chain acyl CoAs and then translocated into the mitochondrial by CPT-1. Activity of CPT-1 is a key regulatory of muscle fatty acid oxidation (338). CPT-1 is highly sensitive to allosteric inhibition by malonyl CoA, the immediate precursor of de novo synthesized fatty acids (339). Insulin and glucose suppress muscle lipid oxidation indirectly by increasing the content of malonyl CoA (344). This would suppress the activity of CPT-1 preventing the transport of LCACoAs into the mitochondria for oxidation. Consequently, LCCoAs would accumulate within the cytosol. A possible link between elevated cytosolic LCCoA levels and insulin resistance may involve the activation of some isoforms of PKC. Chronic glucose infusion to rats causes lipid accumulation and elevations in cytosolic LCCoA levels and chronic activation of PKC-ε. Activation of this PKC isoform is finding common in several rodent models of obesity and insulin resistance (344).

Intramyocellular Lipids

The majority of lipoprotein-derived fatty acids will be sequestered within specific intramuscular lipid pools where they will be available for immediate oxidation as energy needs dictate. Adipocytes located between muscle fibers are termed extramyocellular lipids (EMCL) while intramuscular lipids (IMCL) are triglycerides localized to the cytosol

in close proximity to the mitochondria in myocytes (349,350). Although the intramuscular lipid pool is small compared to whole-body stores, muscle lipids constitute an important source of oxidizable energy. While this pool does not contribute to circulating fatty acids, intramuscular triglycerides can be rapidly hydrolyzed to supply energy to muscles at rest and during exercise (351).

Computed tomography (CT) can be used for the analysis of regional body composition. This method permits the direct visualization and measurement of adipose tissue volume and density, rather than derivation of these values from other measures. CT can also be used to assess the composition of lean tissue based on x-ray attenuation values within each pixel of a CT scan. The attenuation of CT radiation represents the average attenuation or density of the tissue examined. Skeletal muscle and adipose tissue have very different attenuation values on CT; skeletal muscle CT values are positive while those for adipose tissue are negative (352). Furthermore, skeletal muscle with relatively lower attenuation values have been found to contain more lipid deposits. Kelley et al have used CT to assess the effects of obesity on lean tissue density. Mean CT attenuation values were first defined in muscles of lean subjects. Normal density muscle (NDM) was defined as an area having attenuation values between 35 and 100 Hounsfield units (HU). Lean tissue with attenuation values of 0-34 HU was labeled as low density muscle tissue (LDLT). The attenuation values for LDLT were below those for NDM but above the attenuation values of adipose tissue (-200 to -1 HU). In normal weight healthy subjects, NDM accounted for 90±2% of lean tissue volume and 60±6% of overall volume of the midthigh, whereas LDLT accounted for 9±1% of lean tissue volume and 6±1% of midthigh volume. BMI was inversely related to lean-tissue density

($r=-0.73$, $p<0.001$). The relationship between obesity (BMI) and lean tissue density was particularly strong in the posterior flexor muscle ($r=-0.84$, $p<0.001$). There was no association between NDM volume and BMI. However, the volume of LDLT was positively correlated with BMI ($r=0.63$, $p=0.003$). Furthermore, there was a strong positive association between LDLT and adipose tissue volume ($r=0.80$, $p<0.0001$). Thus, the effect of obesity on thigh composition is to selectively increase the volume of LDLT without affecting NDM (353). In obese subjects, muscle attenuation was found to be the strongest positive correlate with insulin sensitivity ($r=0.48$, $p<0.01$). Thus, marbling of muscle with fat deposits may contribute to the progressive deterioration of insulin sensitivity in skeletal muscle of obese subjects.

Importantly, the improvement in insulin sensitivity that accompanies weight loss may be partly due to the depletion of intramyocellular lipid deposits. Intramyocellular lipids are substantially reduced subjects maintaining reduced body weight following gastric by-pass surgery ($p<0.05$). Although fat accumulates within myocytes of morbidly obese subjects, the majority of lipid is deposited between muscle fibers. However, weight loss selectively depletes intramyocellular lipids, without affecting extramyocellular lipid deposits. Furthermore, the depletion of intramyocellular lipids in weight reduced subjects was positively associated with improvements in whole body glucose disposal ($r=0.49$, $p<0.004$), independently of changes in overall adiposity or changes in extramyocellular lipids (354). These findings are consistent with rodent studies in which intramyocellular lipid accumulation induced by high-fat feeding or by inhibition of fat oxidation was found to closely correlate with in vivo insulin-mediated glucose disposal (355).

To gain a clearer understanding of the factors determining IMCL formation and the time course of their deposition, Bachman, et al. (356) subjected lean healthy subjects to a series of lipid infusions during euglycemic, hyperinsulinemic clamp studies. In lean subjects, hyperinsulinemia is insufficient to induce IMCL formation. Similarly, lipid infusion to significantly raise plasma NEFA levels, fails to significantly increase IMCL levels. However, the combination of hyperinsulinemia and elevated plasma NEFA levels significantly increases IMCL in tibialis anterior muscle within 2 hours, while 4 hours is required to elevate IMCL levels above basal in soleus muscle. Maximal IMCL formation occurred within 6 hours in tibialis anterior muscle, and was significantly greater than the maximum IMCL level in soleus muscle. A strong association was noted between IMCL content and GIR during the clamp studies in both the TA ($r=-0.98$, $p<0.003$) and SOL ($r=-0.97$, $p<0.005$) muscles.

In normal subjects, brief periods of dietary intervention have been shown to influence IMCL levels in tibialis anterior muscle. Consumption of a high-fat, low-carbohydrate diet for three days led to a 50% increase in the IMCL level of tibialis anterior muscle, and was associated with reduced insulin sensitivity, as indicated by a reduction in the rate of glucose infusion necessary to maintain euglycemia during hyperinsulinemic clamp studies. In contrast, subjects fed a high-carbohydrate, low-fat diet for 3 days demonstrated a non-significant decrease in IMCL in tibialis anterior and soleus muscle, while insulin sensitivity was unchanged (356).

Nuclear magnetic resonance (NMR) spectroscopy provides a non-invasive method that differentiates between intramyocellular (IMCL) and extramyocellular (EMCL) lipid content (357-359). This technology has been employed by a number of researches to

assess the role of skeletal muscle lipid content in the insulin-resistance associated with obesity. Sinha, et al. (357) were the first to use this method in pediatric subjects to demonstrate that muscle lipid content and insulin sensitivity are closely related and independent of other factors known to influence insulin sensitivity and insulin action in vivo, including total body fat and central adiposity. In obese children, soleus muscle IMCL and EMCL levels were both more than 200% higher ($p<0.01$) than levels in lean children. Using ^{1}H-NMR spectroscopy, it was shown that in soleus muscle both IMCL and EMCL are considerably greater in obese compared to lean children. IMCL and EMCL content were positively associated with overall indices of obesity including BMI ($r=0.665$, $p<0.001$) and percent total body fat ($r=0.69$, $p<0.0001$). Similar positive associations were observed between EMCL and indices of overall adiposity. Furthermore, subcutaneous and visceral fat were shown to be positively associated with IMCL ($r=0.539$ and 0.729, respectively, $p<0.01$) and EMCL ($r=0.795$ and 0.861, respectively, $p<0.01$). Perhaps the most significant finding of this study was that whole body insulin sensitivity (assessed by glucose infusion rate during euglycemic, hyperinsulinemic clamp) varied as a function of IMCL ($r=-0.59$, $p<0.02$) and was more strongly predictive than EMCL ($r=-0.53$, $p<0.05$). Also noteworthy is that the relationship between IMCL and insulin sensitivity is independent of percent body fat and subcutaneous abdominal fat, but not of visceral fat. These relationships suggest that insulin sensitivity and increased intramyocellular lipid accumulation are pathological features of obesity in a young population.

Virkamäki, et al. (360) have found impairments in two specific actions of insulin as a consequence of excessive IMCL accumulation. Healthy, non-obese (BMI: 25.7 ± 0.7

kg/m^2) men were divided into two groups, based on high (HiIMCL) or low (LoIMCL) content as determined by ^{1}H-NMR spectroscopy. The HiIMCL and LoIMCL groups had identical mean BMIs, similar lean body mass, and comparable volumes of visceral and subcutaneous fat. However, men with HiIMCL exhibited significant impairments in insulin-stimulated whole body glucose uptake and insulin-mediated suppression of circulating FFA levels compared to men of comparable BMI but with LoIMCL content. Men with HiIMCL content had basal insulin levels that were 46% higher ($p<0.05$) than LoIMCL subjects. Despite achieving similar levels of glycemia during euglycemic clamps, plasma insulin levels remained ~20% higher in HiIMCL compared to LoIMCL ($p<0.05$) subjects. In the fasting state, serum free fatty acid concentrations were similar in both groups. However, during experimental hyperinsulinemia, FFA levels declined more slowly ($p<0.01$) and remained higher ($p<0.05$) in HiIMCL compared to LoIMCL subjects. IMCL content was positively correlated with mean FFA levels during hyperinsulinemia ($r=0.53$, $p<0.02$) (360). This is consistent with lipid infusion studies which document increases in IMCL during hyperinsulinemia but not under fasting conditions and is speculated to be the result of increased FFA availability and inhibition of intramyocellular lipolysis by insulin facilitating additional lipid accumulation in muscle (356).

Changes In Skeletal Muscle Lipid Metabolism In Response To High Fat Feeding

High fat feeding induces a number of significant changes in fatty acid metabolism in red gastrocnemius muscle of rats. The alterations in lipid metabolism in response to diet has been studies using the fatty acid tracer [9,10-^{3}H]-2-bromopalmitate (^{3}H-R-BrP),

which permits the tissue specific measurement of fatty acid uptake (361). When used in conjunction with ^{14}C-palmitate (^{14}C-P) the fate of fatty acids entering the cell can also be evaluated. Compared to the standard diet, high-fat feeding increased whole body fatty acid clearance, and fatty acid clearance by gastrocnemius muscle and white adipose tissue under basal and insulin stimulated conditions ($p<0.05$ for all). Compared the standard diet, the high fat diet increased lipid storage in gastrocnemius muscle in the basal state ($p<0.05$) and during lipid infusion ($p<0.05$) (361).

One possible mechanism by which high fat feeding enhances fatty acid clearance into red gastrocnemius muscle is by increasing the activity of the enzymes of lipid metabolism. Compared to control, high fat feeding increased ($p<0.01$) the activity of acyl CoA synthase (ACS), the enzyme that catalyzes the first committed step of intracellular fatty acid metabolism by activating fatty acids to long-chain acyl-CoA (362). Furthermore, ACS activity was correlated with both lipids direct to intramuscular storage ($r^2=0.36$, $p<0.05$) and triglyceride content ($r^2=0.49$, $p<0.02$) of the red gastrocnemius muscle. The results of this study suggest that muscle undergoes a series of metabolic adaptations which permit this tissue to more efficiently utilize the most abundant substrate available (363).

Restoration of Insulin Responsiveness With Weight Loss and Energy Restriction

While over nutrition is known to be associated with impaired glucose metabolism and insulin action, moderate caloric restriction (~60-70% of ad libitum intake) improves insulin action in humans and rodents. Because it is the greatest consumer of available glucose, skeletal muscle is potentially the most important target for interventions

designed to improve insulin sensitivity. In addition, skeletal muscle is extremely responsive to alterations in substrate availability and quickly adapts to changes in the availability of glucose, lipids, and protein (363).

To determine the effect of weight loss on in vivo glucose disposal, Friedman, et al. (364) performed euglycemic insulin clamps in subjects before and after elective gastric by-pass surgery. Surgery resulted in loss of 36% of initial body weight and subjects were allowed to stabilize at this reduced weight prior to study. Although glucose disposal increased roughly three-fold following weight loss ($p<0.01$), rates remained well below those observed in lean subjects under identical conditions. Even so, this represents a substantial improvement. Whereas the glucose disposal rate in obese subjects prior to weight loss was 27% of lean subjects, this improved to 78% of normal after weight loss. Furthermore, weight loss induced a 2-fold increase ($p<0.01$) in maximal glucose transport activity, and consequently, restored maximal insulin-stimulated glucose transport activity to 88% of normal, non-obese subjects. These improvements could not be attributed to changes in GLUT-4 expression which was unchanged by weight loss. Given that skeletal muscle is responsible for more than 80% of insulin-stimulated glucose uptake, these findings are consistent with the development of a reversible defect in skeletal muscle insulin sensitivity during the course of obesity (364).

Dean, et al. (365) studied the effects caloric restriction on insulin action in rats. Moderate energy restriction for five days increased insulin-stimulated glucose transport in rat epitrochlearis 38% ($p<0.05$), a level of improvement is maintained for at least 20 days. There was no evidence for changes in total GLUT-4 levels, indicating improved insulin action by a separate mechanism. In a separate study, Cartee, et al. (366), energy

restriction extended for 5 months improved insulin-stimulated glucose uptake in rat muscle by 59%. Thus, it is likely that the bulk of the improvement in glucose transport occurred following the first few days of energy restriction (365,366).

Subsequently, Dean et al. (367) sought to identify the mechanisms by which energy restriction enhances insulin action, independent of changes in total GLUT-4 levels in skeletal muscle. Rats were fed standard rodent chow *ad libitum* or at 60 en% of *ad libitum* intake. In muscle preparations for energy-restricted rats, the rate of insulin-stimulated glucose transport was nearly 2-fold higher ($p<0.05$) than in *ad libitum* fed rats, despite similar levels of cell surface GLUT-4. Energy restriction altered neither the magnitude nor time course of the insulin-stimulated increase in IRS-1-associated PI3-K activity, suggesting energy restriction enhances a more distal step in the insulin signaling cascade, perhaps involving alterations in the subcellular localization of PI3-K activity, known to be an important aspect of insulin signal transduction (367).

VI. Energy Expenditure and Mitochondrial Uncoupling Proteins

Components of Energy Expenditure

In humans, basal energy expenditure is attributable to three components. The resting metabolic rate (RMR) is that energy expending by a resting, fasted subject in bed in the morning under ambient conditions. This component of daily energy expenditure includes the cost of fueling the metabolic systems of the body and maintenance of normal body temperature (368,369). RMR accounts for 60-70% of daily energy expenditure in most adults and as such is the single greatest component of basal energy requirements. Adaptive thermogenesis is defined as the energy required in excess of RMR in response a variety of factors including food intake, cold or heat acclimation, stress, and drug metabolism (368). Physical activity is the last component of daily energy needs and is by far the most variable component in adults. While this component can account for a significant amount of energy needs, physical activity represents only 20-30% of total daily energy expenditure in most adults (369).

Functions of UCPs in Mitochondrial Respiration and ATP Generation

Uncoupling proteins (UCPs) are mitochondrial transport proteins which dissociate mitochondrial respiration from oxidative phosphorylation such that heat, rather than ATP, is generated (370-372). Structurally, UCPs are composed of six membrane spanning α-helices, with both the C- and N-termini oriented in the cytoplasm. Mitochondria are the major sites of cellular ATP production. The metabolism of energy substrates generates electron carriers, such as NADH, that shuttle to the mitochondrial inner membrane, where they donate electrons to the electron transport chain (373). As

electrons move down this chain, protons are pumped out of the mitochondrial matrix by complexes I, III and IV, creating a proton gradient. The UCPs function as metabolic uncouplers by dissipating this electrochemical gradient. Subsequently, protons may re-enter the mitochondrial matrix by using the energy inherent to the proton gradient in a reaction coupled to ATP synthesis via ATP synthase and which requires oxygen consumption (370-373). Alternatively, protons may "leak" across the mitochondrial inner membrane in a process which by-passes ATP synthesis (374,375). Proton leakiness is recognized as a component of resting metabolic rate, with skeletal muscle proton leaks contributing an estimated 20-50% of total energy expenditure (376).

UCP-1 and Brown Adipose Tissue Thermogenesis

UCP-1 is a dimeric protein located in the inner mitochondrial membrane and is expressed exclusively in brown adipocytes (372). UCP-1 catalyzes the purine nucleotide-sensitive transport of monovalent unipolar anions, including fatty acid anions. Purine nucleotide inhibition occurs by binding of the nucleotide to a regulatory region on the cytosolic side of the inner mitochondrial membrane (371,372).

Brown adipose tissue (BAT) differs from white adipose tissue primarily in mitochondrial density and lipid content (377). Adipocytes from brown fat are rich in mitochondria and contain small lipid droplets, while white adipocytes contain large lipid droplets and comparatively fewer mitochondria (377,378). In contrast to its role in humans (379), brown adipose tissue in rodents and most other mammals, is critical for maintaining body temperature via non-shivering thermogenesis (380). The thermogenic capacity of BAT is due to the expression of UCP-1 which disrupts the link between

mitochondrial respiration and oxidative phosphorylation from ATP generation, releasing energy as heat (373). UCP-1 expression in BAT is increased by cold (381), α_1- and β-adrenoceptor activation (382), norepinepherine (382), NEFAs (383) and PPAR agonists (384), while fasting and energy restriction suppress sympathetic stimulation of brown adipose tissue and consequently downregulates expression of UCP-1 (385).

Human Homologs of UCP-1

In humans, BAT is active only transiently during infancy (379). Thus, regulation of body weight by increased energy expenditure due to mitochondrial uncoupling in BAT is unlikely. However, two homologues of UCP-1 have been identified in several human tissues (386,387). UCP-2 is expressed in a variety of human tissues including white adipose tissue, skeletal muscle, heart, liver, and pancreas (388,389). In rodent brown adipose tissue, UCP2 mRNA levels are highest before birth (390). Maximum levels of UCP2 mRNA are observed 48 hours after birth and correspond to the period of greatest mitochondrial biogenesis in BAT (390).

In humans, UCP-3 expression is abundant only in skeletal muscle, although low levels of expression are detectable in brown adipocytes (387). In humans, two species of UCP-3 mRNA have been identified; the fully functional long form, UCP-3L and a short form (UCP-3s) (387). A polyadenylation site at the junction of intron 6 and exon 7 of the UCP-3 gene results in premature truncation of roughly half of all transcripts. UCP-3s lacks the last 37 amino acid residues of the C-terminal region found in UCP-1 and UCP-2 (387), which is required for purine nucleotide inhibition of UCP-1 uncoupling activity (391).

Evidence Against A Thermoregulatory Function for UCP-2 and UCP-3

Acute cold exposure induces expression of UPC-3 in skeletal muscle, which is consistent with involvement of UCP-3 in non-shivering thermogenesis (392). However, expression of both UCP-2 and UCP-3 are elevated during starvation and energy-restriction, physiological conditions during which energy conservation is critical and thermogenesis is suppressed accordingly (393). Expression of UCP-3 in skeletal muscle is also upregulated by conditions which increase free fatty acids, including fasting (394). Lastly, fasting studies conducted at thermoneutral temperatures to prevent the increase in energy needs that necessarily follow weight loss secondary to food deprivation, demonstrate that expression of UCP2 and UCP3 in muscle remain elevated (395).

Role of UCP-2 in Hepatic Lipid Handling

In severe obesity, chronic elevations in serum free fatty acids increase lipid flux to the liver, where fatty acids can uncouple mitochondrial oxidative phosphorylation from ATP generation leading to ATP depletion and ultimately cell necrosis (396). UCP-2 is not normally expressed to an appreciable degree in adult rat hepatocytes. However, lipid treatment of cultured hepatocytes results in a dose-dependent induction of UCP-2 (397). The induction of UCP-2 mediated uncoupling may limit synthesis and accumulation of triglycerides in hepatocytes by depleting cellular ATP levels necessary for triglyceride synthesis (397). Thus, *in vitro* studies demonstrating a stimulatory effect of increased lipid availability on hepatocyte UCP-2 expression, suggest a potential mechanism for disposing of excess substrates by induction of UCP expression in adipose tissue and skeletal muscle in vivo (384,398).

Induction of UCP-2 in Obesity

UCP-2 expression is barely detectable in the livers of lean mice, however, UCP-2 mRNA and protein levels are elevated five- to six-fold in genetically obese *ob/ob* mice (396). Hepatic UCP-2 induction in obesity may be an adaptive mechanism whereby the cell can dispose of excess energy substrates by activating pathways which uncouple substrate oxidation from energy production. There is a general increase in mitochondrial protein leak and mitochondrial oxidative capacity in hepatocytes from *ob/ob* mice. Baseline ATP levels were ~50% lower in *ob/ob* mice compared to littermates. Portal vein occlusion rapidly decreased ATP levels in lean and *ob/ob* mice, but the extent of ATP depletion is more dramatic in *ob/ob* mice.

The implications of this study are two-fold. By decreasing the efficiency of ATP production in the face of excessive substrate supply, the cell is able to balance ATP production with cellular energy needs. The downside of this system is that the ability to respond to acute stresses, such as an ischemic period, may be compromised. This is illustrated by the rapid depletion of cellular ATP stores when blood flow was occluded and the sluggish nature of ATP repletion upon reperfusion in *ob/ob* compared with littermate controls (396,397).

UCP-2 as a Negative Modulator of Insulin Secretion

Normally, the ATP production within the pancreatic β-cell is tightly coupled to glucose metabolism, permitting these processes to proceed in parallel. Within the β-cell, glucose oxidation increases cellular ATP levels and thus, raises the ATP:ADP ratio. The β-cell responds to the increased ATP:ADP ratio by closing membrane ATP-sensitive

potassium (K_{ATP}) channels, resulting in membrane depolarization, calcium (Ca^{2+}) influx through voltage-dependent calcium channels (VDCCs), and ultimately, insulin secretion. Under euglycemic conditions, the β-cell K_{ATP} channels are open, permitting potassium efflux from the cell and the resting membrane potential is clamped at -70 mV. When the glucose concentration to which the β-cell is exposed rises, ATP levels increase and displace ADP bound to K_{ATP} channels, permitting their closure. With regards to the β-cell, the uncoupling of glucose metabolism from ATP generation may impair the ability of the β-cell to secrete insulin in response to glucose (399).

The physiological role of endogenous UCP2 in regulating insulin secretion has been studied in Ucp2 gene knockout (-/-) mice by Zhang, et al. (400). Homozygous -/- mice lack an intact Ucp2 gene and fail to express UCP2 transcripts in all tissues which normally express high levels of UCP-2 mRNA including heart, kidney, spleen, white adipose tissue, and pancreatic islets. When incubated with glucose, islets from UCP-2 deficient mice have higher concentrations of ATP and secrete 2-3 times more insulin than islets from control mice incubated under identical conditions.

In the fed state, serum insulin levels are 2.8-fold higher and blood glucose levels 18% lower in UCP-2 deficient mice compared with wild type controls. Partial UCP-2 deficiency (UCP-$2^{+/-}$ mice), are characterized by a phenotype intermediate between homozygous deficient and wild-type mice, with serum insulin levels elevated 2-fold and blood glucose levels reduced by 11% compared to wild type controls. Fasting reduced blood glucose levels to a similar extent in control and homozygous mice, but insulin levels remained 3-fold higher in fasted homozygous animals compared to wild-type controls as a consequences of higher basal insulin secretion in UCP-2 deficient mice.

UCP-2 deficient mice maintain normal sensitivity to exogenously administered insulin, making it unlikely that the increase in insulin secretion occurred secondarily to peripheral insulin resistance (400).

To assess the physiological consequence of induction of UCP-2 in islets in obese states, Zhang, et al. (400) subsequently crossed UCP-2 knockout mice were with *ob/ob* mice, a mouse model of human obesity and non-insulin dependent diabetes mellitus. As a consequence of obesity, *ob/ob* mice develop extreme insulin resistant and consequently, expand their β-cell mass 10- to 30-fold, in an unsuccessful effort to maintain normoglycemia (224). UCP-2 deficiency did not influence the course of obesity in *ob/ob* mice. Beginning at 5 weeks of age, UCP-2 deficient *ob/ob* mice had significantly higher insulin levels and lower blood glucose levels compared to *ob/ob* mice with an intact UCP-2 gene. The improvement in glucose tolerance observed in UCP-2 deficient *ob/ob* mice was largely due to enhanced β-cell function since UCP-2 deficient *ob/ob* did not demonstrate enhanced sensitivity to exogenously administered insulin.

Following administration of an exogenous glucose load, *ob/ob* mice exhibit markedly elevated plasma glucose levels and significantly lower insulin response to lean littermates. In UCP-2 deficient *ob/ob* mice, insulin levels rose significantly within 15 minutes and consequently, blood glucose levels were significantly reduced relative to *ob/ob* mice. Thus, UCP-2 deficiency ameliorated the abnormalities of the insulin response normally observed in obese *ob/ob* mice and further demonstrates that that UCP-2 negatively regulates glucose-induced insulin secretion (400).

The mechanism by which obesity leads to enhanced UCP-2 expression in the β-cell is unknown. However, Lameloise, et al. (401) have established a link between UCP-2

expression, fatty acid excess and impaired glucose-stimulated insulin secretion. Chronic exposure to elevated free fatty acid levels may lead to the accumulation of long-chain acyl-CoA esters in the β-cell, which may open K_{ATP}-channels and hyperpolarize the membrane. This could prevent glucose-induced closure of K_{ATP}-channels and consequently, membrane depolarization. *In vitro*, incubation of INS-1 cells with glucose for 72 hours is associated with an increase in cellular ATP levels ($p<0.02$) and an increase in the ATP:ADP ratio ($p<0.02$), followed by membrane depolarization. When oleate is added to the incubation medium, neither ATP levels nor the ATP:ADP ratio are increased and the change in membrane potential is less than half that observed in control glucose-treated cells. This suggests that fatty acid exposure results in a low level of depolarization and impairs the capacity of the membrane to undergo further depolarization (401).

UCP-3 as a Determinant of Energy Expenditure

While there is a notable lack of evidence to support a role for UCP-2 and UCP-3 in thermoregulation, an increasing body of evidence suggests UCP-3 expression may be a critical determinant of energy expenditure (402). In non-diabetic male Pima Indians, BMI was positively associated with the level of UCP-3L mRNA in skeletal muscle ($r=-0.53$, $p=0.025$). No association could be demonstrated between the level of UCP-2 expression and BMI in these subjects. Resting metabolic rate is an important determinant of 24-hour energy expenditure and the expression of UCP2 and UCP-3 in skeletal muscle suggests these uncoupling proteins may contribute to 24 hour energy expenditure. After adjusting for fat mass and fat free mass, sleeping metabolic rate

(SMR) was positively correlated with UCP-3L expression in skeletal muscle ($r=0.69$, $p=0.006$). If one assumes that mRNA levels reflect UCP-3 protein content, these findings suggest that reduced expression of UCP-3L in skeletal muscle results in low sleeping metabolic rate. Low RMR is recognized as a factor which predisposes one to weight gain. Thus, it is interesting to speculate that individuals with low skeletal muscle UCP-3 expression may eventually have higher body weight compared to individuals with normal expression levels.

Role for UCP-3 in Insulin Stimulated Glucose Transport

UCP-3 is normally expressed at low levels in L6 myotubules, making this an ideal system for studying the metabolic regulation of UCP-3. Huppertz, et al. (403) overexpressed human UCP-3 in rat L6 myotubules and observed 2-fold increases in glucose transport and GLUT-4 recruitment to the plasma membrane, which is similar to the effects observed in normal L6 myotubules treated with 100 nM insulin, suggesting enhanced UCP-3 expression in skeletal muscle *in vivo* may improve glucose homeostasis. Total cellular GLUT-4 levels are similar in control myotubles and those overexpressing UCP-3, and both cell types respond to 100 nM insulin with similar increases cell surface GLUT-4 content. The effects of UCP-3 to enhance glucose transport seem to be specific for skeletal muscle, since neither basal nor insulin-stimulated glucose uptake is increase by overexpression of UCP-3 in 3T3-L1 adipocytes (403).

L6 myocytes grown in glucose-free medium rely on oxidative phosphorylation, as opposed to glycolysis, to meet energy needs. Overexpression of UCP-3 blocks oxidative phosphorylation and renders these cells unable to survive short term glucose deprivation

(404). Control myocytes begin to lose their characteristic morphological traits following 6 days of exposure to a glucose-free environment. In contrast, L6 myotubules overexpressing UCP-3 lose their tubular structure and die within 2-3 days, which is consistent with the physiological consequences of uncoupling mitochondrial respiration and oxidative phosphorylation (404,405).

Nutritional Modulation of UCP-2 and UCP-3 Expression

Developmental changes in UCP2 and UCP-3 expression in skeletal muscle and alterations in response to nutritional changes have established that UCP-2 is relatively insensitive to nutritional intervention (394). In mice, UCP-2 mRNA is detectable in skeletal muscle at birth and the level of UCP-2 mRNA increases rapidly on the first day following birth with maximum levels of expression observed on the second day of life after which time, there is a progressive decline in UCP-2 expression (390).

In contrast, UCP-3 mRNA is almost undetectable at birth. There is a sudden, rapid increase in UCP-3 mRNA in skeletal muscle during the first day of life which is followed by a slower, progressive induction of UCP-3 expression. Maximum UCP-3 expression occurs on day 15 and corresponds to peak suckling activity of pups. Standard rodent chow is a high carbohydrate formula to which pups are weaned around day 21 of life. The shift in dietary macronutrient content from mother's milk (high fat) to rodent chow is associated with a decline in UCP-3 mRNA. Fasting of 15 or 21 day old mice was associated with an increase in serum NEFA levels and a parallel increase in expression of UCP-3 in muscle. UCP-2 expression was unchanged by fasting. Thus, the changes in

UCP-3 expression closely parallels changes in NEFA levels, while UCP-2 is insensitive to nutritional determinants such as starvation and dietary macronutrient intake (390,394).

To explore the roles of UCP-2 and UCP-3 in body weight regulation and substrate metabolism, Semac, et al. (384,392) examined expression of UCP-2 and UCP-3 in intrascapular brown fat and skeletal muscle in response to 50% energy restriction for 2 weeks. At the end of the 2-week period of energy restriction, were divided into two groups: one group was sacrificed to examine the changes in UCP expression during energy restriction, while the second group was pair-fed to *ad-libitum* controls to determine changes in UCP expression in response to refeeding.

After 2 weeks of energy restriction, serum fatty acids were elevated and body fat of energy restricted animals was 50% below that of control animals ($p<0.01$). Nonetheless, no differences in body weight or energy intake were observed between refed and control animals following the two week refeeding period, suggesting animals were able to recover the weight lost during the period of energy restriction. Refed animals laid down body fat at a rate nearly 3-fold faster than control animals and consequently, on days 7 and 14 of refeeding, body fat mass was significantly greater in refed versus control animals, despite similar body weights for the two groups. Thus, it seems that virtually all excess energy consumed during the refeeding period was directed toward lipogenesis. Energy expenditure was roughly 17% lower in refed animals, which contributed to a 2-fold enhancement in energy efficiency, further promoting the flux of substrates towards lipogenesis.

Expression of all three UCPs in intrascapular brown adipose tissue was reduced by energy restriction, and restored to control levels by day 5 of refeeding. Energy

restriction also increased the levels of UCP-2 and UCP-3 mRNA in gastrocnemius and soleus muscle compared to ad libitum fed control animals. That such significant upregulation of UCP-3 expression occurs during a period of energy restriction, when circulating free fatty acids would represent the predominant fuel utilized by muscle, implicates UCP-3 as a critical factor regulating lipid utilization.

Interestingly, there was a more pronounced enhancement of UCP-3 expression in the gastrocnemius muscle (5-fold elevation, $p<0.01$ vs control) than in the soleus muscle (2.5 fold increase, $p<0.08$ vs control). Following three days of refeeding, UCP3 expression was reduced relative to control animals. During refeeding, the gastrocnemius muscle again demonstrates greater metabolic flexibility compared to the soleus muscle. After 3 days of refeeding, UCP-3 expression in gastrocnemius muscle was 12-fold greater in control compared to refed animals ($p<0.01$). Expression of UCP-3 in soleus muscle was also increased in control compared to refed animals, albeit to a lesser degree (2.6-fold, $p<0.05$). UCP-3 levels in soleus muscle of refed animals were restored to control levels by day 5 of refeeding, while levels in gastrocnemius muscle remained markedly suppressed below control levels following 10 days of refeeding. The pattern of UCP-3 expression in skeletal muscle is consistent with the metabolic characteristics of both soleus and gastrocnemius muscles. UCP-3 expression was increased in both muscles by energy restriction (384,392). This is consistent with the changes in lipid availability and glucose sparing by skeletal muscle during this time.

The enhancement of UCP-3 expression during the energy restricted period was greatest in the gastrocnemius muscle, which is composed of both oxidative and oxidative/glycolytic fiber types. In contrast, the soleus muscle is composed primarily of

slow-twitch oxidative fibers and consequently has very low glycolytic capacity. Thus, soleus muscle relies more heavily on lipids for fuel even in the basal state and therefore, has very little capacity to further increase lipid utilization during a period of energy restriction when free fatty acids are more available (284). Suppression of UCP-3 levels during refeeding is associated with increase glucose availability and less reliance on lipids (384,392). As previously noted, soleus muscle is more heavily reliant on lipids as an energy substrate, which is consistent with the more attenuated effects of refeeding on UCP-3 expression in this tissue.

The regulation of UCP-2 in response to fasting and refeeding followed the same pattern in soleus and gastrocnemius as that observed for UCP-3. Energy restriction was associated with increased levels of UCP-2 mRNA, with a more pronounced effect observed in gastrocnemius muscle. Refeeding restored UCP-2 levels in soleus muscle by day 3 of the refeeding period, while UCP-2 levels in gastrocnemius remained incompletely restored compared to control animals following 10 days of refeeding. Thus, UCP down-regulation during refeeding, effectively "spares" lipids from oxidation directing them instead to replenish adipose tissue stores in advance of a future period of energy deprivation. This study examined extremes in lipid availability. The question then arose as to whether more subtle changes in lipid availability would influence UCP expression (382,394).

Samec, et al. (395) examined the effects of dietary fat level in energy restricted animals upon refeeding. Two groups of energy restricted (50% of *ad libitum* intake) were refed with either a low-fat (6 en%) or high-fat (53 en%) diet for two weeks. Compared to control animals fed the low-fat diet, previously energy restricted animals subsequently

refed (isocalorically) the same diet had markedly lower levels of UCP-2 and UCP-3 mRNA in gastrocnemius muscle ($p<0.001$), despite lower energy expenditure (-16%, $p<0.001$) and a 3-fold ($p<0.001$) greater rate of adipose tissue accumulation. Compared with animals refed the low-fat diet, animals refed isocaloric amounts of the high-fat diet had higher levels of UCP-2 and UCP-3 in muscle, but lower energy expenditure (-12%) and increased (33%) body fat accumulation. Thus, refeeding the high-fat diet overcomes the suppression of UCP-2 and UCP-3 expression observed during refeeding the low-fat diet. Although no differences were detected between groups after 5 days of refeeding, plasma free fatty acid levels were higher in animals refed the high-fat diet ($p<0.01$) than in low-fat refed and control animals (395).

Further support for the role of UCP-3 in lipid handling comes from human studies examining the effects of NEFA on UCP-3 expression (406). In the absence of hyperinsulinemia, infusion of a triglyceride emulsion elevates plasma glycerol, NEFA, triglyceride and β-hydroxybutyrate levels. In addition, there is a significant decrease in the rate of glucose oxidation ($p<0.02$), which is accompanied by a parallel increase in lipid oxidation ($p<0.05$). Triglyceride infusion specifically enhances the expression of UCP-3 in skeletal muscle, without affecting the level of UCP-2 expression. Skeletal muscle UCP-3 mRNA levels were positively associated with plasma NEFA levels ($r=0.53$, $p=0.005$), lipid oxidation rates ($r=0.56$, $p=0.004$), and plasma β-hydroxybutyrate levels ($r=0.48$, $p=0.013$). An inverse relationship between UCP-3 mRNA levels in skeletal muscle and rates of glucose oxidation ($r=-0.61$, $p=0.001$) was also observed (406).

When hyperinsulinemia was induced by exogenous insulin infusion, the rate of whole body lipid oxidation during triglyceride infusion is similar to that observed in the absence of insulin. However, hyperinsulinemia overrode the effect of triglyceride infusion on muscle UCP-3 mRNA and abolished the relationship between UCP-3 mRNA and plasma NEFAs. Thus, plasma NEFA levels and lipid oxidation rates are important regulators of skeletal muscle UCP-3 expression in vivo. The finding that hyperinsulinemia blocks the increase in skeletal muscle UCP-3 expression associated with increased lipid availability, may link obesity-associated hyperinsulinemia and preferential use of lipids, when glucose is readily available (406).

Effects of Obesity and Weight Reduction on the Expression of UCPs

Vidal-Puig, et al. (407) have demonstrated a significant reduction in the levels of skeletal muscle UCP-3L (by 38%, $p<0.0059$) and UCP-3S (by 48%, $p<0.0047$) mRNA in weight-reduced subjects compared to lean and obese individuals. UCP-2 mRNA levels in skeletal muscle were similar in lean and obese subjects, while the level of UCP-2 mRNA was increased by 30% in weight-reduced subjects. During active weight loss resulting in a 10% reduction in body weight, adipose tissue UCP-2 mRNA levels increased by 58%. With four weeks of maintaining the reduced body weight, UCP-2 mRNA levels fell to 26% of their peak level observed during active weight loss. Thus, UCP-2 and UCP-3 mRNA levels respond discordantly to a sustained reduction in body weight. Whereas the levels of UCP-3L and UCP-3S in skeletal muscle were decreased in weight-reduced previously obese individuals, UCP-2 mRNA levels increased. Active weight loss was also associated with an increase in the level of UCP-2 mRNA in adipose

tissue, which moderated as weight stabilized. Based on these findings, the inability to maintain higher levels of UCP-3 in skeletal muscle may contribute to reduced energy expenditure in weight-reduced subjects (408). Furthermore, these findings suggests that the conditions of active weight loss and maintenance of reduced-body weight are physiologically distinct and may be associated with alterations of substrate availability and utilization during these conditions (407).

VII. Metabolic Regulation by Peroxisomal Proliferator-Activated Receptors

Isotypes and General Pattern of Tissue Distribution

Peroxisome proliferators-activated receptors (PPARs), members of the nuclear hormone receptor superfamily, are transcription factors known to be important in the regulation of lipid storage and utilization, adipocyte differentiation, and insulin action (409,410). The PPARs are ligand-activated transcription factors that control gene transcription by binding to the promoter region of target genes in response to small lipophilic compounds including retinoic acid, thyroid hormone, vitamin D, and fatty acids (411-413). Three subtypes have been identified and each has a distinct tissue distribution pattern. In adult rodents, PPAR-α is expressed most abundantly in tissues with high rates of fatty acid utilization including, brown adipose tissue, liver, proximal tubules of the kidney, intestinal mucosa, heart, vascular endothelium and skeletal muscle (414). This isoform has been specifically implicated in the control of lipoprotein metabolism, fatty acid oxidation, and fatty acid uptake. PPAR-γ expression is highest in white and brown adipose tissue, although it is expressed to a lesser degree in the large intestine and retina (414).

Attempts to generate PPAR-γ null mice were unsuccessful; homozygous PPAR-γ-deficient mice were embryonic lethal and identified this isoform as playing a critical role in placental development (415,416). *In vitro* and *in vivo* studies have demonstrated the necessity of this isoform in adipogenesis (417). Alternative splicing yields two distinct products, PPAR-γ1 and PPAR-γ2. PPAR-γ2 contains an N-terminal span of 30 amino acids which is absent in PPAR-γ1 (418). PPAR-β has a broad expression pattern and is

found in higher amounts than PPAR-α and PPAR-γ in all tissues with the exception of adipose tissue (414,419). Recently, PPAR-β activation has been linked to basal lipid metabolism, inflammation, and colon cancer (420-422).

Structural Organization and Mechanism of Activation

The three PPAR isotypes share common structural organization. The amino-terminal A/B domain is poorly conserved between the three isotypes and contains the ligand-independent transactivation domain, the phosphorylation state of which contributes to the regulation of the α- and γ- isoforms (423,424). The central (C-domain) contains the DNA-binding region and is highly conserved between isotypes, with two zinc finger-like regions and an α-helical DNA-binding motif. The ligand binding domain (LBD), composed of 13 α-helices and a small four-stranded β-sheet, of PPAR-α and PPAR-γ is about twice the size of other receptors, which permits binding of a wide range of ligands (425). An additional α-helix at the base of the LBD facilitates the entry of ligands by increasing the size of the pocket as well as conferring ligand specificity (426,427).

Ligand binding to the PPARs induces a conformational change which allows recruitment of co-activators and the release of repressors (426,428). Activation of PPARs requires dimerization with the 9-cis retinoid X receptor (RXR) which results in the formation of a stable complex that is capable of binding PPAR response elements (PPREs) in the promoter region of genes under the transcriptional control of PPARs (425,429,430). The consensus PPRE sequence is 5'-AACT AGGNCA A AGGTCA-3' (431). These elements bind PPAR-RXR heterodimers in a ligand-independent manner, with the PPAR occupying the 5' extended half site while RXR is situated in the 3' half

site with one nucleotide spacing between the two half sites. In the absence of ligand, the PPAR/RXR heterodimer is associated with a multiprotein corepressor complex that contains intrinsic histone deacetylase activity which serves to inhibit active gene transcription (425,426,431). Upon binding of ligand, the corepressor complex dissociates and a coactivator complex, containing histone acetylase activity, is recruited to the PPAR-γ/RXR heterodimer to facilitate gene transcription (432,433).

Tissue Distribution of PPAR Isotypes and Variations in Altered Metabolic States

In nearly all adult rodent tissues, PPAR-β is the most abundantly expressed isotype, with highest levels of expression (by ~4-fold) in the digestive tract, with lower levels of expression in the kidney, heart, diaphragm, and esophagus. The expression of PPAR-α in brown adipose tissue is approximately 4-fold higher than its expression in the liver, heart, diaphragm, skeletal muscle, esophagus and kidney. Expression of PPAR-α in the liver is three-fold higher than the level of expression found in the small intestine and skeletal muscle. In white adipose tissue, PPAR-α expression is detected at negligible levels. PPAR-γ1 mRNA is abundant in both white and brown adipose tissue, where its expression is comparable to PPAR-α. Adipose tissue expression of PPAR-γ1 is three-fold higher than is observed in the colon and cecum. Compared to the adipose tissue, much lower levels of PPAR-γ1 expression are found in the spleen, stomach, lung, heart, small intestine, and liver. Both PPAR-γ isoforms have been consistently found in the lung, white adipose tissue, brown adipose tissue, and skeletal muscle, although the level of PPAR-γ2 expression is 18-33% of the level of PPAR-γ1 (434).

In adult rats fasted overnight, the expression of PPAR-β decreases by 75 and 80% in the liver and kidney, respectively. PPAR-α expression is dramatically increased in the liver, small intestine, and colon following a 12-hour fast. Fasting increases the expression of PPAR-α in the liver by 75%, underscoring the important role of this isoform in hepatic lipid metabolism (434). Of the two PPAR-γ isoforms, only -γ2 appears to be regulated by fasting, resulting in a 50% reduction of PPAR-γ2 in white and brown adipose tissue (434).

To evaluate the tissue distribution and potential role of the PPARs in insulin action in fat and skeletal muscle of humans, muscle biopsies were obtained from lean, obese and subjects with NIDDM. In all subjects, appreciable amounts of all three known PPAR isoforms were present in skeletal muscle. PPAR-α protein expression was consistently greater (5- to 9-fold, $p<0.05$) in muscle than in abdominal adipose tissue from the same subject. The high levels of PPAR-α protein in muscle relative to fat (7.6:1) suggest a role for PPAR-α in muscle fatty acid metabolism. PPAR-β protein levels were equally abundant in muscle and adipose tissue. The relative expression of PPAR-γ protein in fat and muscle was similar in non-diabetic and diabetic subjects, however PPAR-γ2 protein was detectable only in adipose tissue. PPAR-γ1 seems to be the predominant isoform expressed in skeletal muscle, while adipose tissue contains both the -γ1 and -γ2 isoforms. In muscle, no differences in expression of PPAR-α, -β, or -γ protein expression were detected between diabetic and non-diabetic subjects. Similarly, the relative expression of PPAR-γ mRNA and protein was similar in muscle from diabetic and non-diabetic subjects with significant correlations between PPAR-γ protein and mRNA in both non-

diabetic ($r=0.61$, $p<0.05$) and diabetic ($r=0.80$, $p<0.05$) groups. The finding of both the -α and -γ isoforms of PPAR in skeletal muscle suggests these receptors influence different but related aspects of skeletal muscle metabolism (435). However, only PPAR-γ protein expression appears to be influenced by the prevailing metabolic state, with the highest levels of the protein being found in the most insulin resistant subjects. Furthermore, the relationship between PPAR-γ protein and skeletal muscle insulin action and glucose disposal is either a cause or consequence of diabetes as these alterations are not observed in non-diabetic subjects suggesting PPAR-γ is the isoform through which insulin influences glucose and lipid metabolism in skeletal muscle (435).

The liver plays a critical role in glucose homeostasis and lipid metabolism in vivo. While PPAR-α is abundantly expressed in the liver, basal expression of PPAR-γ in the liver is very low (434). PPAR-γ mRNA expression is reportedly increased in the liver of *ob/ob* mice; however, it is unclear if there are any functional consequences of this upregulation (436). PPAR-α mRNA was abundantly expressed in the liver of control animals and was increased by 2.3-fold in *ob/ob* and by 2.2-fold in *db/db* mice relative to their respective controls. Basal hepatic expression of PPAR-γ was low in control animals, but expression is increased by 7.9-fold in *ob/ob* and by 9.4-fold in *db/db* mice compared with littermate controls (437). Similar levels of PPAR-δ mRNA were detected in livers of control, *ob/ob* and *db/db* mice. Adipose tissue PPAR-γ mRNA levels were similar in control, *ob/ob* and *db/db* mice (438). These data suggest that the increased expression of PPAR-α and PPAR-γ in the liver is related to the development of obesity.

Levels of PPAR-α and -γ mRNA in liver were examined in *ob/ob* mice at the age of 5 and 10 weeks to determine the relationship between the onset of obesity and the increase in PPAR isoform expression. At five weeks of age, *ob/ob* mice exhibit a modest increase (of ~40%) in body weight compared to age-matched littermate controls. However, by 10 weeks of age, the weight of *ob/ob* mice is almost 150% of age-matched controls. Comparable levels of PPAR-α mRNA is detected in the liver of control and *ob/ob* mice at 5 weeks of age but levels are increased 2.8-fold in *ob/ob* mice at the age of 10 weeks. Induction of PPAR-γ mRNA in liver appears to be an earlier consequence of obesity with levels increased by 7.7- and 9.3-fold in 5- and 10-week old *ob/ob* mice, respectively (437,438).

Despite the low level of PPAR-γ in human skeletal muscle, its expression in this tissue has been the focus of much attention because of the central role skeletal muscle plays in the development of insulin resistance. Lapsys, et al. (439) determined if PPAR-γ expression in human skeletal muscle was associated with the expression of genes known to be important in lipid metabolism. PPAR-γ mRNA expression was significantly correlated with expression of LPL ($r^2 = 0.54$, $p=0.003$) and FABP ($r^2 = 0.324$, $p=0.034$). Furthermore, PPAR-γ expression was significantly correlated with expression of CPT-1 ($r^2 = 0.42$, $p=0.012$), a key enzyme involved in β-oxidation of fatty acids. Together, these findings suggest that enhanced PPAR-γ activity in skeletal muscle may regulate lipid metabolism by up-regulation of lipid metabolizing genes. Such regulation seems contrary to the improvements in skeletal muscle insulin action attributed to the PPAR-γ agonists (440), since increased activity of lipid metabolizing enzymes may increase

intramyocellular lipid levels. Alternatively, skeletal muscle may adapt to TZD treatment over several weeks so that the net result of this type therapy is to simultaneously increase lipid flux to adipose tissue and reduce circulating lipid levels by enhancing skeletal muscle lipid utilization (440).

Endogenous and Synthetic PPAR Ligands

PPARs have a large ligand-binding pocket which allows the receptors to accommodate a number of different ligands (411). *In vitro* studies have identified a number of fatty acid derivatives capable of binding to and activating PPARs (413,414). PPAR-α have the highest affinity for fatty acids, preferring long chain unsaturated fatty acids such as linoleic, linolenic and arachidonic acid (413,441,442). Linolenic and linoleic acids bind all three PPAR isotypes but have the highest affinity for PPAR-α (442). Inflammatory mediators including 8(S)-hydroxy-eicosatetraenoic acid (8(S)-HETE) and leukotriene B4 are specific for PPAR-α, whereas the linoleic acid metabolite 15-deoxy-Δ12,14-prostaglandin J2 (PGJ2) is the most potent natural ligand for PPAR-γ identified thus far (443). Long chain polyunsaturated fatty acids are effective PPAR-β natural ligands (444).

The fibrates are a class of drugs prescribed for the treatment of dyslipidemia, most of which preferentially activate PPAR-α (445). The class of drugs known as thiazolidinediones (TZDs) was discovered during the screening of a number of compounds for hypolipidemic actions (446-448). These compounds differ primarily in their side chain structures (449). In rodent models of insulin resistance, these drugs were consistently shown to reduce blood glucose levels and improve hyperinsulinemia (449).

Three TZDs, which specifically activate PPAR-γ, have been studied extensively in humans: troglitazone (Rezulin®), rosiglitazone (Avandia®), and pioglitazone (Actos®) (448). Of these rosiglitazone has the highest binding affinity for PPAR-γ. Troglitazone has the weakest binding affinity for PPAR-γ and was the first TZD introduced for the treatment of NIDDM in humans (448). However, troglitazone was withdrawn from the market in March 2000 after its use was linked to severe hepatotoxicity leading to hepatic failure and death (450,451). Rosiglitazone and pioglitazone have been available for clinical use since 1999 (452,453). The regulation of inflammatory processes by PPAR-α and PPAR-γ may be mediated in part by activation of these isotypes with non-steroidal anti-inflammatory drugs, such as indomethacin and fenoprofen (454).

Physiological Functions of PPAR-α Activation

The physiological functions of PPAR-α have been examined using the PPAR-α null mouse (PPAR-$\alpha^{-/-}$). The phenotype of the fasted PPAR-$\alpha^{-/-}$ mouse, which is more severe than the phenotype of fed PPAR-α null mice, includes hypoglycemia, hypothermia, elevated plasma free fatty acids, and fatty liver. That fasting induces a more impaired phenotype than that observed in fed animals underscores the pivotal role of PPAR-α in the adaptive response to fasting. These mice have a reduced capacity for metabolism of long chain fatty acids, which contributes to dyslipidemia, expansion of adipose tissue stores, and excessive hepatic lipid accumulation (455-459). When fasted, PPAR-α null mice have severe and prolonged hypoglycemia (460,461). PPAR-α expression could affect glucose homeostasis by virtue of its expression in several tissues: the liver, pancreatic β-cells, and peripheral insulin-sensitive tissues (419,462). PPAR-$\alpha^{-/-}$ mice

develop fatty liver characterized by pronounced accumulation of large lipid droplets in hepatocytes in response to high-fat feeding. PPAR-$\alpha^{-/-}$ mice maintain numerous lipid droplets following an overnight fast, which may be partially mediated by circulating free fatty acid levels that are 2-fold higher in fasted PPAR-$\alpha^{-/-}$ mice compared with fasted wildtype controls (461).

Plasma β-hydroxybutyrate is an important end product of fatty acid oxidation and is produced exclusively by the liver. Fasting greatly increases plasma β-hydroxybutrate levels in wild-type mice, an effect not observed in PPAR-α deficient mice. Consequently, plasma β-hydroxybutrate levels in fasted wildtype mice were 7-fold higher ($p<0.01$) than in fasted PPAR-α deficient mice, suggesting a marked impairment in induction of fatty acid oxidation in mice lacking functional PPAR-α in liver. Enhanced fatty acid oxidation during fasting ensures that an adequate gluconeogenic response is mounted to maintain blood glucose levels. In the early stages of a prolonged fast, blood glucose levels are largely maintained by glycogenolysis. Within 5 hours following food withdrawal from PPAR-α deficient mice, there is a rapid and precipitous fall in blood glucose levels, compared to the more sustained and less pronounced fluctuations observed in wild-type mice. The rapidity with which hypoglycemia develops in food deprived PPAR-$\alpha^{-/-}$ mice suggests a reduction in glycogen stores in the livers of fed PPAR-$\alpha^{-/-}$ mice. As fasting is prolonged, blood glucose levels continued to fall in PPAR-$\alpha^{-/-}$ mice, suggesting impairments in gluconeogenesis as well. Despite the inability of PPAR-$\alpha^{-/-}$ to maintain normal glucose homeostasis during fasting, glucose response curves following intraperitoneal injection of glucose were similar in PPAR-$\alpha^{-/-}$ and wild-

type mice, implying peripheral insulin sensitive does not suffer as a result of PPAR-α deletion (460,461).

Guerre-Millo, et al. (462) examined the effects of PPAR-α deficiency on glucose homeostasis and insulin resistance induced by high-fat feeding. Compared with wildtype mice fed standard rodent chow, high-fat fed wildtype mice developed hyperglycemia and hyperinsulinemia, a compensatory response reflecting peripheral insulin sensitivity also seen as a contributing factor in obesity induced by high-fat feeding. Conversely, PPAR-$\alpha^{-/-}$ mice fed the high-fat diet maintained normal blood glucose and insulin levels. Despite the absence of hyperinsulinemia in high-fat fed PPAR-$\alpha^{-/-}$ mice, adipose tissue mass ($p<0.05$) and circulating leptin levels ($p<0.05$) were significantly higher than control mice fed standard rodent chow. Liver weight was elevated compared to animals consuming standard chow in both high-fat fed wild-type ($p<0.05$) and PPAR$^{-/-}$ ($p<0.05$) mice, but was more pronounced in the absence of functional PPAR-α.

To more directly determine if insulin resistance developed in PPAR-$\alpha^{-/-}$ mice, intraperitoneal insulin and glucose tolerance tests were preformed. Glucose and insulin response curves were similar in wild type and PPAR-$\alpha^{-/-}$ mice fed the standard diet. In contrast, high-fat fed wild-type mice demonstrated a blunting of the hypoglycemic response to insulin, such that both the $AUC_{glucose}$ and the $AUC_{insulin}$ was significantly greater (by ~50%, $p<0.05$) than in wildtype mice consuming the standard diet. None of these abnormalities were observed in high-fat fed PPAR-$\alpha^{-/-}$ mice. The insulin resistance index (IR index), calculated as the product of the areas under the glucose and insulin curves obtained during the glucose tolerance test, demonstrated that the high-fat diet caused a four-fold increase in the IR index of wild-type mice ($p<0.05$), whereas no

change was observed in PPAR-$\alpha^{-/-}$ mice. Based on these data, a phenotype of insulin resistance emerges in wild-type mice in response to high-fat feeding, whereas lack of a functional PPAR-α appears to protect against high-fat diet related impairments in insulin action. Furthermore, these data suggest a role for PPAR-α in control of peripheral glucose utilization in response to dietary fat. According to the Randle hypothesis (55), increased fatty acid oxidation would impair insulin-stimulated glucose uptake. PPAR-$\alpha^{-/-}$ mice have a reduced capacity for fatty acid utilization in insulin-sensitive tissues, which would favor the increased use of glucose in tissues such as skeletal muscle and intrascapular brown adipose tissue. Thus, the PPAR-α response may contribute to the accumulation of intracellular fatty acid-derived signaling molecules that may subsequently impair insulin-stimulated glucose utilization and promote the development of insulin resistance. No major impairments of β-cell insulin release were observed in PPAR-α null mice, regardless of diet (462). This is somewhat unexpected since one would expect that lack of PPAR-α would reduce fatty acid oxidation in islets, resulting in excessive lipid accumulation which is known to impair the insulin response to glucose (463).

Effects of Synthetic PPAR-α Agonists on Metabolic Regulation

Mancini, et al. (464) examined the effects of fenofibrate treatment on the development of obesity in response to high-fat feeding. Normal rats experienced a 25% increase in body weight following 2 months of high-fat feeding, whereas this increase in body weight was prevented in rats treated with fenofibrate while consuming the same high-fat diet. Consequently, final body weights were similar for control animals

consuming standard rodent chow and fenofibrate-treated animals consuming the high fat diet, with body weight of both groups 16% lower ($p<0.05$) than in untreated high-fat fed rats. In a separate group of animals consuming the high-fat diet, fenofibrate treatment was not initiated until the beginning of the second month, when body weights were already significantly greater ($p<0.05$) than control animals fed standard rodent chow. Fenofibrate was administered to one group solely during the second month of high-fat feeding. However, fenofibrate treatment solely during the second month of high-fat feeding, attenuated weight gain such that these animals weighed 14% less ($p<0.05$) than untreated, high-fat fed animals. Furthermore, the effect of fenofibrate was greater in animals treated only in the second month of high-fat feeding. Visceral fat mass in high fat fed animals was 163% of that from animals consuming the standard diet ($p<0.05$). Fenofibrate treatment of high-fat fed animals for one or two months reduced WAT mass by 42 and 55%, respectively compared to untreated high-fat fed animals ($p<0.05$). There was a tendency for greater resting metabolic rate in fenofibrate-treated animals but this did not reach significance. Hepatic PPAR-α mRNA levels were increased 1.7-fold in fenofibrate-treated animals compared to untreated high-fat fed controls. Gastrocnemius UCP-2 mRNA levels were decreased by 3.3- and 2.2-fold after 1 and 2 months of fenofibrate treatment, respectively, whereas expression of UCP-3 in this tissue was unaffected. The final weight of fenofibrate treated high-fat fed animals was similar to control which suggests that under conditions of enhanced fatty acid availability, fenofibrate prevents excessive weight gain and likely promotes mobilization of adipose tissue lipid stores (464).

Physiological Functions of PPAR-γ Activation

To examine the physiological role of PPAR-γ in vivo, Kubota, et al. (465) generated PPAR-γ-deficient by targeted disruption of the PPAR-γ gene. Homozygous PPAR-γ deficient mice (PPAR-$\gamma^{-/-}$) were embryonic lethal due to placental dysfunction. Heterozygous PPAR-$\gamma^{+/-}$ mice showed normal weight gain and insulin sensitivity on standard rodent chow. To determine the role of PPAR-γ in high-fat diet-induced obesity and insulin resistance, wild type and PPAR$\gamma^{+/-}$ mice were fed either a high-carbohydrate (HC) or high-fat (HF) diet. There was no difference in body weight gain between wild type and PPAR-$\gamma^{+/-}$ animals consuming the HC diet for 15 weeks. Wildtype mice consuming the HF diet gained significantly more body weight than did wildtype mice fed the HC diet. In contrast, PPAR-$\gamma^{+/-}$ mice consuming the HF diet gained very little weight. Wildtype mice fed the HF diet had significantly greater adipose tissue accumulation than either wild type or PPAR-$\gamma^{+/-}$ mice consuming the HC diet. Thus, PPAR-$\gamma^{+/-}$ mice were somewhat protected from excessive adipose tissue accumulation normally induced by chronic consumption of a high-fat diet. Wildtype animals fed the HF diet had marked hepatic lipid infiltration, which was absent in HF-fed PPAR-$\gamma^{+/-}$ animals. Total white adipose tissue mass of epididymal, retroperitoneal, and perirenal fat pads was reduced by 70% ($p<0.01$) in HF-fed PPAR-$\gamma^{+/-}$ mice compared to HF-fed wild type mice. Adipocytes from wild type and PPAR-$\gamma^{+/-}$ mice fed the HF diet were considerably larger than adipocytes from mice fed the HC diet. However, adipocytes from PPAR-$\gamma^{+/-}$ mice fed the high-fat diet were significantly smaller than wild type mice fed the same diet.

After 15 weeks of high fat feeding, the glucose lowering effect of insulin was markedly larger in PPAR-$\gamma^{+/-}$ than in wild type mice. Thus, PPAR-$\gamma^{+/-}$ mice were insulin sensitive while HF-fed wild type mice were insulin resistant. Consistent with greater insulin sensitivity, plasma insulin levels were significantly lower in PPAR-$\gamma^{+/-}$ mice after HF feeding compared to wild-type animals fed the same diet. Glut-4 mRNA levels in epididymal adipose tissue were reduced in both mouse genotypes by high-fat feeding, but remained approximately 2-fold higher in PPAR-$\gamma^{+/-}$ mice compared to wild type, which may partially account for the increased sensitivity to insulin in these animals.

To determine if the physiological benefits of partial PPAR-γ ablation observed in PPAR-$\gamma^{+/-}$ mice could be overridden by increasing the activity of PPAR-γ, wild type and PPAR-$\gamma^{+/-}$ mice were given a low dose (0.004% wt/wt) of pioglitazone, a PPAR-γ agonist, while consuming the high-fat diet. Pioglitazone treatment for 5 weeks increased body weight gain and total white adipose tissue mass of epididymal, retroperitoneal and perirenal fat pads in both mouse genotypes compared to untreated animals. In HF-fed wild type mice, pioglitazone treatment normalized blood glucose and insulin levels, and thus, lessened the severity of insulin resistance induced by the high-fat diet. In contrast, PPAR-$\gamma^{+/-}$ mice treated with pioglitazone experienced increased serum insulin levels without any change in blood glucose levels, indicating a reduction in insulin sensitivity. Thus, pioglitazone treatment further reduced insulin sensitivity in PPAR-$\gamma^{+/-}$ mice, effectively driving them toward a state of insulin resistance similar to that induced by the high-fat diet in untreated wild type mice. In wildtype mice, pioglitazone treatment caused a modest but significant decrease in adipocyte size, associated with an increase in

adipocyte number. Smaller adipocytes are known to be more responsive to insulin than larger adipocytes. In contrast, PPAR-$\gamma^{+/-}$ mice, pioglitazone treatment caused a significant increase in adipocyte size without altering the total number of adipocytes. This suggests that increased PPAR-γ activity in these mice abolished the protection from adipocyte hypertrophy in response to high fat diet in these animals. The net effect of increased PPAR-γ activity in PPAR-$\gamma^{+/-}$ animals by low dose pioglitazone was sufficient to cause adipocyte hypertrophy but unable to stimulate differentiation.

Despite the reductions in adipose tissue mass and adipocyte size, leptin mRNA levels were markedly increased in PPAR-$\gamma^{+/-}$ animals on the high fat diet compared to wild type. Furthermore, serum leptin levels were ~1.8-fold higher in PPAR-$\gamma^{+/-}$ animals on HF-diet compared to wild type. This is attributable to the greater secretory capacity of adipocytes in PPAR-$\gamma^{+/-}$ animals compared with wild type. Despite higher serum leptin levels in high-fat fed PPAR-$\gamma^{+/-}$, daily leptin administration resulted in similar reductions in food intake and body weight in high-fat fed PPAR-$\gamma^{+/-}$ and wild-type mice, suggesting leptin sensitivity was unaltered

Supraphysiological activation of PPAR-γ by TZD markedly increase triglyceride content of white adipose tissue, thereby decreasing the triglyceride content of the liver and muscle, leading to amelioration of insulin resistance. Unfortunately, this occurs at the expense of an expanded adipose tissue mass (466). Heterozygous PPAR-γ deficiency (PPAR-$\gamma^{+/-}$) decreases the triglyceride content of white adipose tissue, skeletal muscle, and liver in association with increased leptin expression, increased fatty acid oxidation, and decreased lipogenesis, thereby ameliorating high-fat diet induced obesity and insulin

resistance. TZDs increase total white adipose tissue mass while at the same time stimulating adipocyte differentiation and apoptosis, which prevents adipocyte hypertrophy and the subsequent secretion of adipocyte-derived factors (eg. TNF-α, free fatty acids) which contribute to the development of insulin resistance. Thus, insulin resistance is improved by two seemingly contradictory states: PPAR-γ deficiency and supraphysiological PPAR-γ activation.

PPAR-γ Expression in Insulin-Resistant Humans and Treatment with TZDs

To determine whether the in vivo metabolic milieu influences the expression of PPARs in muscle, Loviscach, et al. (467) examined the relationships between PPAR protein levels and clinical characteristics of subjects. Multiple regression analysis showed no influence of age, BMI, fasting NEFAs, fasting triglycerides, blood glucose, glycated hemoglobin, and fasting serum insulin on PPAR protein levels in the study group as a whole or in diabetic and non-diabetic subjects individually. Because PPAR-γ has been implicated in skeletal muscle insulin sensitivity, the relationship between these variables was explored. The insulin-stimulated glucose disposal rate was determined during the last 30 minutes of a hyperinsulinemic euglycemic clamp and used as an index of insulin action in skeletal muscle. In diabetic subjects, there was an inverse relationship between muscle PPAR-γ protein and maximum GDR ($r=-0.47$, $p<0.05$). Conversely, in non-diabetic subjects there was a nonsignificant trend for increasing PPAR-γ protein with increasing GDR, the opposite of the association noted in diabetic subjects. This suggests an abnormality in PPAR-γ expression in subjects with NIDDM.

Diabetic subjects were further classified according to severity of insulin resistance estimated by glucose disposal rate during clamp studies. Diabetic subjects with severe insulin resistance showed significantly higher muscle PPAR-γ protein levels compared age and weight matched diabetic subjects with moderate insulin resistance ($p<0.01$) and low insulin responsive non-diabetic subjects ($p<0.01$). Thus, the most insulin resistant diabetic subjects had the highest level of PPAR-γ protein in muscle. Expression of PPAR-γ protein in muscle was not significantly altered from baseline levels by 3 hours of insulin infusion (467).

Insulin (0.1 μmol/l) induces a rapid and transient increase in PPAR-γ2 mRNA, with a maximal effect after 2 hours (77±21%) and return to baseline levels by 3 hours. In contrast, PPAR-γ1 mRNAs increased slowly and steadily during the 6 hour of incubation with insulin, achieving values 57±8% higher than basal by the end of the incubation. Dose response curves clearly demonstrate that induction of PPAR-γ1 and PPAR-γ2 mRNA is dependent on insulin level. The half maximal effect occurred with insulin concentrations of ~1-5 nmol/l for both isoforms (468).

Based on the results of their in vitro studies, Rieusset, et al. (468) characterized the expression of PPAR-γ1 and PPAR-γ2 in subcutaneous adipose tissue and skeletal muscle in subjects over a wide range of BMI. To investigate the acute effect of insulin on PPAR-γ expression, control, obese non diabetic and subjects with NIDDM underwent a 3-hour euglycemic hyperglycemic clamp. GIR required to maintain euglycemia was greatest in control subjects ($p<0.05$ vs. both), and significantly lower in obese subjects and non-obese subjects with NIDDM, indicating profound insulin resistance with

respect to whole body glucose disposal in obese and diabetic subjects. Plasma free fatty acids decreased in all groups during the insulin clamp but remained higher in obese and NIDDM subjects than in control ($p<0.05$).

This study demonstrates that insulin acutely upregulates PPAR-γ mRNA and protein levels in human adipocytes. In subcutaneous abdominal adipose tissue, mRNA levels for both PPAR-γ1 and PPAR-γ2 were increased two-fold following a 3-hour insulin infusion. The stimulatory effect of insulin is observed in lean, obese and subjects with NIDDM. In isolated human adipocytes, insulin induced PPAR-γ mRNA in a dose-dependent manner, with half maximal stimulation achieved at concentrations of 1-5 nmol/l, suggesting this in vivo effect was due to direct action of insulin on adipocytes. In vivo, a 3-hour period of hyperinsulinemia led to a two-fold increase in PPAR-γ1 and PPAR-γ2 mRNA levels in abdominal subcutaneous adipose tissue. This effect is observed in lean and obese subjects, with or without NIDDM, which suggests that these pathologies are not associated with altered regulation by insulin of PPAR-γ gene expression. Furthermore, although obese subjects and those with NIDDM were markedly insulin resistant with regards to whole body glucose uptake, the findings of this study indicate that the pathway involved in regulation of PPAR-γ expression in not resistant to insulin action. This study also confirmed previous reports in which the basal expression level of PPAR-γ in adipose tissue is not altered in obese subjects or those with NIDDM (468).

The *in vivo* regulation of both PPAR-γ splice variants (PPAR-γ1 and -γ2) have been examined in obese and weight-reduced subjects by Vidal-Puig, et al. (469). PPAR-γ1

mRNA expression was highest in adipose tissue with much lower levels of expression detected in liver, skeletal muscle and heart. The expression of PPAR-γ2 mRNA was 10-25% of that seen in adipose tissue, with comparable levels found in lean, obese and diabetic subjects. To determine if obesity is associated with abnormal adipose tissue expression of PPAR-γ, subcutaneous adipose tissue biopsies were obtained from lean and obese subjects and levels of PPAR-γ transcripts, normalized with 18S expression, determined. Adipose tissue expression of PPAR-γ2/18S was increased 43% ($p<0.05$) in obese subjects compared to age- and sex-matched lean controls. In contrast, levels of PPAR-γ1/18S expression were similar or slightly reduced in obesity. There was a strong positive correlation between the ratio of PPAR-γ2/-γ1 and BMI in all subjects ($r=0.70$, $p<0.001$). Sexually dimorphic expression was apparent with increased expression of both PPAR-γ1 ($p<0.05$) and PPAR-γ2 mRNA ($p<0.01$) in subcutaneous adipose tissue of women compared to men with comparable BMI. In response to energy restriction, obese subjects demonstrated improved metabolic control characterized by a 10% reduction in body weight and fasting insulin levels that were 26% lower ($p<0.01$) than before weight loss. These changes were accompanied by a 25% reduction ($p<0.05$) in adipose tissue PPAR-γ2 expression. Reduced PPAR-γ2 expression was a transient effect as pre-weight loss expression levels were re-established after maintaining reduced body weight for four weeks. Expression of PPAR-γ1 was not influenced by weight loss (468).

Cha, et al. (470) examined the influence of short-term (2 days) troglitazone administration in subjects with NIDDM. Troglitazone treatment increased basal glucose uptake by 26% ($p<0.05$) and enhanced insulin-stimulated glucose uptake by 23%

compared with untreated subjects with NIDDM. The improvements in basal and insulin-stimulated glucose uptake in subjects treated with troglitazone occurred in the absence of any changes in the level of GLUT-4 protein. However, troglitazone increased basal and insulin-stimulated glycogen synthase activity by 132 and 77% ($p<0.05$), respectively compared with untreated subjects with NIDDM. Skeletal muscle palmitate oxidation was 68% ($p<0.005$) greater in troglitazone-treated NIDDM compared with untreated diabetic controls. This was partially attributable to the troglitazone-associated increase (113±41% above control, $p<0.025$) in expression of FAT/CD36, a protein which facilitates fatty acid transport by skeletal muscle cells (469).

The level of PPAR-γ1 in skeletal muscle is only 10-15% of the level found in adipose tissue. No differences in basal levels of skeletal muscle PPAR-γ1 expression are detected between lean, obese and diabetic subjects and expression of PPAR-γ1 is not correlated with BMI or WHR regardless of adiposity or diabetic status. However, a strong positive relationship was found between skeletal muscle PPAR-γ1 expression and percent body fat in both lean and obese subjects ($r=0.76$, $p<0.05$), with an even stronger association observed in subjects with NIDDM ($r=0.82$, $p<0.05$). In all subjects, basal skeletal muscle PPAR-γ1 expression was significantly higher in those whose body fat accounted for more than 25% of body weight compared to subjects with less than 25% body fat ($p<0.02$). No relationship was observed between skeletal muscle PPAR-γ1 mRNA expression and fasting insulin levels. During a five-hour euglycemic insulin clamp, skeletal muscle PPAR-γ1 mRNA levels were correlated with the rate of glucose disposal in obese ($r=0.92$, $p<0.01$), but not lean or diabetic subjects. Obese and diabetic subjects

were markedly insulin resistant, with GDR during the last 30 minutes of the clamp that were only 31 and 67%, respectively, of those in lean subjects. Despite obvious reductions in insulin sensitivity in obese and diabetic subjects, no differences in skeletal muscle PPAR-γ1 mRNA levels were found between the three groups (471).

Interplay Between PPAR Isotypes and Mitochondrial Uncoupling Proteins

Although PPAR-α is predominantly expressed in the liver and skeletal muscle, it is also detectable in BAT (407,410). *In vitro* data indicates that stimulation with a PPAR-γ agonist is sufficient to induce differentiation of cultured brown adipocytes, along with induction of UCP-1 expression (472). The potential for regulation of UCP-1 gene expression by PPAR-γ and the involvement of PPAR-γ and PPAR-α in lipid metabolism and homeostasis suggests the in vivo effects of PPAR activation may involve UCP-1.

To examine these possibilities, Kelly, et al. (473) treated animals with PPAR-γ agonists or a specific PPAR-α agonist, followed by determination of expression levels of different UCP isoforms in various tissues. Sprague Dawley rats were given an oral dose (5 mg/kg/day) of the thiazolidinedione (TZD) AD5075, a potent and selective PPAR-γ ligand, for 14 days. Consistent with previous reports, there was a 2- to 4-fold increase in the mass of intrascapular BAT depot. TZD treatment increased UCP-1 expression by 300% compared to untreated control mice. TZD treatment for 14 days also increased intrascapular BAT UCP-2 mRNA (by 3-fold) and UCP-3 mRNA (by 2.5-fold). In contrast to the effects of TZD treatment on UCP gene expression in BAT, the expression of UCP-2 and UCP-3 in skeletal muscle and the epididymal fat pad was unaltered (473).

Berger, et al. (474) demonstrated that administration of selective PPAR-γ agonists to diabetic *db/db* mice for at least 10 days normalizes blood glucose and triglyceride levels and improves insulin sensitivity. Concomitantly, the mass of the intrascapular BAT depot is increased 100-150%. PPAR-γ agonist treatment reduced blood glucose levels by 70-80% and normalized serum triglycerides compared with untreated *db/db* mice. Furthermore, agonist treatment increased the levels of UCP-1 and UCP-3 mRNA in BAT by 200% and 300%, respectively, compared to untreated animals.

The expression patterns of UCP-2 and PPAR-α overlap. Treatment of either obese *db/db* or lean *db/+* mice with a selective PPAR-α agonist for 10 days resulted in UCP-2 mRNA levels that were 400% of values in vehicle-treated control animals. Other consequences of *in vivo* PPAR-α activation included increased levels of acyl CoA oxidase mRNA (2.6-fold, $p=0.01$) and FABP protein (2.8-fold, $p=0.015$) in liver and 50% reduction in serum triglyceride levels in treated *db/db* mice relative to control. In contrast, PPAR-α agonist treatment did not affect UCP-2 mRNA levels in liver in either normal lean or obese Zucker rats. In summary, in vivo administration of a potent and selective TZD PPAR-γ agonist to normal rats for 14 days results in the up-regulation of all three isoforms in BAT without affecting UCP-2 or UCP-3 in WAT or muscle (474).

PPAR-γ2 expression is increased by a low-calorie diet and is down-regulated in adipose tissue of obese subjects. This is also the major isoform involved in activation of adipocyte differentiation. Increased levels of plasma fatty acids caused a marked induction of PPAR-γ2 mRNA above baseline (200±8%, $p<0.05$), suggesting that fatty acid supply may be directly involved in modulating the expression of genes relevant for fat cell differentiation. It has been shown that transcriptional activation of PPAR-γ2

triggers fat cell differentiation by transactivation of adipose-specific genes including those involved in lipid storage and metabolism. Other genes induced during differentiation include leptin, UCP-2, UCP-3 and TNF-α. High fat feeding increases leptin gene expression in rats but it is unclear if an acute increase in NEFA availability has any direct effects on leptin expression in humans (469).

VIII. Dietary Carbohydrate

Dietary recommendations evolve to reflect our current understanding of foods and food constituents and their role in maintaining health. To illustrate this point, consider the Dietary Guidelines for Americans issued by the United States Department of Health and Human Services. The 1980 and 1985 guidelines focused on reducing the overall fat content of the diet while increasing consumption of dietary fiber. Regarding dietary carbohydrate intake, the guidelines encouraged Americans to "eat foods with adequate starch and fiber"(475,476). This statement was revised in 1990 to emphasize the importance of vegetables, fruits, and grains in a healthy diet (477). Furthermore, the revised statement shifted emphasis away from starch, which was increasingly regarded by the public as a component of foods that promoted weight gain. The revised guidelines preceded the issuance of the Food Guide Pyramid in 1992 (478). To emphasize the role of grain products in forming the foundation of the Food Guide Pyramid and as the single greatest contributor to total dietary energy intake, the guideline statement was reissued in 1995 to encourage consumption of "grain products, vegetables, and fruits" (479).

Analysis of the 1994-1996 USDA's Continuing Survey of Food Intakes by Individuals Survey (CSFII) determined that U.S. adults consumed an average of 6.7 servings of grain products per day, yet only one of these was classified by the Food Guide Pyramid as a whole grain food (480). Furthermore, 36% of those surveyed averaged less than one whole grain serving per day. Thus, considerable debate has arisen regarding the formulation and utility of the dietary guidelines as presented in the Food

Guide Pyramid (481-483). Currently, the debate is focused on the nature of dietary carbohydrates and the extent to which they should contribute to total energy intake.

Carbohydrates in the Diet

Carbohydrates form the foundation of the human diet, comprising 40-75% of total energy intake worldwide (484). The major carbohydrate-containing foods in the human diet are cereals, sweeteners, root crops, legumes, vegetables, fruits, and milk products. On a dry weight basis, cereals contain 65-75% carbohydrate, 6-12% protein, and 1-5% fat (484). Cereal grains are also rich sources of other nutrients known to promote good health including fiber, micronutrients, and phytochemicals, making them the most nutrient-dense source of dietary carbohydrate. Corn, wheat and rice, the most widely consumed cereals, account for 75% of the world's cereal crops (484,485). The United States produces 41% of the world's corn crop, 20.6% of which is processed to some extent prior to human consumption. Total grain and cereal consumption approaches 100 grams per day in North America (486).

Sugars, primarily derived from sugar beet and sugar cane, are the second largest contributor to dietary carbohydrate. Both sugar beet and sugar cane typically contain 15-16% sucrose and thus contribute only 12% of the carbohydrate produced worldwide (484). However, discretionary sugar intake in the United States has been estimated to be 11-12% of total energy intake (487).

Roots and tubers make up the third largest group of food carbohydrates with potatoes being the dominant crop of this group. Other widely consumed roots and

tubers include cassava, yams, sweet potatoes and taro. In general, root crops contain 15-30% carbohydrate, of which 70-75% is starch (484).

Legumes in the Western diet include peas, beans, lentils, peanuts, and other podded plants used as foods (488). The most widely consumed legume in the United States is the pinto bean followed by kidney beans, Northern beans, and lima beans. Beans and other legumes, which are typically 50-60% carbohydrate by weight, also contain significant amounts of dietary fiber, vitamins, and minerals. Unfortunately, less than 1/3 of adults in the United States consume one serving of beans in any three-day period (489). Worldwide, fruit and vegetable production far exceeds legumes. However, with the exception of bananas and plantains, legumes contain significantly more carbohydrate than do fruits and vegetables (484).

Digestion and Absorption of Dietary Carbohydrates

Mechanical and enzymatic digestion of carbohydrate-containing foods begins in the mouth where chewing breaks food into smaller pieces and thus, increases the surface area over which salivary α-amylase can act, ultimately influencing the rate of absorption (490). Although the time spent in the mouth is very short and carbohydrates undergo only a negligible amount of enzymatic hydrolysis in the oral cavity, chewing facilitates enzymatic digestion by disrupting the physical structure of foods (490,491). For example, as bread is chewed, it loses its cohesive structure resulting in smaller particles and release of some starch granules from the gluten protein matrix (491). Similarly, shorter strands are produced by chewing spaghetti although the general shape of the pasta is maintained. Despite the brevity of oral digestion (20-30 seconds), Hoebler, et al.

(491) have demonstrated that roughly 50% of starch in bread is hydrolyzed and solubilized into smaller molecular weight oligosacchardies, rendering it more available for further digestion. Because of its dense structure, only 25% of the starch in pasta is hydrolyzed during the oral phase of digestion (492).

The activity of salivary α-amylase is quickly lost in the acidic environment of the stomach. Digestion of amylose and amylopectin is a two-stage process which resumes in the duodenum (490,493). Starch hydrolysis is initiated by pancreatic α-amylase, an enzyme specific for internal α-1,4-glycosidic bonds; α-1,6 bonds are not susceptible to the action of α-amylase, nor are the α-1,4 bonds of glucose units, which form branch points and are sterically inaccessible to the enzyme (490,493,494). Under physiological conditions, little free glucose is formed by the action of α-amylase in the intestinal lumen (490). Starch hydrolysis proceeds as single glucose residues are sequentially removed from the non-reducing end of the α-1,4 chain by the action of sucrase. α-dextrinase is the sole enzyme responsible for hydrolysis of non-reducing, terminal α-1,6 bonds (490,493,494). Following complete hydrolysis of starches and disaccharides, the resulting monosaccharides can be absorbed across the intestinal mucosa (495).

Classification of Dietary Carbohydrates

Monosaccharides and Disaccharides

Historically, dietary carbohydrates have been classified based on degree of polymerization, much as dietary fatty acids are classified based on chain length (496). Monosaccharides are structurally the simplest form of carbohydrates and are often called

"simple sugars" since they cannot be hydrolyzed to yield smaller units (496). The most abundant and nutritionally important monosaccharide found in nature is glucose. In addition to being the sole constituent of starches, glucose is also a component of each of the three major disaccharides. Along with glucose, fructose (fruit sugar) is found in the free form in varying amounts in fruits, berries and vegetables. The third monosaccharide, galactose, is found in its free form only in very small quantities. Sucrose is the most important disaccharide in the diet and upon hydrolysis yields glucose and fructose (496). Lactose, also a disaccharide, is found in the greatest quantities in milk products, with cow's milk containing approximately 50 grams of lactose per liter (496,497). Other foods containing significant amounts of lactose include yogurt, cottage cheese, and ice cream (498)

Dietary Starch

Quantitatively, plant starch is the most important food carbohydrate and, along with glycogen, represents the only polysaccharides susceptible to human digestive enzymes (490). Starch granules are found in seeds, roots, and tubers as well as stems, leaves, and fruits (484,485). Photosynthetic plants synthesize and deposit starch in densely packed, water-insoluble granules ranging from 1 to 100 μm in diameter (485,499). The amyloplast is the organelle responsible for synthesis of both amylose and amylopectin and thus, largely determines starch granule composition (500,501). Starch granules consist of amylose and amylopectin in varying proportions, depending on the botanical species, and small amounts of non-carbohydrate components including lipids, protein and phosphorus (501,502).

Amylose is a long essentially linear polymer of α-1,4-linked D-glucose units (499,503). There is evidence that larger amylose molecules may contain a very small number of α-1,6-linked D-glucose branches; however, the typical amylose molecule contains 500 to 600 anhydrous glucose units and is free of branching (500,503). Most starches contain 15-25% amylose, although there is considerable variation, ranging from wrinkled peas, which contain 85% amylose to waxy maize, which is less than 1% amylose (486,504,505).

In most starches, amylopectin, the larger of the two starch components, is found in much higher quantities than is amylose (500,501,503). Amylopectin has a highly branched bi-model structure of short, linear α1,4-linked D-glucose arrays with α-1,6-branch points, accounting for 5% of the total structure and occurring approximately every 25 glucose units (499,500). Such extensive branching induces significant flexibility and permits efficient packaging of the short chains of amylopectin to form doubles helices, which subsequently associate in clusters forming the crystalline region of the granule (499,500). Alternating with the crystalline regions are amorphous zones created through the branch points of amylopectin with amylose filling in any gaps created in the expanding granule, particularly along the outer surface (501,503). Compared to the crystalline core of the granule, the amorphous, gel-like phase has a more open configuration and thus, is more susceptible to enzymatic attack, chemical modifications and swelling upon hydration (505,506). The core of cereal starch granules is characterized by very densely packed amylopectin double helices (507). Alternatively, tuber starches are less densely packed with amorphous pores, which can accommodate water (505,507). It should be noted that there is no clear line of demarcation between

the amorphous and crystalline phases of starch within the granule. Consequently, larger amylose polymers may participate in double helices with amylopectin and co-localize within the core where they are less likely to leach from the granule when subjected to enzymatic or thermal stress (503,505).

Dietary Fiber

Dietary fiber is a heterogeneous group of non-starch plant polysaccharides (NSP), which resist degradation by human digestive enzymes (505,508,509). The most abundant NSPs, including cellulose, hemicellulose and pectin, are found as structural components of plant cell walls (505). NSPs also include storage polysaccharides such as fructans (e.g. inulin), glucomannans (e.g. konjac-mannan), and galactomannans (e.g. guar gum) (505,508-510).

Dietary fiber is classified into two types, soluble or insoluble, based on its behavior in aqueous solution (511). Water soluble dietary fibers include pectins, gums, mucilages, algal polysaccharides, some hemicelluloses, and some of storage polysaccharides (509,512). Pectin is composed of a linear backbone of 1,4-linked galacturonic acids interrupted by side chains of neutral sugar residues (509,510). Apples are rich sources of pectin, which contribute to the matrix of the plant cell walls. Pectins are insoluble in unripe apples, but become more soluble as the fruit ripens (513). Pectins have high water-holding capacity enabling them to form viscous gel networks in the intestinal tract, which can bind organic compounds, such as bile acids (509,510). Pectins escape enzymatic hydrolysis in the small intestine but are almost completely degraded by colonic microflora (508). Gums and mucilages are non-structural plant polysaccharides, which

like pectin, form viscous networks in aqueous solutions (505,509). One of the more common gums, guar gum, is found in the endosperm of the Indian cluster bean (514).

It has been argued that dietary fiber should also include the fraction of starch in foods that can resist digestion in the human small intestine due to a number of highly variable factors (505,508,509). Englyst, et al. (515-517) have classified resistant starch into three fractions, RS_1, RS_2, and RS_3, based on the means by which starch escapes intestinal degradation. Starch which is physically inaccessible to the actions α-amylase by virtue of its entrapment within the food matrix are assigned to the RS_1 fraction. Inaccessible starch granules are found in whole or partially milled grains, legumes or other starch-containing foods, the size or physical structure of which protects it from digestion. The RS_2 fraction includes starch associated with the crystalline portion of the starch granule, which would necessitate complete gelatinization prior to digestion. Starch rendered resistant to α-amylase as a result of retrogradation constitutes the RS_3 fraction (515-518). Recently, a fourth category of resistant starch, RS_4, was added to include chemically modified starches that are resistant to enzymatic degradation (509).

Physio-Chemical Properties of Dietary Fiber

Numerous physiological effects have been attributed to dietary fiber, reflecting the complexity and diversity of their physical and chemical components. The nutritionally important properties of fiber are its water holding capacity, viscosity, binding and absorptive capacity, bulking capacity, and fermentability (508,509,511). Soluble and insoluble fibers have very different physio-chemical properties and consequently, have different local effects in the gastrointestinal tract (509,518,519). Few foods contain

soluble fiber to the exclusion of insoluble fiber and vice versa. As a result, the extent to which a fiber is soluble in aqueous solution and the rate at which it is absorbed is largely determined by the ratio of soluble fiber to insoluble fiber in a food in much the same way the amylose:amylopectin ratio determines the nature and rate of starch digestion (508).

Hydration capacity is a function of the extent to which the plant cell walls can retain water and in part, determines the fate of ingested fibers (509,511). In general, fibers composed primarily of plant cell wall components have greater water retention capacity than fibers containing secondary structures. Processing techniques such as grinding, drying, heating, and extrusion cooking, can disrupt the physical structure of the plant cell matrix, and thus, influence hydration capacity (508,509). Such mechanical processes increase the porosity of the starch granule exposing more of the matrix components to digestive enzymes (505).

Viscous dietary fibers are those that form thickened gel-like networks in the aqueous environment of the stomach and small intestine (509,518,520). Viscosity is a property that is exclusive to soluble fibers (508). Viscous fibers are known to delay gastric emptying, slow intestinal absorption of sugars, and increase fecal loss of bile acids (519,521-523).

Bulk volume influences a number of local gastrointestinal properties including transit time, colonic fermentation, and fecal excretion (508,509,524). Although both soluble and insoluble fibers can serve as fuel substrates for colonic microflora, insoluble fibers alone retain their structure in the colon and thus, contribute to fecal weight (525). Wheat bran has the greatest capacity to increase fecal bulk by virtue of its poor

fermentability, low hydration capacity and negligible viscosity in the aqueous environment of the intestinal lumen and colon (526). In contrast, pectin is a highly fermentable, highly viscous soluble fiber that contributes little to fecal weight (510).

Physiological Effects of Viscous Soluble Fibers

Viscous soluble fibers form a thickened gel-like matrix in the aqueous environment of the intestinal lumen and thus, increase the viscosity of the digesta (509,518,520). The viscosity of the intestinal contents may play an important part in determining the rate of gastric emptying and small bowel transit, the extent to which digestive enzymes are able to interact with food components, micelle formation, and absorption of nutrients by the intestinal mucosa (509,527-530). The gel network formed by the partial solubilization of viscous fibers in the small intestine effectively increases the thickness of the unstirred water layer which serves as a barrier to nutrient absorption at the intestinal brush border without affecting the total volume of digesta absorbed (520,531).

Addition of viscous fibers to the diet may delay nutrient absorption by influencing the hormonal response to other food components (528,529). Cholecystokinin (CCK) is a gastric hormone released in response to the presence of fat within the intestinal lumen and is thought to be largely responsible for the satiating effects of dietary fat (532-534). Increasing the viscosity of intestinal components retards fat absorption and extends the digestive process further along the length of the small intestine (534). Plasma CCK levels rise following consumption of a mixed meal (533) and Burton-Freeman, et al. (534) observed increases in postprandial CCK levels following the incorporation of viscous fibers into a low-fat mixed meal (20 energy% fat) that were similar to those observed

following consumption of a high fat meal (30 en% fat). Similarly, Bourdon, et al. (535) reported that plasma CCK levels remained significantly elevated for six hours following consumption of high-fiber pasta enriched with β-glucan, CCK levels returned to baseline within three hours of ingesting low-fiber pasta. Although plasma glucose levels were similar, plasma insulin levels were lower ($p<0.05$) following the high-fiber versus the low-fiber pasta.

The Glycemic Index and Dietary Carbohydrates

The classification of foods according to their blood glucose response was first undertaken by Otto and Niklas (536) in order to allow incorporation of a variety of carbohydrate-containing foods into the diabetic diet while keeping the glycemic impact relatively constantly. Wolever, et al. (537) developed the concept of glycemic index (GI) as a physiologically based method of classifying foods according to their post-prandial blood glucose response and as a supplement to information regarding the chemical composition of foods found in food tables.

The glycemic index is defined as the incremental area under the blood glucose curve in response to a standardized carbohydrate load, i.e., the blood glucose response curve above the level of fasting blood glucose (537). The glycemic index for a particular food is derived by expressing the individual glycemic index as a percent of a reference food, typically white bread or glucose (537,538). The portion of food tested generally contains 50 grams of available carbohydrate to allow direct comparison to the same portion of reference food (537-539). The glycemic indices of selected foods are shown in Table 1.

Table 1

Glycemic Index of Selected Foods*

		Reference Food	
		Glucose (GI = 100)	White Bread (GI = 100)
Simple Sugars			
	Fructose	20 ± 5	29 ± 0
	Sucrose	50 ± 9	85 ± 1
	Lactose	43 ± 0	61 ± 0
Legumes & Tubers			
	Black Beans	42 ± 9	59 ± 12
	Kidney Beans	28 ± 4	39 ± 6
	Lentils	26 ± 4	36 ± 5
	Mung Beans	31 ± 0	44 ± 6
	Mung Bean Noodles	33 ± 7	47 ± 10
	Potato, baked	85 ± 9	121 ± 16
Cereal Grains & Grain Based Foods			
	Barley Bread, whole meal	67 ± 1	96 ± 6
	Oat Bran Bread	47 ± 3	68 ± 5
	Rye Bread, whole meal	58 ± 6	83 ± 8
	White Bread, wheat flour	73 ± 2	105 ± 3
	Pita	57 ± 2	82 ± 7
	Spaghetti, durum wheat	57 ± 6	81 ± 8
	Spaghetti, whole meal	37 ± 5	53 ± 7
	Sweet corn, cooked	53 ± 4	78 ± 6
	Rice, long grain, parboiled	47 ± 3	68 ± 4
	Rice, instant	69 ± 8	98 ± 7
Breakfast Cereals			
	All Bran	42 ± 5	60 ± 7
	Cornflakes	81 ± 3	116 ± 5
	Rolled Oats, porridge	58 ± 4	83 ± 5
	Instant Oatmeal, porridge	66 ± 1	94 ± 1
Fruits & Fruit Juice			
	Apple	38 ± 2	52 ± 3
	Apple Juice	40 ± 1	57 ± 1
	Banana, ripe	51 ± 1	73 ± 2
	Banana, slightly under-ripe	42 ± 2	60 ± 3
	Orange	42 ± 3	60 ± 5
	Orange Juice	52 ± 3	74 ± 4
Dairy Products			
	Milk, whole	27 ± 4	38 ± 6
	Milk, skim	32 ± 5	46 ± 4
	Yogurt, low fat, aspartame sweetened	14 ± 4	20 ± 2
	Yogurt, low fat, sucrose sweetened	33 ± 7	47 ± 3

*Adapted from Foster-Powell, K., et al. (2002) International table of glycemic index and glycemic load values: 2002. Am J Clin Nutr 76, 5-56.

To approximate the glycemic index of a meal, the carbohydrate content of each food is determined. The proportion of carbohydrate contributed by each food is then multiplied by its glycemic index and the values are totaled to give the glycemic index of the meal (537-539). Wolever, et al. (538) determined that this method of combining the glycemic indices of meal components yields a glycemic index within 2% of the measured value for the aggregate meal.

Factors Influencing the Glycemic Response

Day-to-Day Physiological Variations

There is considerable controversy regarding the practical application of glycemic index due to the variable nature of responses observed among individuals. After studying diabetic subjects at weekly intervals over a four month period, Wolever, et al. (540) observed significant differences in the absolute glycemic responses between different individuals, as well as significant intra-subject variability to the same food tested on different days, the severity of which was influenced by the degree of glucose intolerance of a given subject. Consequently, Wolever et al suggest that most of the variability in GI values is due to day-to-day physiological variations within the same subject. Subsequently, Wolever, et al. (541) observed that the glycemic response in a given subject is influenced by a number of physiological and hormonal factors which fluctuate throughout a 24-hour period, and the results are most reliable when test meals are consumed following an overnight fast.

Simple Sugars

Given that glucose and fructose are both monosaccharides, it was initially assumed that similar blood glucose response curves would be obtained following ingestion of a solution containing 50 gram of either monosaccharide. However, when compared to a 50 gram oral glucose load (GI = 100), fructose produced a mean score of only 23, while the same amount of sucrose, a mixture of glucose and fructose, produced a mean score of 60 (542). This suggests that the GI for a given sugar can be influenced by the molar ratio of glucose to other monosaccharides (537,542,543). Sucrose and lactose each contain 1 glucose molecule and thus, have glycemic indices lower than an equivalent amount of glucose. On the other hand, maltose, which is formed from two glucose molecules, has a glycemic index of 105 (542,543).

An additional factor influencing the blood glucose response to simple sugars is the mechanism and rate of absorption in vivo (495). While glucose is actively absorbed in the small intestine and appears rapidly in circulation. In contrast, fructose is primarily metabolized by the liver and consequently, little fructose appears in circulation nor does the glucose derived from fructose contribute to the postprandial glycemic response (490).

Naturally Occurring versus Added Sugars

There is debate over the nature of the blood glucose response to naturally occurring sugars in plant foods compared to foods containing added sugars. Brand-Miller, et al. (544) found that in general, the median GI of the foods containing only naturally-occurring sugars was similar to that of foods to which sugars had been added during

processing. No significant differences in GI values were observed for cakes, muffins and most cookies made with or without added sugars. This may be the result of sugars being converted to carbon dioxide by yeast during the baking process (545). However, significant differences were observed among dairy products, canned peaches, and beverages according to the presence or absence of added sugars (544). Sucrose, which is highly water-soluble, ties up water molecules making them unavailable to starch. Consequently, the addition of sucrose inhibits the swelling of starch and retards gelatinization in a dose-dependent manner (546).

Starch versus Sugar

The term complex carbohydrate has been part of the consumer lexicon since the 1970s and has been used to describe a heterogeneous group of foods. When originally proposed as a mandatory component of the Nutrition Facts segment of the nutrition label, the term complex carbohydrates was defined as the sum of digestible polysaccharides with a degree of polymerization greater than nine; little attention was paid to the complex physiological effects of dietary carbohydrates. Lack of a clear definition for what constitutes complex carbohydrates, methodological considerations, and the physiological effects of various dietary carbohydrates led to the omission of the term from the mandatory nutrition labels (547).

Conventional wisdom suggests that the metabolic effects of simple sugars should be more detrimental than an equivalent amount of dietary starch. The substitution of dietary starch for dietary sugar results in significant metabolic changes. Wolever, et al. (548) have demonstrated that starchy meals result in much less variable postprandial

blood glucose levels than do oral glucose loads. In subjects tested over three days, the intra-subject variability in the postprandial blood glucose response was significantly greater following ingestion of 75 grams of glucose compared with 50 g of white bread or 50 grams of carbohydrate derived from an oat bar. The intrasubject variability 2 hours after ingesting the glucose load was 2-3 times higher than for white bread or oat bar, suggesting more accurate determinations of glucose tolerance can be obtained using starch rather than the typical glucose load.

Amylose and Amylopectin Content

Ultimately, all starch is susceptible to digestion by human α-amylase (485,490). Both amylose and amylopectin are susceptible to enzymatic hydrolysis by α-amylase, present in saliva and pancreatic secretions; in addition, the α-1,6 branches of amylopectin are susceptible to isomaltase found in the brush border of the small intestine (485,490). As a result, amylopectin has many more sites of enzymatic attack than does amylose and is more rapidly degraded than is straight-chain amylose (485,490). Amylose is characterized by extensive hydrogen bonding, which results in a more compact structure and effectively reduces the surface area over which α-amylase can act (506). Thus, the rate of starch digestion and the postprandial response are influenced by the ratio of amylose to amylopectin within the starch granule.

Heat Processing and Gelatinization

Starch granules are insoluble in cold water but may be partially solubilized (gelatinized) by heating above 60-70° C in excess water to disrupt the native starch

structure (518,549). The constituent molecules of starch granules are held together primarily by hydrogen bonds, which weaken when heated. Swelling begins in the center of the granule where the hydrogen bonds are less rigid and spreads toward the periphery of the granule (500). Significant swelling begins at the initial gelatinization temperature (~60° C) and increases the porosity of the starch structure allowing further influx of water, disruption of the botanical structure and dispersion of the semi-crystalline amylose-amylopectin complex permitting leaching of smaller amylose polymers from the granule (500,501,503). With continued swelling, fully hydrated granules separate from the protein matrix in which they were encased and may escape into the aqueous environment. Consequently, gelatinization increases the susceptibility of starch to α-amylase (505). As a result of partial gelatinization, α-amylase is able to act throughout the starch granule instead of being restricted to the surface as it was in the native starch granule (500,505). Because of its linear structure, the amylose content of a particular starch largely determines its gelation properties (502,506). Upon cooling of a gelatinized starch, the dispersed amylose and amylopectin molecules may reassociate and partially return to a granular state in a process known as retrogradation (518). Amylose undergoes retrogradation much more rapidly than does amylopectin (518). Thus, amylose becomes more rigid and stable during storage than does amylopectin. Although cooling results in partial restoration of the native starch structure, retrograded starch is generally more susceptible to enzymatic hydrolysis than was the native conformation (518).

Legumes

Typical legume starch granules are embedded in a protein matrix making isolation of the starch *per se* difficult (550,551). Legume starch granules are generally oval in shape and contain 30-40% amylose, significantly more than is found in widely consumed cereal grains (502). In addition to having greater amylose content, legumes contain twice as much protein as cereals and roots or tubers (502).

Legumes, such as lentils, contain significant amounts of fiber and yield flattened glycemic responses, similar to those obtained following ingestion of viscous soluble fibers, such as guar gum and pectin (521,552). The favorable glycemic response to legumes was initially attributed to an increase in intestinal viscosity. To test this hypothesis, viscosity was determined by measuring the rate at which glucose was released from dialyzed leguminous foods compared to dialysate containing only glucose solution as a control. If legumes were acting by increasing viscosity as was believed, the rate of glucose released from the legumes would be reduced in proportion to the amount of lentils in the dialysis bag. However, there were no differences in the rate of glucose released between legumes and the glucose solution, suggesting the flatter blood glucose response to legumes was not mediated by an increase in viscosity of the digesta (492).

Subsequently, Wong, et al. (553) hypothesized that the tight botanical structure characteristic of legumes may limit the extent to which carbohydrate contained in the core granule may be accessed by hydrolytic enzymes. Prior to cooking, whole lentils were subjected to various forms of mechanical distress (mashing, blending, grinding), which increased the surface area to different degrees. The rate of starch hydrolysis was greatest ($p<0.001$) for ground lentils, which had the greatest surface area, and lowest for mashed

lentils, demonstrating the important influence of physical structure and surface area on digestion and absorption (492,553).

O'Dea, et al. (554) have demonstrated greater postprandial glucose responses to ground versus whole lentils ($p<0.001$). There was also a different time course for the postprandial response with peak glucose levels observed at 30 minutes following ground lentils, while the response to whole lentils was delayed until 60 minutes post ingestion. Despite a higher peak glucose level in response to ground lentils ($p<0.025$), the attenuated response to whole lentils led to nearly identical total AUC_G for the two foods. The postprandial insulin response closely paralleled glucose responses with peak values occurring at 30 minutes following mashed lentils and 60 minutes after whole lentils. Although the time course of the insulin responses were different, neither peak insulin values nor AUC_I differed between the foods. The divergence of the postprandial glucose and insulin responses suggests that factors other than the rate of glucose absorption influence the magnitude of the insulin response to leguminous foods and that the metabolic response can not be predicted solely by the rate of starch digestion in vitro (554,555).

Wong, et al. (553) demonstrated that commercially prepared canned beans were more susceptible to hydrolysis by α-amylase compared with home-cooked beans of the same botanical species. Following treatment with α-amylase, nearly half of total starch contained in canned navy beans had been hydrolyzed, whereas the same treatment led to hydrolysis of less than 10% of total starch in home-prepared beans. These differences are presumably due to the method of preparation. Traditional home cooking of dry beans usually involves overnight soaking followed by prolonged boiling, while

commercial methods employ much higher temperatures and pressure treatment, which is more disruptive to the structure of the starch granule (549).

Cereal Grains

Wheat and Corn

Cereal grains are characterized by a multi-layer botanical structure which includes the pericarp, testa, germ, aleurone, and endosperm. The greatest contributor to cereal grain structure is the endosperm which contains the starch, as well as vitamins and minerals. Surrounding the endosperm is the aleurone layer, which is a rich source of B vitamins, minerals and protein (556).

Most cereal grains undergo some form of mechanical or thermal processing prior to consumption or inclusion in other foods. Milling and grinding of cereal grains reduces particle size and increases the surface area over which digestive enzymes can act (557-559). Less severe mechanical treatments, which do not destroy the botanical structure, nonetheless introduce physical imperfections into the otherwise smooth kernel surface, rendering the starch more accessible to hydrolytic enzymes (557,559).

The extent to which physical structure of the grain is disrupted during processing is an important factor influencing postprandial metabolism (500,505,557). For example, intact grains retain the outer bran and germ layers, which serve as physical barriers to enzymatic digestion, and cooking is only partially effective in removing these layers (549,560,561). The milling process by which flour is made separates the bran and germ layers from the starchy endosperm, which is subsequently ground into flour (559). Typical wheat starch is 30% amylose and 70% amylopectin in granules of variable size

(2-35 μm) (556,559). Wheat starch granules are affixed to storage proteins by a matrix of water-soluble proteins, which swells rapidly upon hydration, facilitating starch hydrolysis (546).

Heaton, et al. (562) examined the glucose and insulin responses of normal volunteers to wheat-based meals varying only in the extent to which the wheat had been processed. There was a trend for plasma glucose levels to rise higher and fall lower in response to flour-based meals compared to cracked or whole wheat meals, in which particle size was significantly larger. The area under the glucose curves followed a pattern similar to blood glucose levels, but there were no significant differences for either plasma glucose level or the area under the blood glucose curve. This suggests that with respect to the postprandial glucose response, starch particle size is irrelevant. However, particle size had a significant impact on plasma insulin levels. Stepwise increases in peak plasma insulin concentrations and area under the insulin curve were observed as the particle size of the wheat decreased. The largest differences were noted between fine and coarse flours, with the area under the insulin curve being 38% higher after fine flour ($p = 0.0063$). Similar results were observed for maize-based meals. There were no differences in plasma glucose responses, however, plasma insulin levels rose much more after corn flour than after cracked or whole corn. The area under the insulin curve was 60% higher after the flour than after cracked corn and 89% higher than after whole corn. (562).

Pasta

Among grain-based products, pasta has been consistently reported to yield flattened postprandial glucose responses, with the blood glucose response to spaghetti is 40-60% lower than that of white bread in healthy and diabetic subjects (543,563-565). The blunted glycemic response to pasta may be due to a number of factors. Over the last decade the pasta industry has introduced new production processes which expose the product to higher temperatures and result in more complete gelatinization of starch (566,567). High-temperature drying of spaghetti increases protein denaturation and results in a considerable loss of α-amylase activity in vitro (566,567). Dexter, et al. (568) have also noted that higher processing temperatures yield firmer products, which may further influence the susceptibility of pasta products to enzymatic degradation in vivo.

Most pasta is made of semolina from durum hard wheat. Semolina, which is 60-70% starch, is derived from the endosperm of the wheat kernel. During the production of pasta, the starch contained in semolina undergoes partial hydrolysis due to the presence of naturally occurring amylases (566,567). Prior to cooking, microscopic examination of durum semolina pasta reveals perfectly intact starch granules within a gluten protein matrix. When cooked, a protein matrix encapsulates the starch granules, which become swollen and partially gelatinized (566). However, starch granules remain largely intact, partially attributable to the viso-elastic nature of the associated gluten, which serves as a protective barrier against hydrolysis.

In an attempt to understand the mechanisms influencing the postprandial response to pasta, Granfeldt, et al. (569) compared two pasta products, which differed only in physical structure (spaghetti and macaroni), to bread prepared with pasta ingredients. In

diabetic subjects, macaroni resulted in a higher $AUC_{glucose}$ than spaghetti ($p<0.05$). Furthermore, the peak glucose response above the fasting level was significantly lower ($p<0.05$) for spaghetti than for bread prepared from the same ingredients as the spaghetti. Thus, it appears the physical structure has a significant effect on the postprandial response. The blunted response to spaghetti has been attributed to larger particle size and higher product density, both of which influence the susceptibility of starch to α-amylase (569).

Interestingly, cooking time appears to exert little influence on the blood glucose and insulin responses to a pasta meal in healthy subjects (570). Relative to an oral glucose load, blood glucose and insulin responses were significantly lower following consumption of a pasta meal, regardless of cooking time. Although the exact mechanism to which these findings can be attributed are unclear there are several possibilities. During the manufacturing of pasta, partial gelatinization of starch is known to occur (546). Alternatively, the protein matrix which envelopes the starch granules in pasta may be similar regardless of cooking time and responsible for the blunted response of the pasta meals compared to glucose (566).

The carbohydrate content of pasta can be reduced by the addition of milk or whey proteins, eggs, animal proteins, processed bran, and gluten (563,566,569). However, the industry lacks a standardized method for preparation of commercially available pasta makes it difficult to determine the nature and mechanisms influencing the postprandial response to pasta products. Granfeldt, et al. (569) examined the importance of processing conditions, physical structure and added nutrients on the postprandial response to pasta-based meals. Healthy subjects were given test meals with an

equivalent amount of available carbohydrate from extruded high-temperature dried spaghetti and three varieties of fresh linguine (thick, thin, thin with egg) made from the same ingredients. A reference bread was baked from the same ingredients as in the pasta products. The four pasta products produced significantly lower peak values for glucose, insulin, and c-peptide in the early phase (40, 50, and 60 minutes) compared to the bread made from the pasta ingredients ($p < 0.05$ for all). The early phase (60 and 90 minutes post ingestion) glucose and insulin responses to the thin linguine were significantly higher than the thin linguine prepared with egg ($p < 0.05$). Consistent with this, the addition of egg to the thin linguine produced resulted in lower AUC values for insulin ($p<0.05$) and c-peptide ($p<0.05$) at 120 minutes the same product without added egg. Guillford, et al. (570) demonstrated an increased insulin response to a spaghetti meal upon addition of protein, whereas the glycemic response was unchanged, suggesting the amount of protein contributed by the addition of egg was insufficient to potentiate the insulin secretory response.

Oats and Barley

Commercial preparation of oats involves removal of the outer husk layer followed by two steaming steps, which result in softer, more pliable particles and inactivation of any naturally-occurring lipases. The soft oats are then rolled and exposed to pulses of warm water after which they are dried by exposure to ambient air. This process removes the outer husk which is a rich source of lignin and insoluble nonstarch polysaccharides, including cellulose and arbinoxylans. In addition, this process disrupts the native structure of the aleurone cell, which makes the cereal grain more susceptible to digestive

enzymes (557). The major component of the soluble fraction of oats is the linear polysaccharide, (1,3)(1,4)-β-D-glucan (β-glucan), which is contained in the aleurone layer (571,572). Oat bran is derived by dehulling whole oats and contains 7-19% β-glucan, while rolled oats contain approximately 4% β-glucan (571,572).

Most commercially available breakfast cereals are heat processed; consequently, the starch is more or less completely gelatinized (557). However, steam rolling and processing of oats is carried out at lower temperatures resulting in incomplete starch gelatinization (557,571). Granfeldt, et al. (573) demonstrated the importance of degree of gelatinization and product thickness on post-prandial glucose and insulin responses to rolled oat in healthy subjects. Dehulled oats were subjected to varying degrees of roasting and steaming or used raw to prepare rolled flakes of different thickness. The glycemic (GI = 88-118) and insulin (II = 84-102) indices for thin (0.5 mm) rolled oat flakes were similar to white bread; thicker (1.0 mm) oat flakes produced significantly lower GI (GI = 70-78) and insulin (II = 58-77) values relative to white bread ($p<0.05$).

Oatmeal is most commonly consumed as a breakfast cereal by cooking rolled oats in water to give a thick, highly viscous porridge. There are two common methods for preparing porridge from rolled oats. In the rapid cooking method, rolled oats are added to boiling water, allowed to return to a boil and simmer for 10 to 15 minutes before being consumed. A smoother porridge is obtained using the gradual boil method, whereby rolled oats are added to cold water, gradually brought to a boil and then simmered for 10-15 minutes prior to eating. These two cooking methods appear to be very similar, however, there are significant differences in the cooked mixture based on the method of preparation. In either case, exposure of the oats to boiling water results

in protein denaturation and starch gelatinization and consequently, increases the amount of readily digestible starch. However, the rapid-boil method results in an oatmeal porridge which retains a greater quantity of β-glucan and is markedly more viscous than a porridge prepared by the gradual-cook method. Microscopically, rapid cooking results in swollen, but otherwise largely intact starch granules. Conversely, gradual cooking leads to more extensive gelatinization as evidenced by disruption of cell walls, distorted starch structures and the absence of recognizable starch granules when viewed microscopically Thus, the gradual cooking method results in greater structural disruption of cell wall structures and permits greater release of soluble β-glucan from the cooked product (574). Therefore, while cooking may facilitate digestion of botanical structures otherwise resistant to human digestive enzymes, it may also lead to solubilization and loss of the β-glucan fraction (526,574).

The degree of gelatinization, method of preparation and physical structure is a significant determinant of the postprandial response to oat products. Whole kernel porridges were prepared by boiling intact oat kernels in water. Rolled oats were served raw or as a porridge by cooking in boiling water. The whole oat kernel porridge produced significantly lower glucose ($p<0.05$) and insulin ($p<0.05$) responses compared to rolled oat porridge and white bread. Interestingly, the rolled oat meals, whether served as raw muesli or as oatmeal porridge, resulted in postprandial glucose and insulin levels and similar to white bread (526). Researchers hypothesized that although starch in rolled oats is only partially gelatinized (62%), this coupled with the disruption of the kernel structure during processing, was sufficient to facilitate more efficient digestion and absorption *in vivo* (526,557,571,574).

The swelling and viscosity properties of β-glucan are similar to those of guar gum and significantly greater than psyllium (572). However, oat bran is commercially available, whereas guar gum is not. Braaten, et al. (575) have demonstrated similar physiological effects of oat gum and oat bran containing equivalent amounts of β-glucan. In addition to white bread, wheat farina, which has a texture similar to oatmeal porridge, was included as a reference meal. Normal subjects and subjects with NIDDM were fed porridge meals containing either wheat farina, wheat farina plus an equal amount of β-glucan from either oat gum or oat bran. Fat and carbohydrate content of the meals were equal, however, the wheat farina meal plus oat bran contained a greater amount of protein due to the need to equalize the amounts of β-glucan in the test meals. In normal subjects, plasma glucose levels were higher ($p<0.05$) at 20, 30, and 40 minutes following the wheat farina meal than either of the β-glucan supplemented meals. In all control subjects plasma glucose returned to baseline within 180 minutes. The three hour AUC for plasma glucose was approximately 28% greater ($p<0.05$) after the wheat farina meal compared to meals containing β-glucan as either oat gum or oat bran in control subjects. Similar trends were observed in patients with NIDDM, albeit at higher baseline levels. The exception occurred in NIDDM subjects for which the wheat farina plus oat bran meal resulted in a greater three hour AUC for insulin ($p<0.05$). This may be attributable to the higher protein content of this meal and the potentiation of the insulin secretory response by dietary protein. For both normal and NIDDM subjects, plasma glucose excursions were more rapid and of greater magnitude following the wheat farina meal compared to those meals containing added β-glucan.

Tubers

A wide range of GI values (GI= 56-85) have been reported for potatoes depending on species, method of preparation, and storage (543). Because potatoes contribute 15-20% of total starch consumed in industrialized countries, there is considerable interest in determining the factors to which the variable glycemic indices can be attributed (486).

Starch grains are most concentrated in the cortex, lying just below the skin of potatoes. Within the cortex, numerous round and oval shaped granules are housed in a thin layer of parenchymal cells. In raw potatoes, the cell walls of the parenchymal layer are quite strong and embedded in a polysaccharide matrix, making them highly resistant to α-amylase (576,577).

Roughly 50% of the starch in raw potatoes is resistant to enzymatic digestion compared to only 3% in freshly cooked potatoes (576-578). Heat treatment does not completely disrupt granule structure but results in significant swelling that may permit leaching of smaller amylose molecules (578). Heating of potatoes also leads to other changes including protein denaturation, reduced cell adhesion properties, and loss of cell membrane integrity, which may facilitate diffusion of some cellular contents (578,579). The net result of cooking is gelatinization of the starch and softer, more easily separated cells in the tissue. Upon cooling, retrogradation occurs, altering the nature of the starch previously rendered susceptible to α-amylase by cooking such that 12% of the total starch in the potato is now resistant to enzymatic digestion (577,580).

Englyst, et al. (580) conducted studies to determine the changes in digestibility and carbohydrate absorption resulting from cooking and storage of potatoes by collection and measurement of effluent collected from ileostomy patients. Overall starch recovery

in effluent increased significantly following consumption of a cooled, cooked potato ($p<0.05$) compared to a freshly cooked, hot potato and a control (non-plant polysaccharide containing) diet. Similarly, total carbohydrate recovered was significantly lower following the control and fresh cooked, hot potato compared to the cooled potato ($p<0.05$). Overall, 9% of the carbohydrate in the fresh cooked potato and 18% of that in the cooled potato was recovered in the effluent. Thus, the retrogradation that occurs upon cooling, renders the starch in potatoes less available for digestion and absorption by the gut compared to the effects of gelatinization. Reheating the cooled potato by microwave oven or steaming also improved the digestibility of the cooled potato resulting in significantly less total starch excretion compared to the cooled potato ($p<0.05$).

Fruits

Influence of Ripeness and Botanical Structure

Fruits are generally harvested prior to prime ripeness to allow for transport and acquisition by consumers. Prior to ripening, fruit has a very rigid structure with well-defined cellular structures. As fruit ripens, intrinsic hydrolytic enzymes are activated which result in softer tissue and diffuse cell walls (513).

As consumed, bananas are a rich source of raw, largely indigestible starch (581). The starch content of bananas is known to decrease as the fruit ripens; consequently, a relatively unripe banana (yellow skin with small amounts of green at the tip) contains roughly 37% starch on a dry weight basis, whereas an overripe banana contains only 3% starch. Free sugars, which constitute 56-75% of total carbohydrate content on a dry

weight basis, increases as bananas ripen. Consequently, the ratio of starch to free sugars can be as much as 20-fold higher in an unripe compared to an overripe banana (513,582). Consistent with the changes in starch and glucose availability during ripening, Wolever, et al. (583) observed that the glucose response to ripe and overripe bananas were 25% and 50% greater ($p<0.05$), respectively, than the response to an under-ripe banana. Thus, the changes in carbohydrate composition which occur during ripening, clearly impact the metabolic response to fruits.

To further examine the influence of botanical structure on the glycemic response, Haber, et al. (584) compared whole apples to fiber-free apple puree and fiber-free apple juice. Despite the relatively small amount of total fiber in apples (1.5% by weight), it is the fiber that is responsible for the physical structure and appearance of the fruit. Apple juice, which contains a significant amount of carbohydrate, can be considered fiber-free apples since the fibrous structure has been completely removed. On the other hand, apple puree retains the fiber but the physical structure has been significantly disrupted. There were no significant differences in the peak blood glucose levels after whole apples compared to juice or puree. However, both apple juice and apple puree resulted in higher serum insulin levels and less satiety than did whole apples ($p<0.05$). There are several potential explanations for these findings. First, the physical form of a whole apple reduces the rate at which it can be consumed compared with juice and puree, which can be swallowed relatively quickly. Although the postprandial glucose responses were similar after consumption of whole apples, fiber-free juice, and fiber-free puree, consumption of juice and puree led to rebound hypoglycemia, which was not observed following ingestion of whole apples. This may be partially attributable to the disruption

of the fibrous structure of whole apples in preparing apple juice and puree. Fiber depleted foods are known to be more rapidly absorbed than high fiber foods (24,37,43). That apple puree gave an intermediate response suggests that particle size, in addition to intact structure, influences postprandial responses. The peak insulin level occurred more rapidly and was almost 2-fold higher ($p<0.001$) following apple juice compared to the whole apple. Consequently, the area under the insulin curve was also greater following juice compared to apples ($p<0.01$). Apple puree resulted in an intermediate peak insulin level, which was higher than after whole apples ($p<0.01$) yet lower than after juice ($p<0.01$).

Role of Gastrointestinal Hormones and Co-ingested Macronutrients

The interaction between increased availability of glucose, fatty acids and amino acids complicates the understanding of those factors that determine the postprandial response to a mixed meal. The term "enteroinsulinar axis" is used to include all metabolic factors, which contribute to enhanced insulin secretion following a meal. Several gut peptides have been demonstrated to be insulin secretagogues in vitro including gastrin, secretin, cholecystokinin (CCK), gastric inhibitory peptide (GIP) and glucagon like peptide 1 (GLP-1) (585-587).

The rate at which the stomach empties is regulated by stimuli generated in the stomach and small intestine. At least 15 different endocrine cell types, many of which secrete more than one type of hormone, have been identified in the gastrointestinal tract (586). Furthermore, the hormones secreted by the gastrointestinal tract are not confined to the area in which they are secreted but rather have endocrine and neurocrine effects as

well. The signals arising from the various gastrointestinal hormones are especially important in the duodenum, where the rate at which chyme is emptied by the stomach must be carefully regulated such that the absorptive capacity of the small intestine is not exceeded (532).

Progressive increases in dietary protein up to 50 grams enhances the insulin response in a dose-dependent manner and extends the time course of the insulin response such that plasma levels are elevated above baseline for longer periods compared to a carbohydrate load which does not contain protein (588). As the amount of dietary protein in a mixed meal increases, the mean incremental serum glucose level is reduced in parallel (588,589).

Dietary fat modifies gastrointestinal function by multiple mechanisms. Fat is known to delay gastric emptying, which may exert beneficial metabolic effects on postprandial glycemia by reducing the rate of carbohydrate delivery to the absorptive cells of the small intestine (590). However, the same combination of carbohydrate and fat reportedly attenuates postprandial glycemia via the stimulatory effect of dietary fat on gastric inhibitory peptide (GIP) secretion, which may subsequently augment the insulin response (591). The infusion of GIP resulting in supraphysiological plasma concentrations is insulinotropic at basal glucose levels in animals; however, in man GIP acts as an insulin secretagogue only under hyperglycemic conditions (587). This has been demonstrated by Verdonk, et al. (591). Fat ingestion under hypoglycemic and euglycemic conditions resulted in significant elevations of GIP above basal levels ($p<0.01$) without influencing insulin levels. Despite similar elevations in GIP levels

during hyperglycemic conditions, plasma insulin levels following fat ingestion were significantly greater than those observed prior to fat ingestion ($p<0.01$).

There are conflicting findings regarding the co-ingestion of fat and carbohydrate. Compared with the response to boiled potatoes, Collier, et al. (592) observed a reduction in the postprandial glucose response ($p<0.05$) when butter was given with boiled potatoes, which is consistent with the effects of dietary fat in delaying gastric emptying. However, serum GIP levels were 8-fold higher ($p<0.005$) in subjects consuming the potato with added butter, and was accompanied by an enhanced insulin secretory response ($p<0.01$) compared to subjects consuming the potato alone.

Siddhu, et al. (590) designed a more elaborate study to evaluate the effect of macronutrient content on the postprandial response. The glycemic and insulin response to glucose (G) were compared to isocaloric meals of variable macronutrient composition. The glycemic response to glucose plus corn oil (GCo) was similar to that of glucose alone; however, the insulin response to GCo was significantly lower than the response to glucose at 120 minutes ($p<0.01$). Consistent with this, the area under the insulin curve (AUC_I) was significantly lower following the GCo meal compared to glucose alone ($p<0.01$). Thus, it appears fat alone exerts little effect on postprandial glycemia, although it does reduce the insulin response. This conflicts with other reports indicating a blunting of the glycemic response when fat is included test meals (591,593). Partial isocaloric substitution of casein for corn oil (GCoCs) led to a significant blunting of the overall glycemic response as demonstrated by a lower AUC_G compared to glucose alone ($p<0.01$). Although the AUC_I for GCoCs was similar to G, the insulin level at 2 hours was significantly lower for the GCoCS meal ($p<0.01$). In this case, the

improvement in postprandial glycemia may be the result of increased insulin secretion in response to the inclusion of protein. A significant reduction in both the glycemic and insulin responses were observed following the addition of pectin (P) to the GCo meal. The glycemic response to GCoP (AUC_G) was roughly 45% lower than following GCo ($p<0.05$). There was an even more profound reduction in the insulin response with lower insulin levels ($p<0.01$) being apparent at 1.5-and 2-hours following the GCoP meals in addition to an overall reduction in the insulin response compared to GCo ($p<0.01$). The addition of dietary fiber as cellulose (GcoCsCl) reduced the glycemic response at 1-hr and 1.5-hr and in terms of AUC_G ($p<0.01$). Cellulose was without effect on insulin levels. The lowest glycemic and insulin responses were achieved with the combination of glucose, corn oil, casein and pectin (GCoCsP). Compared to all other meals, GCoCsP resulted in significantly lower blood glucose responses at all times during the 2-hour glucose tolerance test ($p<0.05$), lower plasma insulin levels at 2-hours ($p<0.01$), smaller AUC_G ($p<0.05$) and smaller AUC_I ($p<0.05$). Thus, the hypoinsulinemic effects of pectin were able to overcome the stimulatory effects of casein on insulin secretion.

Metabolic Benefits of a Low Glycemic Index Diet

Influence of Dietary Carbohydrate Source on Postprandial Metabolism

The metabolic effects of glucose, fructose, sucrose and starch (corn starch, 27% amylose, 73% amylopectin) on postprandial glucose and insulin responses, energy expenditure and substrate utilization were investigated in lean males following a 75 gram carbohydrate load (594). Plasma glucose levels peaked at similar levels 30 minutes

following ingestion of glucose and sucrose and at 60 minutes following ingestion of starch. Peak plasma glucose levels obtained 30 minutes after fructose ingestion were significantly lower ($p<0.01$) than for all other carbohydrates tested. After achieving peak values, plasma glucose levels returned to baseline at 60 minutes with fructose, at 90 minutes with glucose and sucrose, and 120 minutes with starch. Postprandial reactive hypoglycemia was observed from 150-300 minutes following ingestion of glucose and sucrose, and from 240 to 300 minutes following the fructose load. In contrast, postprandial blood glucose levels remained above baseline levels from 2 to 6 hours following ingestion of the starch load. The 6-hour integrated glycemic response above baseline was higher with glucose and starch than with fructose ($p<0.05$), with an intermediate response observed with sucrose ($p<0.05$ vs. fructose).

Plasma insulin levels followed a pattern similar to glucose levels following carbohydrate ingestion. Peak insulin levels were highest at 30 minutes following glucose ingestion ($p<0.01$ vs. all other carbohydrates) and lowest 30 minutes following fructose ($p<0.05$ vs. all other carbohydrates). Intermediate peak insulin levels were observed 60 minutes following ingestion of starch and 30 minutes after sucrose. Insulin levels returned to baseline at 150 minutes with fructose and sucrose, at 180 minutes for glucose, and at 240 minutes with starch. Plasma insulin levels fell below basal levels ($p<0.05$) from 240 to 360 minutes following fructose, glucose, and sucrose. The incremental AUC for insulin was highest for glucose ($p<0.001$ vs. all carbohydrates), lowest for fructose ($p<0.01$ vs. sucrose and starch), with intermediate values following sucrose and starch.

For all carbohydrates, there was a significant increase ($p<0.0001$) in energy expenditure following ingestion. The peak increase in energy expenditure was higher ($p<0.05$) with sucrose compared with all other carbohydrates. Energy expenditure returned to baseline at 150 minutes with sucrose, 180 minutes with glucose and starch, and at 210 minutes with fructose, and remained at baseline levels for the remainder of the study. The total integrated increment in energy expenditure above baseline was similar with fructose and sucrose, and higher with sucrose compared with starch ($p<0.05$) and glucose ($p<0.01$). The fact that sucrose and fructose yielded the lowest insulin responses but resulted in the greatest increase in thermogenesis is somewhat at odds with previous studies, which suggest that insulin is an important determinant of carbohydrate-induced thermogenesis.

Following ingestion of all carbohydrates, there was an increase ($p<0.001$) in carbohydrate oxidation above basal levels. Carbohydrate oxidation increased to similar peak levels at 60 minutes with fructose and sucrose. These values were significantly greater ($p<0.01$) and occurred more rapidly ($p<0.05$) than with glucose and starch. Carbohydrate oxidation returned to baseline levels at 210 minutes following ingestion of glucose, starch and sucrose, and at 240 minutes after ingestion of fructose. Total carbohydrate oxidation over 6 hours was significantly higher ($p = 0.008$) with fructose and sucrose compared with glucose and starch. The integrated exogenous carbohydrate oxidation over 6 hours, which represents the amount of ingested carbohydrate that was directly oxidized, was significantly higher ($p<0.001$) with fructose and sucrose compared with glucose and starch. Exogenous carbohydrate oxidation with fructose was also significantly greater ($p<0.01$) than with sucrose. The contribution of endogenous

glycogen stores to total carbohydrate oxidation was estimated by determining the difference in cumulative total carbohydrate oxidation and cumulative exogenous carbohydrate oxidation. There was a tendency for higher endogenous carbohydrate oxidation with fructose and sucrose compared with glucose and starch, but these differences did not reach the level of significance.

The decline in fat oxidation below basal levels was greatest 30 minutes following ingestion of fructose and 60 minutes after sucrose. The decrement occurred much later and to a lesser degree following starch (at 150 min) and glucose (at 120 min) compared with fructose and starch ($p<0.01$ for both). After achieving nadir, lipid oxidation returned to baseline at 210 minutes with fructose and sucrose and at 270 minutes following glucose and starch.

These results demonstrate that the postprandial metabolic response is influenced by the type of carbohydrate ingested. That these effects are most pronounced when integrated over a 6-hour period following carbohydrate ingestion, suggest that the carbohydrate composition of the diet may have important implications for substrate utilization and energy balance (594).

Using continuous indirect calorimetry, Ritz, et al. (595) have demonstrated different patterns of substrate utilization in the postprandial period following consumption of a test meal containing carbohydrate content equivalent to 50 g of glucose as a high (glucose) compared with a low (manioc starch, 16% amylose) glycemic index carbohydrate. Peak glucose levels were achieved 30 minutes after the glucose meal and were significantly greater ($p<0.01$) than following ingestion of the starch meal. After reaching peak values, blood glucose levels in subjects consuming the high-glucose meal

fell below postabsorptive levels at 180 minutes prior to returning to baseline. In contrast, plasma glucose levels beyond peak levels remained above baseline and were significantly higher ($p<0.01$) from 180 to 270 minutes in subjects consuming the starch meal than after the glucose meal. Insulin levels peaked at 30 minutes and returned to baseline at 360 minutes following ingestion of both meals. However, insulin levels remained significantly higher ($p<0.01$) after the starch-containing meal between 150 to 270 minutes compared with glucose. Plasma free fatty acid levels decreased following ingestion of both meals, reaching nadir at 150 minutes following the glucose meal and 180 minutes following the starch-containing meal. No significant differences were detected until 180 minutes, after which time plasma free fatty acid levels were significantly lower ($p<0.01$) after the starch meal. With both meals, free fatty acid levels rose above baseline from 270 to 360 minutes.

Glucose oxidation rose above basal levels following the glucose and starch meals. Rates of glucose oxidation remained relatively constant in both groups until 200 minutes when rates began to decrease but remained significantly higher ($p<0.01$) after the starch meal until the end of the test. Consequently, total glucose oxidation over the 6-hour period was approximately 21% higher in subjects consuming the starch ($p = 0.0002$) compared with the glucose meal. Carbohydrate oxidation was negatively correlated with plasma free fatty acid and glucose levels after both the glucose ($r=0.63$, $p = 0.0001$; $r=0.39$, $p = 0.0007$; respectively) and starch ($r=0.33$, $p=0.01$; $r=0.35$, $p=0.03$; respectively) meals. Glucose oxidation was correlated with plasma insulin levels ($r=0.47$, $p=0.0001$) following the glucose meal only.

Fat oxidation fell following both the glucose and starch meals. Fat oxidation remained significantly lower ($p<0.01$) for the starch compared with the glucose meal from 220 minutes until the end of the test. At the conclusion of the test, fat oxidation had returned to baseline in subjects consuming the starch meal, while remaining above baseline from 240 to 360 minutes in subjects consuming the glucose meal. There was a tendency for higher total fat oxidation over 6 hours after glucose compared with starch, but these values did not reach significance.

The Metabolic Response to Mixed Meals Containing Sucrose versus Starch

In a study by Storlein, et al. (596) male Wistar rats were randomly assigned to consume either a high-starch or high-sucrose diet for one month to evaluate the metabolic effects of different dietary carbohydrates on insulin action. Basal heat production was similar in starch and sucrose fed rats. Dietary carbohydrate source appeared to have no effect on heat production in response to a glucose gavage or noradrenaline administration, as similar increases in heat production were observed in both groups in response to these stimuli.

While basal blood glucose levels were similar in both groups, sucrose feeding significantly increased basal insulin levels by 35% ($p<0.05$) compared with animals fed the high-starch diet. Sucrose feeding impaired the ability of insulin to suppress hepatic glucose product, consequently, rats fed the sucrose-rich diet required a significantly lower rate of glucose infusion ($p<0.05$) to maintain euglycemia during hyperinsulinemic clamp studies compared with starch-fed rats. Hyperinsulinemia suppressed hepatic glucose output by 76% in starch-fed animals but only 34% suppression was achieved in

the high-sucrose group ($p<0.01$), and the difference in endogenous glucose production between the high-sucrose and high-starch groups accounted for 64% of the difference in glucose infusion rates during clamp studies. Sucrose feeding also significantly reduced ($p<0.01$) peripheral glucose disposal, which accounted for the remaining difference in glucose infusion rates between the two groups.

No differences in tissue-specific glucose metabolism were detected between groups at basal insulin levels. However, at clamp insulin levels glucose metabolism was reduced in sucrose compared with starch-fed animals in muscle (white gastrocnemius, plantaris), white adipose tissue (epididymal, inguinal, subcutaneous) and interscapular brown adipose tissue. While none of the decrements in glucose metabolism in individual tissues of sucrose-fed animals reached the level of significance, the cumulative effect was to reduce glucose metabolism in these tissues by 10% compared with starch-fed animals. Thus, four weeks of sucrose feeding is associated with impaired insulin action in several tissues, which reduces total body glucose metabolism. Furthermore, these impairments precede any detectable changes in body weight or adiposity, underscores the subtle nature of metabolic disturbances arising from chronic ingestion of refined carbohydrate diet.

Pawlak, et al. (597) investigated the development of insulin resistance and obesity in rats fed diets identical in macronutrient composition (35 en% fat, 20 en% protein, 45 en% carbohydrate), but containing either a low (amylose) or high (amylopectin) glycemic index (GI) starch. For comparison purposes, a third group was fed a high (60 en%) fat diet, which is known to induce hepatic and peripheral insulin resistance. To ensure that any changes in adiposity and insulin sensitivity were due to diet, rather than body weight,

animals in the low and high GI diet groups were fed for 7 weeks, while animals consuming the high fat diet were fed for only 4 weeks so that final body weights in all groups were similar.

Epididymal fat pad mass was lower ($p<0.05$) in the low-GI group compared with both the high-GI and high-fat groups. When expressed relative to body weight (g/100 g body weight), epididymal fat pad mass in animals consuming the low-GI diet was 22 and 41% lower ($p<0.05$) than in high-GI and high-fat fed animals, respectively. Thus, despite any detectable differences in body weight, animals consuming the low-GI diet accumulated significantly less adipose tissue than animals consuming the high-GI and high-fat diets.

There were no differences in fasting insulin levels between groups, but basal plasma glucose levels were greater in the high-fat ($p<0.05$) compared with the low- and high-GI groups. During an intravenous glucose tolerance test high-fat fed rats had higher plasma glucose levels ($p<0.05$) compared with the low- and high-GI groups at all time points measured, suggesting high-fat feeding impaired glucose uptake, making these animals less able to clear the glucose load. In contrast, both the incremental $AUC_{glucose}$ and peak glucose levels achieved during the IVGTT were not different in low- and high-GI fed rats. During the IVGTT, peak insulin levels in the high-GI group were not different from high-fat fed rats, but both were approximately 25% greater than animals consuming the low-GI diet ($p<0.05$). Furthermore, the integrated insulin response over the first 30 minutes was 31% higher in the high-GI ($p<0.05$) compared with low-GI group. There were no significant differences in the $AUC_{insulin}$ calculated for high-fat vs. high-GI fed rats.

The type of starch in the diet failed to influence glucose sensitivity in the liver or peripheral tissues under basal or clamp conditions. Rates of insulin-stimulated glucose turnover were similar in all three groups, which suggests diet did not significantly influence whole-body glucose utilization. High-fat feeding lowered the glucose infusion rate (GIR) required to maintain euglycemia by approximately 30% ($p<0.05$) compared with both the high- and low-GI diets. Insulin-mediated suppression of hepatic glucose output was also reduced by 70% ($p<0.05$) in high-fat compared with high- and low-GI groups.

Thus, it appears that despite higher postprandial glucose and insulin levels, the high-GI diet was not associated with changes in peripheral and hepatic glucose disposal. However, despite containing similar amounts of fat, high-GI fed rats demonstrated an exaggerated first-phase insulin response during the IVGTT compared with low-GI fed rats. One can speculate that insulin hypersecretion after only 7 weeks of high-GI feeding may alter the pattern of substrate utilization even in the absence of changes in insulin sensitivity. Furthermore, rats fed the high-GI diet remained glucose tolerant yet epididymal fat mass was increased, implicating high-GI carbohydrates in the pathogenesis of obesity (597).

To track the development of hyperinsulinemia and obesity in response to diet, female Sprague Dawley rats were fed either a high-fat sucrose (HFS; 39 en% fat, 40 en% sucrose) or a low-fat, complex carbohydrate (LFCC; 9 en% fat, 68 en% starch) diet and the influence of these diets on adiposity and insulin sensitivity assessed at 2 weeks, 2 months, and 2 years (598). After only two weeks of *ad libitum* consumption of the HFS diet, plasma insulin levels were significantly elevated ($p<0.05$) compared with animals

consuming the LFCC diet. HFS fed animals would maintain higher insulin levels ($p<0.05$) than LFCC animals after 2 months, 6 months, and 2 years on the diets, which demonstrates the influence of diet on insulin levels beyond the effects of aging.

Consistent with the hyperinsulinemia induced by only 2 weeks of HFS feeding, sarcolemmal vesicle insulin-stimulated glucose transport was reduced by approximately 16% ($p<0.05$) compared with the LFCC group. This trend continued, such that glucose transport remained 21-23% lower in HFS compared with LFCC fed animals at 2 months ($p<0.05$) and at 2 years ($p<0.05$). These differences cannot be attributed to differences in muscle protein, since protein yield in sarcolemma preparations were similar for both groups at each point in time.

Total body fat, expressed as percent body weight, was approximately 30% greater ($p<0.05$) following 6 months of HFS-feeding compared with the LFCC diet. Significant adipocyte hypertrophy was apparent at 2 months in HFS ($p<0.05$) rats and adipocytes continued to enlarge ($p<0.05$) compared with LFCC animals at 6 months.

This study further demonstrates the insidious nature of diet-induced hyperinsulinemia, which was present within 2 weeks in the HFS animals, and confirms its establishment prior to other aspects of the metabolic syndrome, including obesity. Furthermore, it seems that hyperinsulinemia and insulin resistance are not a consequence of obesity, but rather, can be attributed to consumption of a sucrose-rich diet (598).

To further examine the relationships between sucrose-induced insulin resistance and changes in lipid metabolism which may favor the development of obesity, female Sprague Dawley rats were fed the same diets (described above) for 6 months and observed for changes in adipocyte size and hormone-sensitive lipase (HSL) activity (599).

Consistent with previous findings, consumption of the HFS diet for only two weeks significantly elevated ($p<0.01$) plasma insulin levels compared with the LFCC diet. Mean adipocyte cell volume was not significantly different in rats consuming the HFS compared with LFCC diet after two weeks. However, at 2 months, adipocyte volume of the HFS rats was 83% ($p<0.01$) greater than in the LFCC group. Consequently, mean cell number per gram of tissue for LFCC rats was 75.3% ($p<0.01$) greater than in HFS rats. Adipocyte volume in HFS rats further increased beyond 2 months, reaching 149.8% ($p<0.01$) of LFCC rats at 6 months. Due to the increase in cell volume, there was a further reduction in mean cell number per gram of tissue in HFS animals, such that cell number per gram of tissue was only 40.87% ($p<0.01$) of that seen in LFCC-fed animals. Interestingly, adipocytes of LFCC rats did not increase in volume or decrease in number during the 6 month feeding period. These findings demonstrate that adipocyte hypertrophy is one consequence of a refined-sugar diet that can be prevented by the consumption of a low-fat, complex carbohydrate diet.

Plasma glycerol levels, which are indicative of in vivo lipolysis, were not significantly different at 2 weeks, however, by 2 months, glycerol levels in the HFS were 47.3% ($p<0.05$) greater than in the LFCC group. Thus, in vivo lipolysis was increased within 2 months by consumption of a diet known to induce hyperinsulinemia. Furthermore, plasma glycerol levels continued to increase in HFS animals, such that they were 91.4% ($p<0.01$) higher than in LFCC animals at 6 months.

Neither basal nor isoproterenol-stimulated HSL activity was significantly different between diets at 2 weeks. At 2 months, basal HSL activity was nearly two-fold higher in adipocytes from HFS ($p<0.01$) compared with LFCC animals. To determine if the

increase in basal lipolytic rate in HFS- fed animals was due to increased sympathetic nervous system activity, animals were injected with the β-adrenergic receptor blocker, propranolol, and the effect on basal glycerol release determined. In control cells, basal glycerol release was similar to that observed following administration of propranolol, demonstrating the effects of the HFS diet to increase basal lipolysis are not mediated by enhanced sympathetic activity. Isoproterenol-stimulated lipolysis in adipocytes from HFS animals at 2 months was 1.72-times ($p<0.01$) the rate of LFCC animals. This trend continued, resulting in basal HSL activity in adipocytes from HFS animals at 6 months being nearly three-fold greater ($p<0.01$) than in adipocytes from LFCC animals. Isoproterenol-stimulated HSL activity at 6 months in adipocytes from HFS fed animals was 1.71-times ($p<0.01$) that observed in LFCC animals. Fat cell size was positively correlated with both basal ($r^2=0.98$, $p<0.01$) and isoproterenol-stimulated ($r^2 = 0.88$, $p<0.01$) lipolysis. These findings of elevated lipolysis in enlarged adipocytes are consistent with studies previously performed in both rodents and humans (205,208) and demonstrates that feeding a diet rich in sucrose leads to increases in basal and isoproterenol-stimulated HSL activity and adipocyte hypertrophy in as little as 2 months (599).

To assess the response of adipose tissue lipoprotein lipase (LPL) activity to a refined-sugar diet, normal Sprague-Dawley rats were rendered overweight and insulin resistant by 4 weeks of ad libitum consumption of a high-fat, high-sucrose (HFS) diet and compared with control (chow-fed) insulin-sensitive animals (600). Despite similar initial body weights in both control and HFS animals, final body weight was modestly greater

($p<0.05$) in HFS than control animals. The inguinal, epididymal, and retroperitoneal fat pads were 50-70% larger ($p<0.05$) in HFS fed animals compared with controls.

Fasting plasma glucose levels were modestly (by 16%, $p<0.04$) higher in HFS-fed animals, while plasma insulin levels were 2-fold higher ($p<0.003$) in animals fed the HFS diet compared to control. During hyperinsulinemic clamp studies, the steady-state GIR needed to maintain euglycemia in HFS-fed animals was approximately 50% lower ($p<0.0001$) than in control animals.

Following a 24-hour fast, plasma glucose and insulin levels were similar in HFS and control animals. To determine the effects of diet on postprandial metabolism, both groups of animals were refed standard rodent chow. Between 3 and 6 hours after refeeding, plasma glucose levels were 3-fold higher ($p<0.001$) in HFS compared with control animals, despite similar plasma insulin levels in both groups. Hyperglycemia in the absence of elevations in plasma insulin levels suggest that the HFS diet impaired the normal insulin secretory response. Following a 24-hour fast, the activity of LPL in three adipose tissue depots (inguinal, retroperitoneal, and epididymal) was similar for control and HFS-fed animals. Refeeding increased LPL activity in all three fat depots ($p<0.0001$) in control animals, with peak activity occurring at 3 hours in the inguinal and epididymal depots and at 6-hours in the retroperitoneal fat pad. In contrast, only a slight increase (30% above fasting) in LPL activity was observed in HSF-fed animals following refeeding. Three hours following refeeding, LPL activity in all three depots was 2-fold higher ($p<0.003$) in control compared with HFS-fed animals, which coincided with the time when HFS-fed animals demonstrated significantly elevated ($p<0.05$) plasma

triglyceride levels compared with chow-fed controls, suggesting a link between LPL activity and the known hypertriglyceridemic effects of refined carbohydrates.

Activity of soleus muscle LPL was consistently lower in HFS- compared with control-fed animals. These differences reached significance ($p<0.05$) at 1 and 3 hours post-refeeding. In the vastus lateralis muscle, refeeding significantly increased ($p<0.05$) LPL activity only in control animals. As in the soleus muscle, LPL activity was consistently lower in vastus lateralis of HFS- compared to control-fed animals at all points in time, and was nearly 4-fold lower one hour following onset of refeeding. Thus, this study demonstrates that HFS feeding causes significant impairments in the postprandial LPL response in both adipose tissue and skeletal muscle. The HFS diet induced profound insulin resistance with respect to glucose uptake within 2 weeks. Refeeding fasted animals standard rodent chow results in elevations in plasma insulin levels, which should be sufficient to stimulate LPL activity in adipose tissue and simultaneously decrease its activity in skeletal muscle. However, this study clearly demonstrates that chronic consumption of a diet containing a refined carbohydrate (sucrose) rendered LPL in adipose tissue refractory to the influence of insulin. Nonetheless, insulin resistant HSF-fed animals appeared to maintain the ability to decrease muscle LPL activity in response to elevations in plasma insulin levels resulting from refeeding. However, the activity of skeletal muscle LPL was blunted in HFS compared with control-fed animals, which may have contributed to the higher postprandial triglyceride levels in these animals (600).

To determine if the deleterious effects of HFS feeding could be overcome by dietary change, female Sprague Dawley rats were fed the HFS and LFCC diets for 22 months,

with a subgroup of animals consuming the HFS diet switched to the LFCC diet (HFS/LFCC) at 20 months for the remainder of the study (2 months) (601). There were no differences in energy intake between the HFS and LFCC groups during the first 20 months. Nonetheless, energy efficiency was 46% lower ($p<0.01$) in the LFCC compared with the HFS animals. However, the difference in energy intake between the LFCC and HFS groups accounted for only 2.5% of the 123 g difference in body weight between the groups. After 22 months, body weight of HFS-fed animals was 44% ($p<0.01$) greater than for animals consuming the LFCC diet. At 20 months, a subgroup of HFS-fed animals was switched to the LFCC for 2 months, which resulted in a 16% ($p<0.05$) reduction in body weight.

Heparin-releasable LPL activity in abdominal adipose tissue was 50% ($p<0.05$) greater in HFS compared with LFCC animals. Conversely, heparin-releasable LPL activity in the plantaris muscle was reduced by 58% ($p<0.05$) in the HFS compared with the LFCC group. A 33% reduction ($p<0.05$) in LPL activity was observed in the soleus muscle of HFS- compared with LFCC-fed animals.

In the abdominal depot, abundance of LPL protein was similar in both groups, indicating the elevation in LPL activity of HFS-fed animals is not merely a by-product of adipocyte hypertrophy. However, gastrocnemius muscle LPL protein levels were significantly reduced ($p<0.01$) by the HFS diet and was restored ($p<0.02$) in animals switched to the LFCC diet for 2 months.

In summary, chronic consumption of the HFS diet induced reciprocal changes in adipose tissue and skeletal muscle LPL activity. Enhanced LPL activity in adipose tissue occurring concomitantly with attenuated activity in skeletal muscle established a

metabolic environment favoring obesity. However, conversion from the HFS to the LFCC diet for only two months induced significant improvements in body weight, plasma lipids, and LPL activity, suggesting that dietary manipulation should constitute a first-line defense in the treatment or prevention of obesity (601).

In addition to the influence of dietary carbohydrate *per se* on whole body lipid metabolism mediated via hormone-sensitive lipase and lipoprotein lipase, Kabir, et al. (602) have demonstrated an influence on fatty acid synthase, a key enzyme of de novo lipogenesis. Rats that chronically consumed a diet high in waxy cornstarch (0.5% amylose) exhibited significantly elevated rates of basal and insulin-stimulated glucose metabolism in adipocytes compared with rats that consumed a diet rich in mung bean starch (32% amylose). Consequently, rats that consumed the diet rich in waxy cornstarch had adipocytes of larger diameter compared with those that consumed mung bean starch. Although insulin responses did not differ significantly between the diets, animals consuming mung bean starch had lower postprandial insulin peaks than did rats consuming waxy corn starch. Persistently low insulin levels may partially explain differences in adipocyte size observed in this study. Kabir, et al. (603) further determined that varying the glycemic index of dietary carbohydrates appears to stimulate adipocyte *de novo* lipogenesis specifically. Chronic consumption of waxy corn starch significantly increased adipocyte fatty acid synthase in both normal and streptozotocin-induced diabetic rats compared to rats fed a low glycemic index starch diet. The high glycemic index diet was also associated with increased adipocyte size and reductions in insulin sensitivity, whereas the low glycemic index diet resulted in reduced activity of

fatty acid synthase and corresponding decreases in adipocyte diameter, as well as improved insulin sensitivity.

Similarly, Byrnes, et al. (604) demonstrated lower insulin sensitivity in rats fed high amylopectin (waxy cornstarch, 0% amylose) compared to high amylose cornstarch (61% amylose) diets. After 1 week of feeding, animals consuming the high amylopectin diet gained 25% ($p<0.01$) more weight than animals consuming the high amylose diet. This disparity was no longer apparent after two weeks of feeding and by the end of the 9 week feeding period, weight was similar for both groups. However, rats fed the high-amylose diet demonstrated faster clearance of a glucose load compared to high-amylopectin fed rats ($p<0.001$). Furthermore, the rate of glucose disappearance after 10 minutes was significantly lower in rats consuming the high amylopectin diet compared to those consuming the high-amylose diet ($p<0.05$). During the first 15 minutes of an intravenous glucose infusion, plasma insulin levels were significantly higher in rats fed the high-amylopectin diet compared to the amylose-fed animals ($p<0.05$). Although this discrepancy was no longer observed beyond 15 minutes, the peak insulin response in rats fed the high-amylopectin diet was almost twice ($p<0.05$) that of rats fed the high-amylose diet. These findings suggest that chronic consumption of a high glycemic index diet by rats promotes insulin resistance as demonstrated by slower clearance of infused glucose and hypersecretion of insulin.

In a study similar to those of Kabir, et al. (602,603), Lerer-Metzger, et al. (605) the metabolic effects of chronic ingestion of high (wheat) and low (mung bean) glycemic index starches on glycemic control, lipid metabolism and adiposity, were compared in normal and diabetic rats fed diets composed of a single carbohydrate source for five

weeks. Body weight was comparable for both diets at the end of 5 weeks. Plasma glucose levels were 26% lower ($p<0.05$) in animals fed the mung bean diet compared with the wheat diet, regardless of diabetic status. Plasma insulin levels were ~32% higher ($p<0.05$) in diabetic animals fed the wheat versus the mung bean starch diet. In nondiabetic animals there was a nonsignificant trend for higher insulin levels with the wheat compared with the mung bean diet. Plasma triglyceride and free fatty acid levels were 17 and 45% higher ($p<0.05$ for both), respectively, in wheat fed animals compared with those consuming the mung bean diet.

Adipocytes isolated from animals fed the mung bean starch diet were 28% smaller ($p<0.01$) than adipocytes from animals fed the wheat starch diet. Dietary carbohydrate source influenced both size and number of adipocytes in normal rats. Consistent with adipocyte hypertrophy induced by the wheat starch diet, adipocyte number was reduced by 21% ($p<0.05$) compared with animals fed the mung bean diet.

Improved Lipoprotein Metabolism by Low-GI Foods in Hyperlipidemic Subjects

Obesity is believed to increase the risk of cardiovascular disease via its influence on lipid metabolism. Jenkins, et al. (606) designed a three-month study to determine if the plasma lipid profile of hyperlipidemic subjects could be improved by reducing the overall glycemic index of the diet by 20% by manipulation of dietary carbohydrate source, without altering the overall macronutrient composition of the diet. Subjects reduced the mean glycemic index of their diet by approximately 13 units ($p<0.01$ vs. usual diet) by replacing high-glycemic index foods (wheat bread, potatoes, cold breakfast cereals) with low-glycemic index foods (rye bread, oat bran, beans, spaghetti) and consequently,

approximately 24% of total dietary energy was derived from low glycemic index foods and a small but significant reduction (by 8%, $p<0.01$) in total energy intake. Compared to baseline levels, the low glycemic index diet reduced serum and total and LDL-cholesterol by approximately 10% ($p<0.005$), while serum triglycerides fell ~16% ($p<0.001$). The reduction in dietary glycemic index was significantly related to the decrements in both total ($r=0.634$, $p<0.05$) and LDL-cholesterol ($r=0.794$, $p<0.001$).

Fiber intake during the low glycemic index period was inversely ($r=-0.583$, $p<0.05$) associated with HDL-cholesterol. Interestingly, the low glycemic index diet was associated with a decrease in the ratio of polyunsaturated to saturated (P/S ratio) fat intake compared to the control periods yet significant relationships were noted for P/S ratio and total ($r=-0.659$, $p<0.05$) and LDL-cholesterol ($r=-0.644$, $p<0.05$) during the low glycemic index phase. When considering the mean level of dietary macronutrient intake throughout the entire three month period, as opposed to small differences between the low glycemic index and control phases, the lipid lowering effects were most dramatic in subjects whose diet tended to have a higher P/S ratio and higher dietary fiber content. In this context, the most significant association was found between the fall in serum triglycerides and the mean (3-month) dietary fiber content of the diet ($r=0.803$, $p<0.01$). In addition, the fall in LDL ($r=0.616$, $p<0.05$) and HDL-cholesterol ($r=0.617$, $p<0.05$) and the overall P/S ratio of the diet were significant. A small but significant reduction in body weight of 0.4 kg ($p<0.05$) occurred between the fourth week of the pre-test period and the fourth week of the low glycemic index phase; however, this reduction in body weight was not significantly associated with the improvements in lipid profile seen during the low glycemic index phase (606).

Comparison of a Low-GI Diet with the AHA Step I Diet

It has been suggested that conventional dietary recommendations, which promote the consumption of a low-fat, high-carbohydrate diet may have had the unintended consequence of increasing intake of simple sugars and refined starches, which are known to adversely influence risk of developing chronic diseases including obesity, non-insulin dependent diabetes mellitus, and cardiovascular disease. The American Heart Association (AHA) step 1 diet is a reduced fat (30 en%) diet, which emphasizes fat reduction over carbohydrate selection (607). A study of abdominally obese (WC: 117.4±8.4 cm) subjects compared the short-term (6 days) effects of an *ad libitum* low-glycemic index (low-fat, high-protein) diet, in which carbohydrates with a glycemic index greater than 55 were excluded, with the American Heart Association phase I diet consumed *ad libitum* (608). A pair-fed group was included in which the energy content of the diet was matched to the low-glycemic index group but with macronutrient composition of the AHA step 1 diet.

During the *ad libitum* phase of the study, energy intake of the low-glycemic index diet group was spontaneously reduced by 25% ($p<0.05$) compared with subjects consuming the AHA diet. Although the low glycemic index diet was less energy ($p<0.05$) dense than the AHA diet, the differences in energy intake between the two groups remained significant after adjustment for energy density. In the short-term, *ad libitum* consumption of the AHA diet had no influence on body weight, however, significant decreases in several anthropometric indices were noted following the consumption of the low-glycemic index diet *ad libitum* for the same length of time. Weight loss on the low GI diet ($p<0.001$) was accompanied by reductions in both waist girth ($p<0.001$) and hip

circumference ($p<0.001$). Similar reductions in body weight ($p<0.001$ vs. AHA) and waist circumference ($p<0.02$ vs. AHA) were observed during the pair-feeding phase. While these changes are significant, it is important to note that initial changes in body weight in response to a change in diet do not necessarily reflect loss of body fat, nor are they predictive of long-term changes in body composition.

Dietary regimen had no effect on plasma cholesterol levels, however, the consumption of the AHA phase I diet ad libitum resulted in a 28% increase in fasting triacylglycerol levels ($p<0.05$) and a 10% reduction in HDL-cholesterol levels ($p<0.01$). Consequently, the AHA diet, either *ad libitum* or with energy restriction, was associated with a significant increase in the cholesterol:HDL ratio ($p<0.05$), an accepted index of cardiovascular disease risk. In contrast, *ad libitum* consumption of the low-glycemic index diet was associated with a 35% reduction in plasma triacylglycerol levels ($p<0.0005$), while plasma HDL-cholesterol levels and the cholesterol:HDL ratio were unchanged.

The AHA step 1 diet, either in its *ad libitum* or hypoenergetic form, failed to influence fasting plasma insulin and apolipoprotein B levels, nor was LDL particle diameter affected. In contrast, the *ad libitum* low glycemic index diet resulted in significant reductions in fasting plasma insulin levels ($p<0.05$ versus baseline) and increased LDL particle size ($p<0.05$). Postprandial glucose excursions were also reduced by the *ad libitum* low glycemic index diet compared with either AHA diet. Fasting plasma insulin levels were significantly lower ($p<0.05$) in subjects consuming the low-glycemic index diet compared with the AHA diet after 6 days. Furthermore, the insulin response to an oral glucose load was reduced by the low glycemic index diet, yet remained unchanged

with either AHA diet regimen. In summary, the benefits of a low-glycemic index diet are rapid and dramatic, and include a spontaneous reduction in energy intake, reduced fasting plasma insulin levels, and a less atherogenic lipid profile, characterized by an increase in LDL particle size, in abdominally obese subjects. These findings indicate a significant reduction in spontaneous energy intake and improvements in the metabolic risk profile of abdominally obese subjects following consumption of a low glycemic index diet for only seven days. Based on these results, dietary advice given to subjects at risk for chronic disease must be carefully evaluated.

Acute Effects of Dietary Carbohydrate on Insulin Sensitivity

To establish whether individual insulin sensitive responses are acutely affected by major alterations in dietary carbohydrate source, a crossover study was conducted in which subjects consumed a high-sucrose (50 en% sucrose) or high-starch (50 en% starch) diet as four meals over a 24–hour period (609). Rates of glucose disappearance determined during an insulin tolerance test were similar for both meals, suggesting no alterations in insulin-stimulated glucose uptake. Similarly, insulin-mediated suppression of plasma NEFA levels were comparable for both meals. Nonetheless, the brevity of this study does not permit speculation regarding the long term effects of dietary manipulation of this sort.

Despite the apparent failure of dietary manipulation to influence insulin sensitivity in fasting subjects in the short-term, several parameters of postprandial metabolism were responsive to alterations in dietary carbohydrate source. Following the high-sucrose diet, blood glucose levels rose more rapidly and to higher peaks ($p<0.05$) after each meal

compared with the high-starch diet. After achieving peak postprandial levels, blood glucose levels fell more rapidly ($p<0.05$) with the high-sucrose diet and reactive hypoglycemia was observed after breakfast and lunch in sucrose-fed subjects. In contrast, postprandial blood glucose levels remained above fasting levels at all times with high-starch feeding.

Both diets suppressed postprandial NEFA levels, while causing a gradual rise in triacylglycerol levels after breakfast, with a further increase in the hour immediately following the noon meal. Subsequently, the similarities in the postprandial triacylglycerol response are lost. Following the noon high-starch meal, plasma triacylglycerol levels gradually declined towards fasting levels. In contrast, plasma triglycerides continued to rise following the noon high-sucrose meal, and peak levels were delayed until following the afternoon meal, before beginning a gradual decline. Nonetheless, an additional peak in triglyceride levels occurred two hours following the evening sucrose meal, and consequently baseline levels were achieved only following an overnight fast. Although blood lactate and pyruvate concentrations peaked approximately one hour after each meal in both diet groups, postprandial levels were as much as three-fold higher ($p<0.05$) with the high-sucrose diet than with the high-starch diet (609).

Alterations in Insulin Action Resulting from Chronic Changes in Dietary Carbohydrate Source

The influence of dietary carbohydrate source on insulin action and muscle substrate utilization was investigated by feeding healthy men isoenergetic diets containing 46 en% carbohydrate, as either low or high glycemic index carbohydrates, for 30 days (610). Fasting glucose and insulin levels were unchanged from baseline following thirty days of

consuming either the low or high glycemic index diets. Fasting plasma fatty acids rose by 52% from baseline ($p<0.05$) after three days of consuming the low glycemic index diet and remained elevated above baseline in these subjects for three weeks. However, at the conclusion of the study, there were no differences in plasma fatty acid levels between low and high glycemic index diet groups. After the lunch meal, serum insulin levels in the low glycemic index diet group on day three were significantly lower ($p<0.05$) than those encountered with the high GI diet.

Following thirty days of consuming the low glycemic index diet, there was a significant decrease (~13%, $p<0.05$) in muscle glycogen concentration compared to baseline. In contrast, muscle glycogen concentration remained constant with the high glycemic index diet, but was significantly greater ($p<0.05$) than in subjects consuming the low glycemic index diet.

Metabolic Effects of a Low-GI Diet in Normal Weight and Obese Subjects

While the exact mechanism by which dietary glycemic load influences energy metabolism is unknown, it has been suggested that the high insulin levels associated with consumption of high-glycemic index foods may favor deposition of fuels for storage as opposed to promoting their oxidation.

Bisschop, et al. (611) evaluated the dose response effects of isocaloric variations in dietary carbohydrate and fat content on postabsorptive free fatty acid release and fat oxidation in healthy subjects. Subjects consumed a sequence of three diets for seven days each containing 15 en% protein but differing in carbohydrate and fat content. The control diet provided 44 en% carbohydrate and 41 en% fat. The high-carbohydrate diet

provided 85 en% as carbohydrate, whereas the high fat diet provided 83 en% as fat and 2 en% carbohydrate. Fasting insulin levels were 39% lower ($p<0.01$) after the high-fat compared with the high-carbohydrate and control diet periods. Lower insulin levels in the high-fat group may have influenced the appearance rate for palmitate which was 66% greater ($p<0.01$) than the high-carbohydrate and control diets. Consequently, plasma free fatty acid levels, which were similar between the high-carbohydrate and control diets, were 67% greater after the high-fat diet ($p<0.01$).

Similar respiratory quotients (RQ) were observed after the high-carbohydrate and control diets, but RQ was slightly but significantly lower (10%, $p<0.05$) after the high-fat diet. Whole-body fat oxidation increased 47% ($p<0.01$) with the high-fat diet compared with both the high-carbohydrate and control diets. Because the diets used in this study were isoenergetic and isonitrogenous, the observed effects are solely attributable to the variations in dietary carbohydrate and fat. These findings in conjunction with results from Poppitt, et al. (612) who demonstrated that chronic consumption of a high-fat diet is associated with increased plasma fatty acid levels during hyperinsulinemic euglycemic clamps, suggest that high-fat feeding reduces plasma insulin levels and the reduces the ability of insulin to suppress free fatty acid release (611).

Metabolic Benefits of Diets Rich in Whole Grain Foods

To examine the potential benefits of a diet rich in whole-grain rather than refined-grain foods on insulin sensitivity, independent of changes in body weight, overweight hyperinsulinemic adults were used in a randomized, crossover controlled feeding trial consisting of two 6 week periods (613). The habitual diets of the subjects prior to

beginning the study indicated relatively low median intake of fruit (1.0 serving/day), vegetables (2.2 servings/d), carbohydrate (40 en%/d), and high intake of total fat (40 en%/d) and saturated fat (40% of fat).

Body weight was not significantly different between the whole-grain and refined-grain periods. There was a nonsignificant trend for lower blood glucose levels following the whole grain period. Fasting insulin levels were modest (10%) but significantly ($p<0.05$) lower following the whole-grain versus the refined-grain period.

The homeostasis model for insulin resistance was computed using fasting insulin and glucose levels as a means for estimating insulin sensitivity (12). Based on this model, insulin sensitivity was significantly improved ($p<0.01$) by consumption of whole-grains compared with refined sugars consumed for the same duration. A greater rate of glucose infusion ($p<0.01$) was required to maintain euglycemia under hyperinsulinemic conditions following the whole-grain compared with the refined grain diet, suggesting a diet rich in whole grains improves insulin sensitivity. This study suggests that a whole grain diet can improve insulin sensitivity independently of changes in body weight in obese, hyperinsulinemic adults.

Schwarz, et al. (614) examined the influence of diet on hepatic *de novo* lipogenesis (DNL) was compared in weight-stable subjects consuming a balanced, low-fat, high (68 en%) carbohydrate (46% complex carbohydrates, 54% simple sugars) compared with a standard high-fat (40 en%) diet for five days. *De novo* lipogenesis was minimal in lean and obese, normoinsulinemic subjects consuming the high-fat, low-carbohydrate diet for five days, whereas a significantly higher rate of *de novo* lipogenesis ($p<0.05$) was observed in obese hyperinsulinemic subjects following the same diet protocol. DNL was similar

in lean and obese normoinsulinemic subjects after consuming the low-fat, high-carbohydrate diet for five days. However, DNL was significantly higher ($p<0.05$) in obese hyperinsulinemic subjects compared with weight-matched normoinsulinemic subjects consuming the same diet. Thus, obese hyperinsulinemic subjects demonstrate higher rates of de novo lipogenesis following consumption of high-fat and low-fat diets, and this appears to be an insulin-mediated effect since elevated DNL is not observed in obese subjects with normal plasma insulin levels.

These findings are also consistent with the proposed benefits of reduced glycemic load since hepatic DNL was minimal on the high fat diet and was significantly enhanced by a low-fat diet containing simple sugars (614).

Efficacy of a Low-GI Diet in the Prevention or Treatment of Obesity

To evaluate the extent to which dietary glycemic index can modulate body weight, Bouche, et al. (615) assigned healthy subjects to consume either a high- or low-glycemic index for five weeks. During the low-glycemic index (LGI) period, carbohydrates with a GI of less than 45 were recommended, whereas foods with a GI greater than 60 were recommended during the high-GI (HGI) period. Total daily energy intake and macronutrient composition was similar at the end of the two dietary periods.

The 24-hour plasma glucose profile was compared for an effect of diet. The incremental AUCs for plasma glucose after the LGI breakfast and lunch were consistently lower (by ~61%, $p<0.001$) than those observed after the HGI meals. Furthermore, reactive hypoglycemia was observed four hours following consumption of the HGI breakfast. Postprandial insulin levels were consistently lower following the

LGI than after the HGI diet. Peak morning insulin levels and morning insulin incremental AUCs were approximately 41 and 48% lower, respectively ($p<0.01$ for both), for the LGI versus the HGI diet. Likewise, afternoon insulin peaks and the afternoon insulin incremental AUC were ~18 and 31% lower ($p<0.001$), respectively, following the LGI.

Five weeks of the LGI diet induced a significant reduction in total fat mass (700 - 1000 g) and consequently, the change in adiposity (compared with baseline) was significantly greater in the LGI group compared with the HGI group ($p<0.05$). The greatest reduction in adiposity occurred in the truncal region, accounting for at least 500 grams of total fat loss in all subjects consuming the LGI diet. Moreover, these was a tendency for subjects consuming the low glycemic index diet to increase total lean mass ($p = 0.07$), an effect which was notably absent in subjects consuming the HGI diet. Thus, the failure of the LGI diet to reduce body weight appears to be the result of compensatory changes in fat and lean tissue mass.

Expression of genes involved in lipid metabolism and adiposity was examined in abdominal subcutaneous adipose tissue. Compared with the level of expression prior to dietary intervention, consumption of the LGI diet reduced expression of *ob* and LPL by 36 and 38% ($p<0.05$), respectively. In contrast, the HGI diet increased LPL expression by 48% ($p<0.05$ vs. baseline) and was accompanied by trend for increased *ob* expression (12% above baseline, $p=0.07$).

This study demonstrates that the simple substitution of low glycemic index carbohydrates for those with a high glycemic response for five weeks specifically reduces abdominal adiposity, reduces the expression of lipid related genes in the abdominal

depot and improves daily postprandial blood glucose and insulin responses. Although the decrements in plasma glucose and insulin levels were not associated with any changes in whole-body glucose metabolism or insulin sensitivity it is conceivable that maintaining a reduction in total fat mass with the associated increase in lean body mass as observed with the LGI diet, would contribute to improved glucose homeostasis and insulin action over the long-term (615).

In a similar study, Slabber, et al. (616) compared two conventional, fat-reduced diets of identical macronutrient distribution, but which differed in the type of dietary carbohydrate. The low insulin response diet contained only carbohydrates known to evoke a low insulin response, such as lentils, pasta, rolled oats, corn, and long grain rice. In contrast, the control diet contained potatoes, white bread, and processed carbohydrates; all of which are known to result in higher postprandial glucose and insulin responses. In the 12-week parallel study, both diets resulted in similar reductions in body weight. Fasting glucose levels were not influenced by diet. Both diets reduced fasting insulin levels ($p<0.01$) compared with pre-study levels, however, the decrement induced by the low-insulin response diet was 3-fold greater than that caused by the control diet. There were no significant effects of diet on the 30- and 120-minute glucose-stimulated insulin concentration. At the end of the 12 week intervention, the control diet significantly increased fasting c-peptide levels, resulting in a reduction in the insulin:c-peptide ratio. The low-insulin response diet failed to influence c-peptide levels, but the insulin:c-peptide ratio was reduced nonetheless, suggesting the low-insulin response diet improved hepatic insulin extraction without affecting insulin secretion.

Following the parallel study, each group was crossed over to the other diet for an addition 12 weeks. In subjects crossed over to the low insulin response diet, fasting insulin levels were significantly reduced ($p<0.05$ vs. pre-crossover), whereas a non-significant increase in plasma insulin level was observed in subjects crossed-over to the conventional diet. Changing from the control to the low-insulin response diet also decreased ($p<0.05$) insulin levels 120 minutes after meal consumption, whereas no such effect was observed in subjects crossed over to the control diet, despite the similar energy content of the diets and the equivalent reduction in body weight induced by the diets. These findings suggest that while weight loss in response to energy restriction is similar between the two diets, the low insulin response diet resulted in a more substantial improvement in the postprandial insulin response in hyperinsulinemic, obese women. Furthermore, the deterioration of the insulin:c-peptide ratio in subjects consuming the conventional diet, implicates refined sugars and processed carbohydrates specifically in the pathogenesis of islet secretory dysfunction in this population (616).

Lowering Glycemic Load by Manipulation of Dietary Carbohydrate-to-Fat Ratio

The modest success of dietary recommendations such as the AHA step 1 diet led investigators to examine other dietary manipulations. The CARMEN (Carbohydrate Ratio Management in European National diets) Study was a multi-center randomized *ad libitum* feeding trial, which examined the effects of alterations in the ratio of fat to carbohydrate, as well as the ratio of simple to complex carbohydrates *per se* on body composition and lipid profile in overweight and moderately obese adults (BMI: 26-35 kg/m^2) (617).

Subjects were randomly assigned to control or one of two intervention groups for 6 months. Subjects in the low-fat, high-complex carbohydrate group were to reduce fat intake by 10 en%, while reducing the ratio of simple to complex carbohydrates from 1.0 to 0.5. Subjects in the low-fat, high-simple carbohydrate group also reduced fat intake by 10en% were to make the same changes in dietary fat intake but were to increase the ratio of simple to complex carbohydrates from 1.0 to 1.5.

At the conclusion of the study, energy intake was significantly lower ($p<0.05$) in the low-fat, high-complex carbohydrate group compared with both the low-fat, high-simple carbohydrate and control diets. While energy density was significantly decreased from baseline in both the low-fat, simple- and low-fat, complex-carbohydrate groups compared with control ($p<0.001$), the decrease was significantly greater ($p<0.001$) in the low-fat, complex carbohydrate group compared with the low-fat, simple-carbohydrate diet.

The low-fat diets, without regard to carbohydrate type, produced similar reductions ($p<0.0001$) in body weight and total fat mass. In contrast, modest but significant increases ($p<0.001$) in both body weight and fat mass were observed in the control group. This study confirms the benefits of a fat-reduced diet with respect to body weight regulation but more importantly, is the finding that dietary carbohydrate source *per se* significantly influences body weight and adiposity. Although the low-fat, simple-carbohydrate diet reduced body weight and fat mass relative to the moderate fat control diet, ad libitum consumption of a low-fat, high-complex carbohydrate diet induced a produced more significant reductions in body weight and adiposity than the low-fat, simple-carbohydrate diet (617).

The mechanism by which a low-fat, high-carbohydrate diet reduces body weight remains unclear. In some cases, weight loss occurring with the long-term consumption of a reduced fat diet is due to a reduction in energy intake. Alternatively, a high-carbohydrate diet may increase diet-induced thermogenesis compared with a high-fat diet, suggesting the resulting weight loss is due in part to an increase in energy expenditure. As a follow-up to the CARMEN study and to clarify the mechanism by which improvements in body weight and adiposity were induced by a low-fat, high-complex carbohydrate diet, Vasilaras, et al. (618) examined 24-hour energy expenditure (EE) and substrate oxidation in overweight and obese subjects prior to and following 6 months ad libitum intake of a low-fat diet rich in either simple or complex carbohydrates. For comparison purposes, a control diet with macronutrient composition corresponding to the typical European diet was included.

During the intervention period, average *ad libitum* energy intake was similar for the three diet groups and there were no differences in energy expenditure or basal metabolic rate attributable to diet. However, diet intervention was associated with alterations in the pattern of substrate oxidation. Carbohydrate oxidation was significantly higher in subjects consuming the high-complex carbohydrate diet compared with the control diet. Fat oxidation was significantly higher ($p = 0.032$) in the control group compared with the complex carbohydrate group, with an intermediate rate of fat oxidation observed in the simple carbohydrate group. Compared with baseline, fat oxidation decreased by 17 and 25% in the simple and complex carbohydrate groups, respectively, while the control diet increased fat oxidation by 3%. Differences in substrate utilization between diets are reflected in body weight. The complex carbohydrate diet reduced body weight by 3%,

while a comparable increase in body weight was observed in the control and simple carbohydrate groups.

Efficacy of a Low-GI Diet in the Treatment of Obesity in Adolescents and Children

In a study of obese (> 120% IBW) adolescents, Ludwig, et al. (619) report differential effects of mixed meals varying in both amount and type of carbohydrate. This was a crossover study consisting of three separate 24-hour admission periods separated by a 1- to 2-week wash-out period. A different test meal (low-, medium- or high-GI) was given at each admission. Subjects were admitted at 6:00 pm, consumed a low-GI dinner and bedtime snack prior to sleeping. The next morning, subjects completed a hunger scale rating. An isocaloric low-, medium-, or high-GI test meal was given for breakfast and consumed completely within 20 minutes. The high-GI (instant oatmeal) and medium-GI (rolled oats) breakfast meals were isoenergetic and macronutrient distribution was identical. In the third breakfast (low-GI meal) a vegetable omelet was served, thereby, partially replacing carbohydrate with protein and fat.

The mean area under the glycemic response curve for the high-GI meal was twice that of the medium-GI meal ($p<0.001$) and nearly four-fold higher ($p<0.001$) than the low-GI meal. The mean plasma glucose concentration nadir fell below baseline and consequently was lower after the high-GI meal than after the medium- and low-GI meals ($p=0.02$ for both). The area under the insulin response curve after the high-GI meal was 56 and 115% higher ($p<0.01$ for both) than after the medium-GI and low-GI meals, respectively. Plasma glucagon levels, which rose after the low-GI meal, were suppressed

after both the medium- and high-GI meals, an effect likely mediated by the protein content of the meals and the inhibitory effects of hyperglycemia on glucagon secretion.

The combination of increased insulin levels and suppressed glucagon levels should favor glucose uptake in muscle and liver while restraining hepatic glucose production and suppressing adipose tissue lipolysis. Indeed, serum free fatty acids from 2.5 to 4.5 hours after meal ingestion were suppressed to a greater degree following the high-GI compared with the low-GI meal ($p<0.05$).

Effects of a Low-GI Diet in Diabetic Subjects

Obesity, insulin resistance, hypertension, hypertriglyceridemia, and low HDL levels are hallmarks of non-insulin dependent diabetes mellitus (NIDDM). The current dietary prescription for subjects with NIDDM advises a diet containing less than 30 en% total fat (10 en% saturated fat, up to 10 en% polyunsaturated fat, at least 10 en% monounsaturated fats), 15-20 en% protein, with the remainder of energy needs (55-60 en%) provided by carbohydrate (620). However, many studies suggest this macronutrient composition actually accentuates the hypertriglyceridemia associated with NIDDM. Furthermore, because weight loss is known to improve insulin sensitivity, blood pressure and plasma lipid profile in obese subjects, researchers have begun investigating the potential benefits of an energy-restricted, low-glycemic index, high-carbohydrate in subjects with NIDDM.

Increasing the carbohydrate content of the diet often results in an increase in dietary fiber, making it difficult to discriminate between the influence of these two dietary components on metabolism. There is substantial evidence supporting the benefits of

dietary fiber *per se* on glycemic control and lipid metabolism, yet fewer studies exist which clearly demonstrate improvements in these metabolic variables by dietary carbohydrate, independently of dietary fiber (621-623).

To more clearly elucidate the separate influences of dietary carbohydrate and fiber on glycemic control and lipid profile, diabetic subjects (Type 1 and Type 2) consumed three weight-maintaining (2200 kcal/d) diets for 10 days each: low-carbohydrate (42 en%), low-fiber (20 g/d); high-carbohydrate (53 en%), low-fiber (16 g/d); high-carbohydrate (53 en%), high-fiber (53 g/d) (623). There were no significant changes in fasting blood glucose levels during any of the dietary periods. The 2-hour post-prandial blood glucose level were similar after the high-carbohydrate/low-fiber and the low-carbohydrate/low-fiber diets, yet both were significantly higher than after the high-carbohydrate/high-fiber ($p<0.01$) diet. Variations in blood glucose levels observed when changing from the low-carbohydrate/low-fiber diet to either the high-carbohydrate/low-fiber or high-carbohydrate/high-fiber diet were evaluated to determine whether the two high-carbohydrate diets, with different amounts of vegetable fiber, differentially influenced blood glucose levels. In the end, neither type of diabetes nor diet significantly influenced fasting blood glucose levels.

In subjects with type 1 diabetes, a greater reduction in 2-hour post-prandial blood glucose level was observed after the high-carbohydrate/high-fiber than following the high-carbohydrate/low-fiber diet. In subjects with NIDDM, there was a modest increase in the 2-hour post-prandial blood glucose concentration following the high-carbohydrate/low-fiber diet, whereas the high-carbohydrate/high-fiber diet was associated with a comparable decrease in postprandial glycemia. Dietary treatment

exerted a significant ($p<0.02$) influence on 2-hour glucose levels, independent of type of diabetes. Thus, increasing the content of digestible carbohydrate in the diet (up to 53 en%) without a coincident increase in dietary fiber intake, does not substantially improve glycemic control in diabetic subjects. Conversely, increasing both carbohydrate and fiber intake favorably influences post-prandial glucose metabolism in subjects with type 2 diabetes.

LDL levels were reduced to a greater extent ($p<0.001$) following consumption of the high-carbohydrate/high-fiber diet than after the low-carbohydrate/low-fiber diet or the high-carbohydrate/low-fiber diet in both groups of subjects. Total cholesterol levels followed a similar pattern with the lowest levels occurring with the high-carbohydrate/high-fiber diet compared with the low-carbohydrate/low-fiber diet ($p<0.001$) and the high-carbohydrate/low-fiber diet ($p<0.01$).

While diet had no effect on total serum triglyceride levels, VLDL levels were significantly reduced after the high-carbohydrate/high-fiber diet ($p<0.05$) compared with the high-carbohydrate/low-fiber diet. Thus, in addition to improving glycemic control, a concomitant increase in dietary carbohydrate and fiber intake induces a less-atherogenic lipid profile in diabetic subjects (624).

A long-term study of increased intake of fiber-rich, low-glycemic index natural foods on glycemic control has been conducted in type 1 diabetic subjects (625). Subjects were assigned to consume a weight-maintaining high- (HF) or low-fiber (LF) diet of similar macronutrient composition (20 en% protein, 30 en% fat, 50 en% carbohydrate), but containing different amounts of soluble fiber from fresh foods (HF: 50 g/d, LF: 15 g/d) for 24 weeks.

Compared with the LF diet, chronic consumption of the HF diet reduced the mean daily blood glucose concentration by 24% ($p<0.001$). In addition, subjects consuming the HF diet reported a reduction in the number of hypoglycemic episodes per month ($p<0.01$) compared with the LF diet. Thus, in type 1 diabetic subjects, chronically increasing consumption of fresh foods rich in dietary fiber and with a low glycemic index favorably influences blood glucose control and reduces the frequency of hypoglycemic events. Importantly, these effects occurred in the absence of any significant effect of diet on body weight or required daily insulin dosage, which were similar in both groups at the conclusion of the study (625).

The beneficial effects of a low-glycemic index diet are not universally accepted. Hellibron, et al. (626) sought to determine the influence of glycemic index on glycemic control and lipid profile, in overweight, type 2 diabetic subjects managing their condition through diet. After consuming an energy-restricted (1500 kcal/d), high-saturated fat (SFA; 17 en% saturated fat, 50 en% carbohydrate) diet for four weeks, subjects were randomly assigned to either a high- (75 GI units) or low- (43 GI units) GI, energy-restricted (1500 kcal/d) diet (60 en% carbohydrate) for eight weeks.

All subjects lost a modest amount of weight during the first four weeks of energy restriction, despite consuming a diet relatively high in saturated fat. All subjects experienced significant reductions in mid-upper arm, hip and waist circumference, as well as subscapular and suprailiac skin-fold measurements following 12 weeks of energy restriction. Despite similar levels of energy intake, subjects with low glucose tolerance lost 34% ($p<0.01$) less weight than subjects with a greater degree of glucose tolerance. Consistent with the influence of glucose tolerance on overall body weight, more modest

reductions in hip ($p<0.01$) and waist ($p<0.01$) circumferences, as well as subscapular skin-fold thickness ($p = 0.01$) were observed in subjects with low glucose tolerance.

During the first four weeks of energy restriction, subjects experienced significant improvements in glycemia, as evidenced by a 6% reduction ($p=0.008$) in fasting blood glucose levels and a 13% reduction ($p = 0.001$) in $AUC_{glucose}$. Triglyceride levels were reduced by 14% ($p=0.007$) and total cholesterol levels fell by 4% ($p=0.004$), while LDL and HDL concentrations were unchanged. Weight loss was significantly correlated with changes in $AUC_{glucose}$ ($r=0.381$, $p = 0.01$), triglycerides ($r=0.306$, $p=0.04$), and total cholesterol ($r=0.547$, $p<0.001$).

The diets varying in glycemic index were initiated at week four. Between weeks 4 and 12, weight loss did not differ according to diet. Fasting plasma glucose levels were reduced an additional 4-5% ($p<0.01$), and $AUC_{glucose}$ was further reduced by 8-12% ($p<0.001$), without regards to dietary glycemic index. Similarly, fasting triglyceride levels were reduced an additional 6-10% ($p=0.03$) between weeks 4 and 12, independently of diet. When examined separately, subjects with low glucose tolerance consuming the low GI diet had a greater (18%, $p=0.02$) reduction in LDL levels compared with the high GI group. However, with the exception of LDL levels, glycemic index of the diet had no independent influence on glycemic control or lipid metabolism, suggesting that the major factor responsible for the improvements in glycemic control and lipid profile was the reduction in body weight, rather than dietary macronutrient composition (625).

Because of the controversy surrounding the physiological factors influencing glycemic index and the lack of agreement regarding the importance of dietary carbohydrate source in the prevention and treatment of obesity in humans, we designed studies to evaluate

the effects of high-fat diets of identical macronutrient composition but varying in the source of dietary carbohydrate on the development of obesity and overall metabolic control in a rodent model of diet-induced obesity.

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PART 3

EXPERIMENTAL INVESTIGATIONS

I. Effect of Dietary Carbohydrate on the Development of Obesity in Heterozygous Zucker (*fa/+*) Rats[1]

Abstract

Rats carrying one copy of the *fa* allele are predisposed to diet-induced metabolic disturbances which contribute to hyperinsulinemia, obesity and dyslipidemia. To investigate the role of dietary carbohydrate and fat in the development of these conditions, we fed 6-week old male heterozygous (*fa/+*) lean rats carbohydrate-free diets containing primarily saturated fat either *ad libitum* or pair-fed. These diets were compared to standard chow and to a high saturated fat mixed diet containing 10% energy from sucrose for 4 weeks. The carbohydrate-free diet resulted in significantly lower circulating glucose levels compared to all other groups ($p < 0.05$). Weight gain was negligible in the carbohydrate free groups compared to standard diet and 10% sucrose diet ($p<0.05$). This was reflected in energy efficiency which was markedly reduced (90%) in the carbohydrate-free groups compared to the other groups ($p<0.05$). Corresponding changes were noted in fat pad mass. The subscapular and epididymal fat pads were increased 42% and 44%, respectively, in animals consuming the 10% sucrose diet compared to all other groups ($p<0.01$). Comparable changes in fatty acid synthase (FAS) mRNA were observed in response to the carbohydrate-free diet, which resulted in a 53% decrease in adipocyte FAS mRNA ($p<0.01$). Addition of 10% sucrose to the diet completely reversed this effect resulting in a 69% increase in adipocyte FAS mRNA compared to the carbohydrate-free groups ($p<0.05$). Similarly, hepatic FAS mRNA was elevated by 51% and 66% in the 10% sucrose and standard diet groups respectively,

compared to the carbohydrate-free groups. Therefore, diets that contain minimal carbohydrate may minimize net lipid storage and adiposity.

Introduction

During the last 40 years, the prevalence of obesity among adults aged 20 to 74 years in the United States increased from 13.4% to 30.9% (1-3). Results from the Behavioral Risk Factor Surveillance Survey indicate that roughly one-third of U.S. adults are actively attempting to lose weight (4). While it is not possible to ascertain the various methods employed by those currently attempting to lose weight, the increasing popularity of low-carbohydrate diets suggests Americans are straying from the conventional low-fat, high-complex carbohydrate diet prescription. Those advocating the use of low-carbohydrate diets claim such a diet results in rapid and significant reductions in body weight (5,6). In addition to the USDA Dietary Guidelines (7), professional organizations, including the American Heart Association (8,9), the American Diabetes Association (10), and the American Dietetic Association (11), are concerned that low carbohydrate diets may have serious long term health consequences, including increased risk for cardiovascular disease, non-insulin dependent diabetes mellitus, dyslipidemia, and hypertension. A systematic review of the literature on the efficacy of low-carbohydrate diets in the treatment of obesity found that weight loss occurring with carbohydrate-restricted ($\leq$ 60 g/d carbohydrate) diets was predicted by energy intake, duration of diet, and baseline body weight, but not by carbohydrate intake (12).

There is evidence that rats adapted to a high-protein, carbohydrate-free diet have normal blood glucose levels, and decreased lipogenesis in both the liver and adipose

tissue (13-15). Similar metabolic adaptations may contribute to the weight reducing effects of low-carbohydrate diets in humans. The conclusions drawn from studies examining the metabolic consequences of high-protein diets in rats are largely based on comparisons with balanced diets. Consequently, there is little information available regarding the physiological consequences arising from the inclusion of small amounts of refined carbohydrate in these otherwise high-fat, high-protein diets.

The role of liver and adipose tissue in total lipid synthesis was compared in rats fed either a high protein (70% casein w/w) or nutritionally balanced, control (17% casein) diet (13). While the contribution of the liver to total carcass triacylglycerol synthesis was similar in rats fed the high protein (24%) and control (20%) diets, adipose tissue lipogenesis accounted for 57% of total triacylglycerol synthesis in control-fed rats, but only 26% in animals fed the high-protein, carbohydrate-free diet. There was a marked reduction in the in vivo rates of triacylglycerol-fatty acids synthesized in epididymal, retroperitoneal, intermuscular, and subcutaneous adipose tissue depots in animals fed the high-protein compared with the control diet. Despite the absence of carbohydrate in the high-protein diet, plasma insulin levels were similar with both diets (13). Other studies have documented plasma insulin levels that are similar or somewhat lower in animals fed high-protein, carbohydrate-free diets (15,16). Brito, et al. (17) sought to clarify the adaptive changes in adipose tissue metabolism induced by the high-protein, carbohydrate-free diet. The rate of glucose uptake *in vivo* of rats fed the high-protein diet was reduced to 40% and 34% of control values in the epididymal and retroperitoneal adipose tissue depots, respectively. Moreover, glucose uptake was normalized and the activity of pyruvate kinase restored when the high-protein diet was replaced with the

balanced diet for a brief (12 hours) period of time. While these adaptations were accompanied by an increase in plasma insulin levels and a reduction in plasma glucagon levels, the possibility of a direct stimulatory effect of increased substrate (glucose) availability cannot be excluded. Similarly, *in vitro* rates of glucose uptake in fat cells isolated from rats adapted to the high-protein diet incubated with 1 mmol/L glucose were only 49% of the rate in cells isolated from control-fed animals. This was accompanied by a significant reduction in the activity of key glycolytic enzymes: hexokinase, phosphofructo-1-kinase, and pyruvate kinase (17).

While it is well accepted that dietary macronutrient composition can influence the development of obesity, the contribution of a specific macronutrient (carbohydrate or fat) remains controversial. Consequently, the purpose of this study was to examine the role of the level of dietary carbohydrate on the development and treatment of obesity in lean rats susceptible to diet-induced obesity. Specifically, we assessed the metabolic effects of a high-fat diet devoid of dietary carbohydrate to a high-fat diet containing a minimal (10%) amount of sucrose on body weight regulation, glucose tolerance, de novo lipogenesis and lipid metabolism. We propose that the effects of dietary fat in conjunction with a minimal amount of sucrose will exert synergistic effects on adiposity and that their concomitant ingestion may significantly increase total body weight.

Materials and Methods

Animal Model

Rats were produced in two crosses segregating *fa*. F2 litters were obtained by BN/Crl X Crl:ZUC-*fa* intercross (18). F3 progeny of Brown Norway (BN)/Crl times Crl times

Crl: (ZUC)BR-*fa*. In cross 1, both dam and sire were *fa*/+, so that three genotypes, +/+, *fa*/+, *fa*/*fa*, were included among the progeny; however, obese (*fa*/*fa*) were excluded from this study. Subsequent generations were produced by crossing BNZ F2-*fa*/+ males with ZUC +/+ females, so that only *fa*/+ and +/+ were generated in cross 2 (19).

The current study was designed to evaluate the synergistic effects of dietary fat and carbohydrate in a rodent model of obesity. Zucker fatty rats (*fa*/*fa*) have a missense point mutation in the gene for the leptin receptor, which results in the substitution of proline for glutamine at position 269 in the extracellular ligand binding domain (20). This mutation impairs leptin binding and impedes the signaling mechanisms regulating food intake and satiety, resulting in hyperphagia-induced obesity (20). Studies of the physiological consequences of impaired leptin signaling were initially conducted in animals homozygous for the *fa* allele (*fa*/*fa*). As research continued, it was determined that the *fa* mutation, like many other mutations, exhibits haploin-sufficiency, such that animals heterozygous for the *fa* allele (*fa*/*fa*) have half the normal level of functional leptin receptors and are characterized by a phenotype the severity of which is intermediate between *fa*/*fa* and *Fa*/*Fa* animals (19,21,22). Consequently, we chose to investigate the interaction between dietary carbohydrate and fat in heterozygous Zucker (*fa*/+) rats.

Animals were phenotyped at 4 weeks of age on the basis of body weight. Forty heterozygous male Zucker lean rats (*fa*/+) of 6 to 8 weeks in age were adapted to handling for use in this study. Animals were housed in individual stainless steel hanging cages in a climate-controlled environment with a 12-h light-dark cycle.

Experimental Diets

Prior to this study, animals had been maintained on standard rodent chow. All diets were semi-purified and were based on AIN-93 guidelines for rodent diets as shown in Table 2 (23). Animals were randomly assigned to one of three dietary treatment groups: standard diet, carbohydrate free diet, 10% sucrose diet. Animals were allowed *ad libitum* access to food and water throughout the feeding period. A fourth group was included as a pair fed control group. These animals consumed the carbohydrate-free diet adjusted for the energy intake of the standard diet group on the previous day. This group also had *ad libitum* access to water throughout the feeding period. Animals spilled only minimal amounts of food; all spilled food was collected and recorded. Food intake was measured and replaced daily. Body weight was recorded every two days. This protocol was approved by the University of Tennessee Institutional Animal Care and Use Committee. At the completion of the study, animals were anesthetized with sodium pentobarbital (50 mg/kg body weight) and killed by exsanguination while under anesthesia.

Tissue Collection

The liver, selected fat pads, soleus muscle, and gastrocnemius muscle were dissected and immediately weighed. Tissues were then snap frozen in liquid nitrogen and stored at -80° C until time of analysis.

Table 2

Composition of AIN-93 Based Semi-Purified Diets

Component	Standard		Carbohydrate Free		10 % Sucrose	
	g/kg	% energy	g/kg	% energy	g/kg	% energy
Cornstarch	550	57	0	0	0	0
Soybean oil	50	12	77	11	71.5	11
Coconut oil	0	0	482	69	383.5	59
Casein	200	21	314	20	292.5	20
Sucrose	100	10	0	0	146.25	10
AIN mineral mix[1]	35		35		35	
AIN vitamin mix[2]	10		10		10	
Cellulose[3]	50		77		56.25	
DL-methionine	3		3		3	
Choline bitartrate	2		2		2	

[1]American Institute of Nutrition Mineral Mixture 76 (ICN Pharmaceuticals, Costa Mesa, CA)
[2]American Institute of Nutrition Vitamin Mixture 76 (ICN Pharmaceuticals, Costa Mesa, CA)
[3]Fiber used was Alphacel Non-nutritive Bulk (ICN Pharmaceuticals, Costa Mesa, CA)
Diets were isonitrogenous for percent total energy contribution. Energy content: Standard diet, 16.15 kJ/g; carbohydrate-free diet, 26.32 kJ/g; 10% sucrose diet, 24.48 kJ/g.

Blood Collection

Blood was obtained at sacrifice in non-fasted animals by cardiac puncture of the left ventricle using a heparinized 5 cc syringe and a 20 gauge, 1-inch needle. To prevent hemolysis of red blood cells, the needle was removed from the syringe prior to transfer of blood into an EDTA-containing vacutainer blood collection tube. Blood samples were kept on ice prior to centrifugation at 1000 rpm for 15 minutes at 4° C to collect plasma. Plasma samples were subsequently stored at -80°C until time of analysis.

Extraction of Total Cellular RNA from Adipose Tissue

Total RNA was extracted from adipose tissue by the guanidine-HCl method (24,25). Adipose tissue samples (0.5 to 1.0 g) were homogenized using a polytron homogenizer in 4 M guanidine isothiocynate (GTC) containing β-mercaptoethanol (14 μl per ml of GTC). The homogenate was then passed through a 26 gauge, 1-inch needle into a 5 cc syringe. Subsequently, the homogenate was carefully layered over 4 ml of 5.7 M cesium chloride (CsCl; pH 7.0) and centrifuged at 29,000 rpm for 20 hours at 20° C (26). Following centrifugation, the supernatant was removed using a 5 ml transfer pipet and the pellet allowed to air dry before being resuspended in 25 μl of DEPC-treated water and transferred to an autoclaved 1.5 ml eppendorf tube. The sample was allowed to precipitate in 1 ml 100% ethanol (EtOH) overnight at -20° C. Following precipitation, samples were centrifuged at 10,000 rpm for 30 minutes at 4° C. The supernatant was removed and the pellet washed in 1 ml of 70% EtOH. After washing, the pellet was allowed to air dry prior to being resuspended in 10-20 μl of DEPC-treated water and

quantified using dual wavelength (260/280 nm) spectrophotometry (27). The sample was then stored at -20° for further analysis.

Extraction of RNA from the Liver

Total RNA was extracted from the liver by the phenol-chloroform extraction method (25). A sample of the medial lobe of the liver (0.5 to 2 g) was homogenized using a polytron homogenizer in 10 ml of 4 M guanidine isothiocynate (GTC) with β-mercaptoethanol (14 µl per ml of GTC). The homogenate was then transferred to a polypropylene centrifuge tube and one starting volume of phenol:choloroform:isoamyl alcohol (25:24:1, pH 7.9) added prior to vortexing. The homogenate was placed on ice for 15 minutes prior to centrifugation at 10,000 x g for 15 minutes at 4° C. Following centrifugation, the aqueous layer was transferred to a clean polypropylene centrifuge tube containing 1 ml of 3 M sodium acetate and samples mixed by inversion. Subsequently, 5 ml of acid phenol:chloroform:isoamyl alcohol (125:24:1, pH 4.5) was added to each sample. The samples were vortexed and allowed to sit on ice for 15 minutes prior to centrifugation at 10,000 x g for 20 minutes at 4° C. Following centrifugation, the aqueous layer was transferred to a 15 ml centrifuge tube. Samples were allowed to precipitate overnight in 100% isopropanol at -20° C.

Samples were transferred to clean polypropylene centrifuge tubes prior to centrifugation at 10,000 x g for 20 minutes at 4° C. Following centrifugation, the supernatant was decanted and the pellet allowed to air dry for 15 minutes at room temperature. The pellet was washed in 4 M lithium chloride (LiCl). Subsequently, the pellet was resuspended in nuclease-free water containing 0.1 mM EDTA. The samples

were allowed to precipitate for 30 minutes at -20° C prior to centrifugation at 10,000 x g for 30 minutes at 4° C. The supernatant was decanted and the pellet resuspended in 100% ethanol. The samples were allowed to incubate overnight at -20° C.

Samples were transferred to clean polypropylene centrifuge tubes and centrifuged at 10,000 x g for 20 minutes at 4° C. The supernatant was decanted and the pellet resuspended in formamide prior to quantification using dual wave length (260/280 nm) spectrophotometry (27).

Gel Electrophoresis and Northern Blotting

Gel electrophoresis and Northern blotting was performed as described by Herrin, et al. (28). All supplies were cleaned with RNase ZAP (Ambion, Austin, TX) prior to use. For Northern blot analysis, RNA was size fractionated on 1% agarose gels prepared by boiling 1 g agarose (Life Technologies, Carlsbad, CA) in 82 ml of 1 X 4-morpholinepropanesulfonic acid (1 X MOPS) in a conventional microwave oven set on medium (80%) heat for 1 minute. The solution was then cooled at room temperature to 50° C. After cooling, 18 ml of 37% (12.3 M) formaldehyde was added under the fume hood to yield a final concentration of 2.2 M formaldehyde. The solution was poured into a Horizon 11-14 medium gel electrophoresis apparatus (Life Technologies, Carlsbad, CA.) containing a 10-well comb and allowed to cool at room temperature until the gel was cloudy in appearance. When the gel had cooled, the buffer tanks were filled with 1X MOPS to approximately 3-5 mm above the gel. The gel comb was then carefully removed.

Denaturing buffer (67.5% formamide, 1X MOPS, 22.5% formaldehyde) was added to each sample of RNA to yield a final volume of 18 µl. The samples were denatured by boiling for 5 minutes and then immediately placed on ice to cool. When the samples had cooled, 2 µl of loading dye (50% glycerol, 1 mM EDTA, 0.4% bromophenol blue) was added to each sample. Samples were mixed by vortexing prior to being pipetted into wells of the gel.

The gel was run at 100 volts for 3 to 4 hours or until the blue dye front had migrated approximately ¾ of the length of the gel. The gel was stained in ethidium bromide (1 µg/ml) with gentle shaking for 20 minutes. Following this period, the ethidium bromide was decanted and the gel inspected for quality using an ultraviolet light box. To remove the ethidium bromide, the gel was transferred to a tray containing DEPC-treated water for 10 minutes with gentle shaking.

RNA was transferred to Hybond nitrocellulose membrane (Amersham, Piscataway, NJ) by Northern blotting. A buffer tray was filled with 20X SSC and a wetting wick prepared using 3 mm Whatman filter paper saturated with 20X SSC, which was then draped over a glass plate into the buffer tray. The destained gel was placed upside down on the wetting wick. Plastic wrap was used to seal all four edges of the gel. A piece of nitrocellulose membrane was cut to slightly smaller than the size of the gel and the upper right hand corner cut away to denote the position of the first well. The membrane was placed on top of the gel and a 2 ml serological pipet gently rolled over the membrane to remove any bubbles which may have formed between the membrane and gel. Six pieces of Whatman filter paper, roughly the size of the gel, were placed on top of the membrane. Brown tri-fold paper towels were stacked (approximately 1 inch) on top of

the filter paper and a glass plate placed over them. Two water bottles collectively weighing 100 g were placed on top of the glass plate to ensure adequate transfer over 20-24 hours.

After transfer was complete, the membrane was rinsed in 10X SSC for 10 minutes. The membrane was crosslinked under ultraviolet light for 2 minutes and then wrapped in Whatmann filter paper and allowed to dry overnight prior to hybridization (28).

Labeling of Probe and Membrane Hybridization

This procedure used the Random Primers DNA Labeling System (Life Technologies, Carlsbad, CA) and was performed as described by Feinberg, et al. (29). 1 μl of murine cDNA probe for fatty acid synthase (kindly provided by Dr. Naima Moustaid-Moussa) was added to a microcentrifuge tube containing 23 μl of DEPC-treated water and boiled for 5 minutes. The mixture was then immediately transferred to ice and allowed to cool. When sufficiently cool, 2 μl of unlabelled deoxynucleotides (0.5 mM dATP, dGTP, dTTP in 1 mM Tris-HCL), 20 μl Random Primers Buffer Solution (0.67 M HEPES, 0.17 M Tris-HCl, 17 mM $MgCl_2$, 33 mM β-mercaptoethanol, 1.33 mg/ml BSA), and 5 μl of [α-^{32}P]dCTP (Perkin-Elmer, Boston, MA) were added and the solution vortexed prior to the addition of 1 μl of Klenow fragment. The solution was gently vortexed and allowed to incubate for 1 hour at 68° C. After incubation, 200 μl DEPC-treated water was added and the solution mixed by vortexing.

To purify the probe, the plunger was removed from a 3 cc syringe, and a small piece of glass wool placed in the syringe barrel to cover the opening in the bottom of the syringe barrel. The syringe was then placed in a 13 ml centrifuge tube and sephadex

added until the meniscus reached the 2 cc demarcation line, prior to centrifugation at 1000 rpm for 1 minute. The syringe was transferred to a clean 13 ml centrifuge tube and the probe added to the sephadex column prior to centrifugation at 1000 rpm for 1 minute. The syringe was discarded and the probe collected and transferred to a 1.5 ml eppendorf tube. The probe was denatured by boiling for 5 minutes and immediately placed on ice to cool. The probe was considered to be sufficiently labeled when a count of 2 to 3 kb was detected by Geiger counter.

The dry nitrocellulose membrane was transferred to a hybridization bottle containing 2X SSC and placed in a hybrization oven, preheated to 68° C, for 10 minutes. ULTRAhyb™ (Ambion, Austin, TX) hybridization buffer was preheated to 68° C until completely dissolved. The 2X SSC was decanted and the membrane was prehybridized in 6-10 ml of ULTRAhyb™ for 30 minutes at 68° C. Following the prehybridization period, the ULTRAhyb™ was decanted and the probe added to the hybridization bottle. The membrane was allowed to rotate for 24 hours at 68° C.

Following hybridization, the membrane was washed in 2X SSC/0.1% SDS for 15 minutes at room temperature. Following this wash, a Geiger counter was used to assess the level of radioactivity of the membrane. If the count was greater than 1 kb, the membrane was washed again in 2X SSC/0.1% SDS for 30 minutes. If the radioactive count was still in excess of 1 kb, the membrane underwent 5 minute washings in 0.5% SSC/0.5% SDS at 65° C until a count below 1 kb was achieved. Once sufficiently washed, the membrane was wrapped in plastic wrap and exposed to 5 x 7 Kodak x-ray film in an autoradiogram cassette for 24 hours at -20° C. After this period, the autoradiogram cassette was allowed to reach room temperature prior to developing the

film. Autoradiograms were quantitated using the Ultra Lum Electronic UV Transilluminator system (Ultra Lum, Inc., Paramount, CA) and Zero-D scan image analysis software (Scanalytics, Inc., Fairfax, VA), which performs volume densitometry on bands in the gel images.

Expression of fatty acid synthase was normalized for 18s using the same membrane. After obtaining a suitable autoradiogram for FAS, the membrane was boiled for 30 minutes in membrane stripping solution (1 M Tris-HCl, 500 mM EDTA, 1% SDS). The membrane was then probed for 18s and quantitated according to the procedures described above (29). Values are reported as FAS:18s ratio.

Cardiac Exsanguination and Preparation of Plasma

Blood was collected from unfasted animals by cardiac puncture using a heparinzed 5 cc syringe and a 20 gauge 1-inch needle. To prevent hemolysis, the needle was removed from the barrel of the syringe prior to transferring the blood to an EDTA-treated vacutainer blood collection tube. All samples were stored on ice until centrifuged at 1000 rpm for 15 minutes to collect plasma. Plasma was stored at -20 °C until time of analysis.

Determination of Plasma Glucose Levels

Final plasma glucose levels were determined using the glucose oxidase method as first described by Washko, et al. (30) and modified by Marks (31). This procedure is based on the following coupled enzymatic reactions: In the presence of water and oxygen, glucose oxidase catalyzes the conversion of glucose to gluconic acid and hydrogen peroxide.

With hydrogen peroxide and peroxidase available, the otherwise colorless o-diansidine dihydrochloride is oxidized and the product of the reaction is brown in color. The intensity of the brown color measured spectrophotometrically at 475 nm is proportional to the original glucose concentration of the sample.

Reagents for determination of plasma glucose levels were obtained from Sigma-Aldrich Diagnostics (St. Louis, MO). The enzyme solution was prepared by adding 1 capsule of PGO (500 U glucose oxidase from *Aspergillus niger*, 100 U horseradish peroxidase) enzymes to 100 ml distilled water in an amber bottle. The color reagent solution was prepared by reconstituting one vial (50 mg) of o-dianisidine dihydrochloride with 20 ml of distilled water. To prepare the combined enzyme-color reagent solution, 1.6 ml of color reagent solution was added to 100 ml of enzyme solution and mixed by inversion.

For each sample, 25 µl of plasma was transferred to an appropriately labeled borosilicate glass culture tube containing 5 ml of combined enzyme-color reagent solution. A standard was similarly prepared using a known glucose standard (100 mg/dL, Sigma-Alrich Diagnostics, St. Louis, MO). The tubes were vortexed, covered in foil to protect them from exposure to ambient light and allowed to incubate at room temperature for 45 minutes. At the end of the incubation period, the absorbance was read at 475 nm against a water blank. The concentration of glucose in each sample was calculated by dividing the absorbance of the samples by the absorbance of the glucose standard, and multiplying by the concentration of the glucose standard.

Determination of Plasma Triglyceride Levels

Plasma triglyceride levels were determined according to the technique described by Buccolo, et al. (32). Using this method, triglycerides are first hydrolyzed by lipoprotein lipase to glycerol and free fatty acids. Glycerol is then phosphorylated by in an ATP-dependent reaction by glycerol kinase yielding glycerol-1-phosphate. The glycerol-1-phosphate is oxidized to dihydroxyacetone phosphate, with the concomitant reduction of NAD to NADH in a reaction catalyzed by glycerol-1-phosphate dehydrogenase. The resulting NADH is then oxidized coupled to reduction of 2-(p-iodophenyl)-3-p-nitrophenyl-5-phenyltetrazolium chloride (INT) to formazan, which is pink in color, in the presence of diaphorase. The intensity of the color produced is directly proportional to the triglyceride concentration in plasma.

The reagents used in this assay were obtained from Sigma-Aldrich Diagnostics (St. Louis, MO). Plasma (10 μl) was transferred to a borosilicate glass culture tube containing 1 ml reconstituted triglyceride INT reagent (2.0 mM ATP, 2.0 mM NAD, 3.0 M Mg^{2+}, 1.0 mM 2-(p-iodophenyl)-3-p-nitrophenyl-5-phenyltetrazolium chloride, 200 U/L glycerol kinase, 4000 U/L glycerol-1-phosphate dehydrogenase, 455 U/L diaphorase, pH 7.8). A standard was similarly prepared using a known triglyceride standard (250 mg/dL, Sigma-Aldrich, St. Louis, MO). The samples were vortexed and allowed to sit at room temperature for 15 minutes. The absorbance of each sample was measured at 500 nm against a water blank. The concentration of triglycerides in each sample was calculated by dividing the absorbance of the samples by the absorbance of the triglyceride standard, and multiplying by the concentration of the triglyceride standard.

Determination of Total Cholesterol in Plasma

Total plasma cholesterol levels were determined using according to the method of Allain, et al. (33) using reagents obtained from Sigma-Aldrich Diagnostics. This assay is based on a series of enzymatic reactions beginning with the hydrolysis of cholesterol esters present in plasma by cholesterol esterase to yield cholesterol and free fatty acids. The cholesterol produced during the previous reaction was oxidized by cholesterol oxidase resulting in production of cholest-4-en-3-one and hydrogen peroxide (H_2O_2). The resulting H_2O_2 is then coupled with 4-aminoantipyrine and p-hydroxybenzenesulfonate contained in the cholesterol reagent, in the presence of peroxidase. This reaction produces a quinoneimine dye which has an absorbance maximum of 500 nm. The intensity of the color produced is directly proportional to the total cholesterol concentration in plasma.

Plasma (10 μl) was transferred to borosilicate glass culture tubes containing 1 ml of reconstituted cholesterol reagent (300 U/L cholesterol oxidase, >100 U/l cholesterol esterase, 1000 U/L horseradish peroxidase, 0.3 mM 4-aminoantipyrine, 30 mM p-hydroxybenzenesulfonate, pH 6.5). A standard was similarly prepared using a known cholesterol standard (200 mg/dL, Sigma-Aldrich, St. Louis, MO). The samples were vortexed and allowed to sit at room temperature for 5 minutes prior to measuring the absorbance at 500 nm against a water blank. The concentration of cholesterol in each sample was calculated by dividing the absorbance of the samples by the absorbance of the standard and multiplying by the concentration of the cholesterol standard.

Determination of Plasma HDL-Cholesterol Levels

Plasma (500 µl) was added to 50 µl reconstituted HDL cholesterol reagent (Sigma-Aldrich; 10 g/L dextran sulfate, 0.5 mol/l Mg^{2+}, pH 7.0). This assay was developed by Warnick, et al. (34) and is based on the ability of dextran sulfate and Mg^{2+} to precipitate LDL and VLDL, leaving the HDL fraction in the supernatant. The samples were vortexed and allowed to sit at room temperature for 5 minutes. Subsequently, the samples were vortexed and centrifuged at 3000 rpm for 15 minutes. Following centrifugation, 50 µl of the supernatant was added to appropriately labeled borosilicate glass tubes containing 1 ml of cholesterol reagent and cholesterol levels determined as described above.

Determination of Plasma Insulin Levels

This assay is based on the double antibody technique for determination of plasma insulin levels in rats as described by Morgan, et al. (35) using rat insulin radioimmunoassay kit obtained from Linco Research (St. Charles, MO). A standard curve was prepared using purified rat insulin in insulin standard buffer at the following concentrations: 0.1, 0.2, 0.5, 1.0, 2.0, 5.0, and 10.0 ng/ml. For each standard and sample, 50 µl of standard/sample, 50µl of ^{125}I-labeled insulin, and 50 µl guinea pig anti-rat insulin serum in assay buffer were transferred to an appropriately labeled glass culture tube. All tubes were vortexed, covered with foil and allowed to incubate for 20 hours at 4°C. Following incubation, 500 µl of precipitating reagent containing goat anti-guinea pig IgG serum was added to each standard and sample. All tubes were vortexed and allowed to incubate for 30 minutes at 4° C. Subsequently, all tubes were centrifuged at

3000 rpm for 30 minutes and carefully decanted by inversion for 1 minute to separate antibody-bound from free label. Subsequently, the percentage of total binding was determined by counting each sample for 5 minutes in an automated gamma counter. The calculations necessary for determination of plasma insulin concentration was performed automatically using the data reduction capabilities of the gamma counter, and involved plotting the percentage of total binding for each sample on the y-axis and the known concentrations of the standards on the x-axis (36).

Determination of Plasma Leptin Levels

This assay is based on the double antibody technique for determination of plasma leptin levels in human plasma described by Ma, et al. (37) using mouse leptin radioimmunoassay kit obtained from Linco Research. A standard curve was prepared using purified rat leptin in leptin standard buffer at the following concentrations: 0.2, 0.5, 1.0, 2.0, 5.0, 10.0 and 20.0 ng/ml. For each standard and sample, 50 μl of standard/sample and 50 μl rabbit anti-rat leptin serum in assay buffer were transferred to an appropriately labeled glass culture tube. All tubes were vortexed, covered with foil and allowed to incubate for 20 hours at 4°C. Following incubation, 50 μl of ^{125}I-labelled leptin was added to each standard/sample. The tubes were vortexed, covered with foil and allowed to incubate an additional 20 hours at 4° C. Following this period, 500 μl of precipitating reagent containing goat anti-rabbit IgG serum was added to each standard and sample. All tubes were vortexed and allowed to incubate for 30 minutes at 4° C. Subsequently, all tubes were centrifuged at 3000 rpm for 30 minutes to separate antibody-bound from free label. The supernatant was decanted by inverting the tubes

for 45 seconds and the percentage of total binding determined for each sample by counting in a gamma counter for 5 minutes. The calculations necessary for determination of plasma leptin concentration was performed automatically using the data reduction capabilities of the gamma counter, which involved plotting the percentage of total binding for each sample on the y-axis and the known concentrations of the standards on the x-axis (36).

Statistical Analysis

Data were analyzed by analysis of variance (ANOVA) to compare overall group means with subsequent analysis via least significant difference test to identify significantly different group means using the SPSS Statistics Package (38,39). Variables which did not follow a normal distribution were transformed according to standard statistical practices (log base 10, reciprocal, square root, inverse square root, square) to achieve normal distribution prior to ANOVA (39). When normality could not be achieved by accepted statistical transformations, significantly different group means were identified by non-parametric tests (39).

Results

Food Consumption and Energy Efficiency

Data regarding food and energy intake during the four week study is summarized in Table 3. Animals consuming the standard diet consumed a slightly greater ($p<0.05$) quantity of food. However, the energy density of the carbohydrate-free and 10 en% sucrose diets resulted in greater energy intake by these groups ($p<0.05$).

Table 3

Cumulative Food and Energy Intake

	Grams	kcal
Standard	1387.51 ± 23.58[a]	5355.40 ± 90.80[a]
Carbohydrate-Free	1157.15 ± 16.64[b]	7280.83 ± 124.31[b]
Carbohydrate-Free (restricted)	884.16 ± 22.36[c]	5429.97 ± 167.08[a]
10 en% Sucrose	1206.68 ± 49.64[b]	7059.08 ± 290.37[b]

Data is expressed as Mean±SEM. [a,b,c]Non-similar superscripts indicate significant differences at $p<0.05$.

Weight Gain and Body Composition

The data regarding body weight and adiposity are summarized in Table 4. Animals consuming the standard or mixed (10 en% sucrose) diets gained 90% more weight ($p < 0.05$) than animals consuming the carbohydrate-free diet ad libitum (Figure 1A). Changes in body weight were reflected in energy efficiency (weight gain/energy intake ratio) which was 90% lower ($p < 0.05$) in the carbohydrate-free groups compared to standard and 10en% sucrose diet groups (Figure 1B).

Changes in body weight are partially reflected in fat pad mass. Epididymal fat pad mass was 23-44% lower ($p<0.05$, Figure 2A) in the carbohydrate-free groups relative to the standard or 10 en% sucrose groups. Subscapular fat pad mass was approximately 30 and 79% greater ($p<0.05$, Figure 2B) in animals fed the standard and 10 en% sucrose diets, respectively, compared with the carbohydrate-free diet fed ad libitum or pair fed to the standard diet.

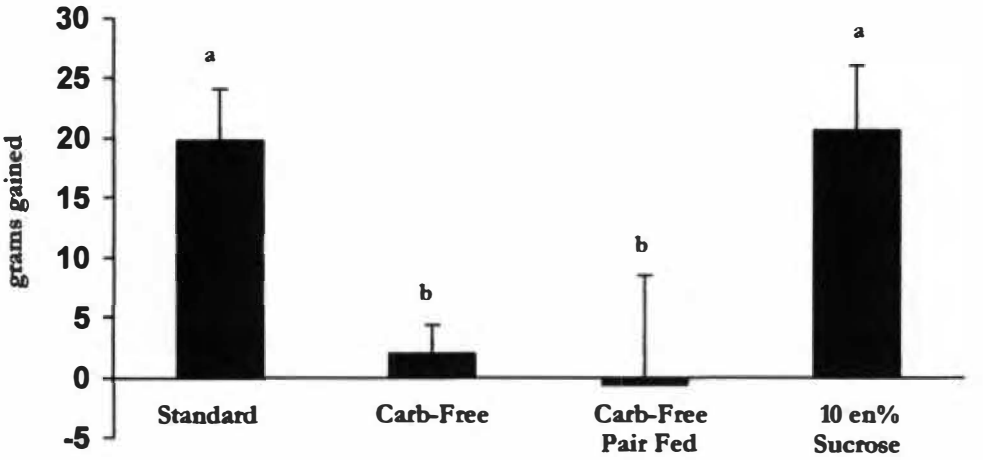
Table 4

Effect of Carbohydrate Restriction on Measures of Body Composition in Heterozygous (*fa/+*) Zucker Rats

	Body Weight	Selected Subcutaneous Fat Pads (g)			Selected Visceral Fat Pads (g)			Combined Subcutaneous & Visceral Fat
	Gain/Loss	Individual Tissues		Combined	Indivdual Tissues		Combined	
	Grams	Subscapular	Epididymal	Mass	Abdominal	Perirenal	Mass	
Standard	19.80 ± 4.26[a]	0.61 ± 0.12[a]	0.18 ± 0.02[a]	0.79 ± 0.12[a]	6.09 ± 0.31[a]	1.02 ± 0.14	7.11 ± 0.42	7.90 ± 0.51
Carb-Free	2.00 ± 2.35[b]	0.47 ± 0.03[b]	0.17 ± 0.04[a]	0.64 ± 0.06[a]	5.55 ± 0.35[a,b]	0.94 ± 0.11	6.49 ± 0.40	7.13 ± 0.41
Carb-Free (pair fed)	-0.60 ± 9.08[b]	0.47 ± 0.08[b]	0.17 ± 0.01[a]	0.61 ± 0.07[a]	4.79 ± 0.54[b]	0.89 ± 0.12	5.68 ± 0.66	6.29 ± 0.71
10% Sucrose	20.60 ± 5.38[a]	0.84 ± 0.11[a]	0.29 ± 0.04[b]	1.13 ± 0.14[b]	6.04 ± 0.75[a,b]	1.03 ± 0.10	7.07 ± 0.82	8.20 ± 0.85

Data is expressed as Mean±SEM. [a,b,c]Non-similar superscripts indicate significant differences at $p<0.05$

A



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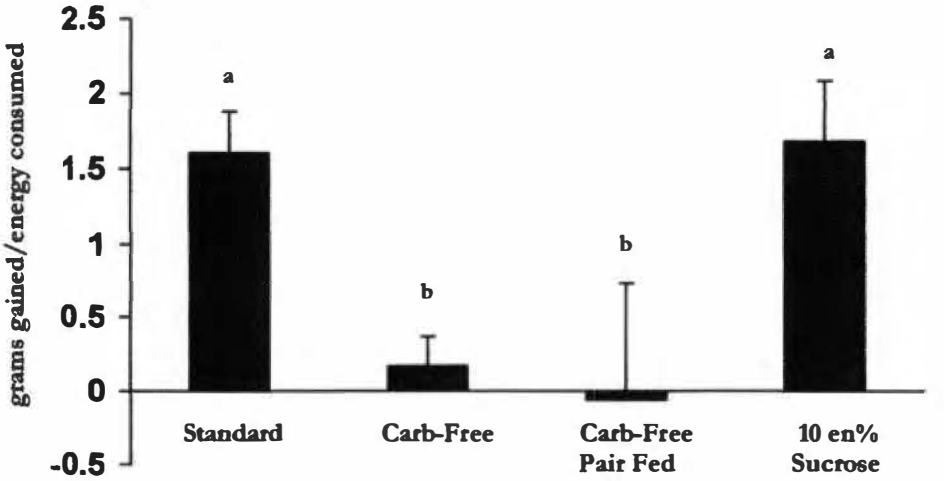
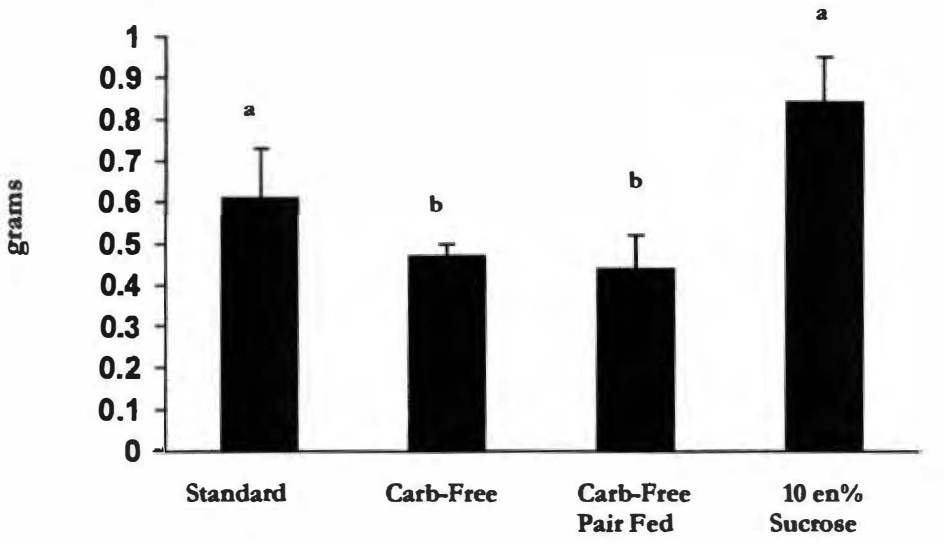


Figure 1. Effect of diet on weight gain (A) and energy efficiency ratio (B) in heterozygous Zucker rats. Data is expressed as Mean±SEM. Non-similar superscripts indicate significant differences at $p<0.05$.

A



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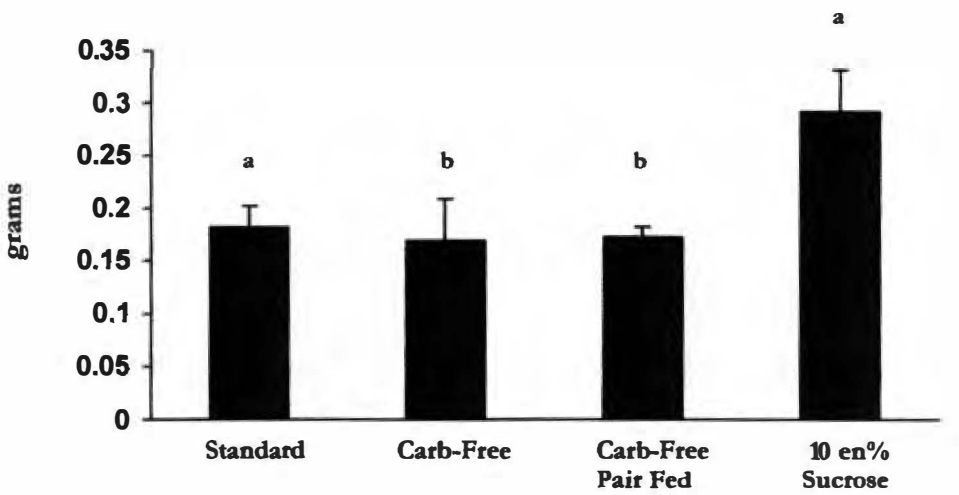


Figure 2. Effect of diet on subscapular (A) and epididymal (B) fat pad mass in heterozygous Zucker rats. Data is expressed as Mean±SEM. Non-similar superscripts indicate significant differences at $p<0.05$.

Non-Fasting Plasma Metabolites

Non-fasting plasma metabolite levels are summarized in Table 5. The diet-induced variations in adipose tissue deposition are reflected in circulating leptin levels, which were significantly greater ($p<0.05$) in animals consuming the 10 en% sucrose diet compared with the standard and carbohydrate-free pair fed groups.

Non-fasting plasma glucose levels were significantly ($p<0.05$) greater in animals consuming the standard diet compared with the 10 en% sucrose and carbohydrate-free diet fed ad libitum or pair fed to the standard diet. Compared with animals consuming the 10 en% sucrose diet, plasma glucose levels were 18% lower ($p<0.05$) when animals were pair fed the carbohydrate-free diet. Regardless of energy intake, the carbohydrate-free diet resulted in plasma HDL-cholesterol levels that were significantly greater ($p<0.05$) than in animals fed either the standard or 10 en% sucrose diet. Diet was without influence on plasma insulin levels.

Fatty Acid Synthase Expression

Adipose tissue fatty acid synthase (FAS) mRNA levels were 53% lower ($p<0.001$) in animals consuming the carbohydrate-free diet compared to those consuming the semi-purified standard diet (Figure 3A). This effect was completely reversed upon addition of 10 en% sucrose to the diet, which instead resulted in a 69% increase ($p<0.01$) in epididymal fatty acid synthase mRNA compared to the carbohydrate-free diet. Similarly, hepatic FAS mRNA levels were 51% and 66% higher ($p<0.01$) in animals consuming the mixed diet (10 en% sucrose) and standard diet, respectively, compared to animals receiving the carbohydrate-free diet (Figure 3B).

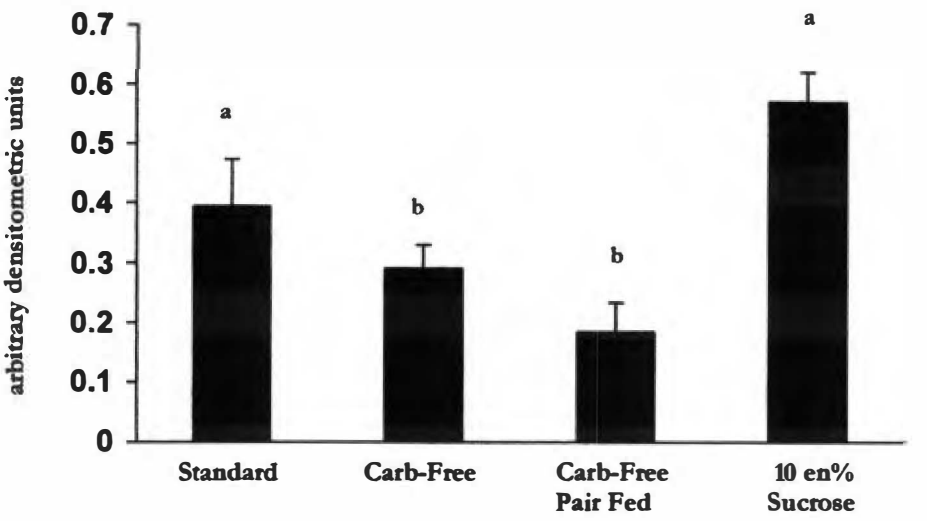
Table 5

Effect of Carbohydrate Restriction on Non-Fasting Plasma Metabolites in Heterozygous (*fa/+*) Zucker Rats

	Glucose	Insulin	Leptin	Triglycerides	Cholesterol	HDL
	(mg/dL)	(μU/ml)	(ng/mL)	(mg/dL)	(mg/dL)	(mg/dL)
Standard	354.54 ± 21.88[a]	11.86 ± 5.13	3.44 ± 1.02[a]	119.57 ± 5.98	143.27 ± 11.49	51.98 ± 3.90[a]
Carb-Free	260.45 ± 8.00[b,c]	17.92 ± 5.89	3.99 ± 0.60[a,b]	124.40 ± 6.50	183.27 ± 12.27	71.68 ± 3.58[b]
Carb-Free (pair fed)	250.21 ± 30.19[c]	12.29 ± 2.20	3.21 ± 0.59[a]	123.37 ± 8.02	183.66 ± 16.26	82.78 ± 6.91[b]
10% Sucrose	305.35 ± 9.59[b]	23.82 ± 7.19	5.91 ± 1.27[b]	109.83 ± 7.49	171.35 ± 17.59	67.03 ± 2.25[a]

Values are means ± SEM. Non-similar superscripts indicate significant differences at $p<0.05$.

A



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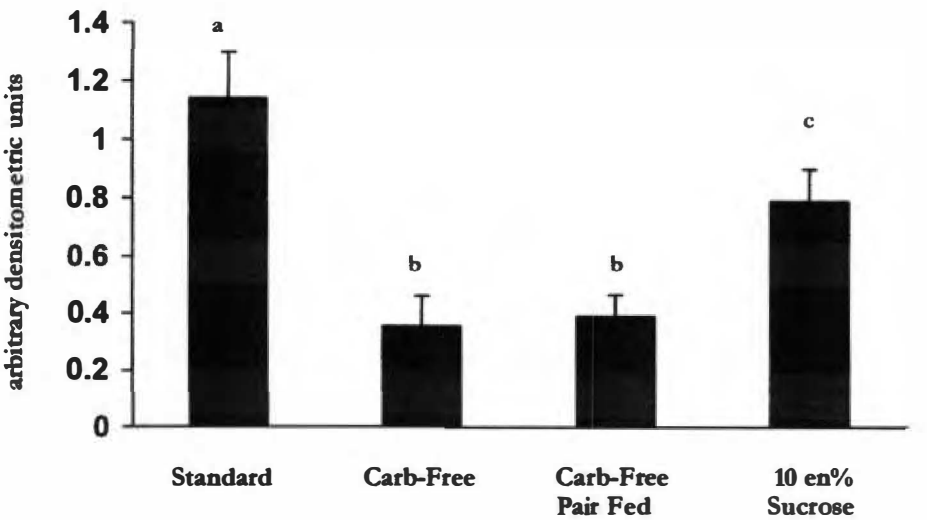

Figure 3. Effect of diet on expression of fatty acid synthase in the epididymal fat pad (A) and liver (B) in heterozygous Zucker rats. Data is expressed as Mean±SEM. Non-similar superscripts indicate significant differences at $p<0.05$.

Discussion

The purpose of this study was to investigate the effects of a dietary carbohydrate on body weight, adiposity, and the expression of fatty acid synthase in a rodent model susceptible to diet-induced obesity. The results of this study suggest that consumption of a carbohydrate-free diet significantly blunts fat accumulation in heterozygous Zucker rats (*fa*/+). This effect is mediated, in part, by reduced expression of fatty acid synthase in both the liver and adipose tissue.

The differences in weight gain and adiposity in animals fed the carbohydrate-free diet and 10 en% sucrose diets *ad libitum* can be attributed to diet composition since the level of energy intake between these groups was similar. The same can be said for animals fed the standard diet and those fed the carbohydrate-free diet at the same level of energy intake. While final body weight was similar regardless of diet, adipose tissue deposition was reduced specifically in the subcutaneous depots (subscapular and epididymal) without influencing visceral adiposity. Furthermore, diet did not influence the individual or combined mass of selected contralateral muscles (soleus and gastrocnemius). Our findings are not inconsistent with those of Botion, et al. (13), who found that total carcass fatty acid content is maintained and loss of body fat is minimal in animals fed high-protein, carbohydrate-free diets despite marked reduced in vivo lipogenesis, as assessed by tritium into liver and carcass fatty acids.

Based on the present studies, we can infer that the marked reduction in subcutaneous fat pad mass in rats consuming the carbohydrate-free diets is at least partially explained by reduced *de novo* lipogenesis in the liver and adipose tissue. Moreover, the effect of the carbohydrate-free diet on FAS expression in adipose tissue FAS was completely reversed

by the substitution of 10 en% sucrose for dietary fat. The lower level of FAS expression in adipose tissue of animals fed the carbohydrate-free diet relative to the 10 en% sucrose diet appears to be a consequence of diet composition since energy intake was similar *ad libitum* fed animals.

In the liver, expression of FAS was higher in animals fed the standard diet compared to both the carbohydrate-free and 10 en% sucrose groups. Induction of FAS expression in the liver of animals fed the standard diet may have been enhanced by the chronic exposure of the liver to lipogenic substrates provided by the higher carbohydrate content of the standard diet.

While we cannot determine the exact mechanism(s) mediating the observed changes in adiposity and lipogenic gene expression induced by the carbohydrate-free and 10 en% sucrose diets, our data together with other studies of rats fed high-protein, carbohydrate-free diets permit some plausible hypotheses (13-17).

The level of dietary sucrose in these diets did not significantly influence non-fasting plasma insulin levels. While others report higher plasma insulin levels in rats adapted to high-protein diets, Botion, et al. (13) report that insulin levels of high-protein fed rats are much less affected by feeding and are markedly resistant to fasting (13-15,17). In addition, our diets contain significantly less protein (20 en%) than the carbohydrate-free, high-protein (70 en%) diets typically used to study the adaptive mechanisms in energy-linked metabolic processes (13-17). Since we did not measure plasma glucagon levels, we cannot exclude the possibility that a low insulin:glucagon ratio at the tissue level was partly responsible for suppressing lipogenesis in animals fed the carbohydrate-free diet. We also cannot exclude that a direct stimulatory effect of increased substrate availability

(carbohydrate) accelerated glucose uptake and enhanced lipogenesis in animals fed the standard or 10 en% sucrose diets.

It is possible that the beneficial effects of the carbohydrate-free diet were due to our decision to supply saturated fat in the form of hydrogenated coconut oil, rather than lard or beef tallow, both of which contain significant amounts of cholesterol. According the USDA nutrient database (40), hydrogenated coconut oil is 86.5% (by weight) saturated fat, with 45% occurring as the medium chain fatty acid, lauric acid (12:0). The metabolic response to ingestion of medium chain triglycerides (MCT, 8-12 carbons) is quite different from the response to long chain triglycerides (LCT, >12 carbons). MCT are more rapidly hydrolyzed to medium chain fatty acids (MCFA) by the action of pancreatic lipase than are LCT. Subsequently, MCFA are absorbed directly into portal circulation for rapid transport to the liver. Conversely, long chain fatty acids (LCFA) liberated by the hydrolysis of LCT are incorporated into chylomicrons, which circulate peripherally via the lymphatic system prior to reaching the liver. Once in the liver, LCFA must be transported across the mitochondrial membrane by carnitine palmitoyl transferase (CPT) 1 prior to undergoing β-oxidation. In contrast, MCFA do not require a transport system to penetrate the outer mitochondrial membrane. Consequently, MCFA gain access to the oxidative machinery more efficiently, and are oxidized more extensively, than are LCFA (41). Consistent with this, human studies have demonstrated that fat oxidation increases more quickly and remains elevated for a longer duration following an MCT versus and LCT load given at lunchtime (42). Furthermore, in the subsequent four to six hours before the next meal, subjects had oxidized almost twice as much lipid after the MCT lunch compared with the LCT lunch (42).

The use of coconut oil may partially explain the differences in adipose tissue accumulation and body weight in our studies. Animals consuming the carbohydrate-free and 10 en% sucrose diets ad libitum consumed 30-36% more energy than did animals consuming the standard diet ad libitum. Nonetheless, there were no significant differences in final body weight between groups. The carbohydrate-free and 10 en% sucrose diets derived 69 and 59 en%, respectively, from coconut oil, while the standard diet contained 12 en% soybean oil. Portillo, et al. (43) have previously demonstrated an increase in interscapular brown adipose tissue (IBAT) and the induction of UCP-1 in IBAT in rats fed a high-fat coconut oil (56 en% coconut oil) diet compared with control (12 en% fat) diet. Moreover, final body weight rats fed the high-fat coconut oil diet was only 7.8% higher than rats fed the standard diet. In comparison, other studies (44,45) demonstrate a range of excess body weight (20-55%) in animals fed high polyunsaturated fat diets compared with control diets. Nonetheless, the use of coconut oil in the present studies cannot be the sole factor mediating the reduction in body weight, as the addition of a minimal amount of sucrose to the coconut oil-based diet significantly increased body weight.

The induction of UCP-1 in IBAT with coconut oil feeding suggests there is a compensatory increase in thermogenesis which minimizes the extent to which excess energy is stored in adipose tissue (43,45,46). Similarly, dietary MCT have been shown to suppress accumulation of body fat and reduce body weight compared with LCT in humans with a BMI exceeding 23 kg/m^2 (47)

In summary, we have demonstrated suppression of weight gain and subcutaneous adipose tissue deposition in rats fed a carbohydrate-free, high-fat diet compared with

semi-purified standard rodent diet or a high-fat diet in which 10 en% sucrose is substituted for dietary fat. These changes occurred independently of energy intake and in the absence of measurable changes in plasma insulin levels. The reduced subcutaneous adipose tissue mass in animals fed the carbohydrate-free diet are mediated, in part, by lower FAS expression in both the liver and adipose tissue. Moreover, the beneficial effects of the carbohydrate-free diet on lipogenic gene expression are completely reversed upon addition of a small (10 en%) amount of sucrose to the high-fat diet. To clarify the mechanisms responsible for these changes, additional studies are required. Future studies should include the assessment of total carcass fatty acid content to clarify the contributions of adipose tissue to changes in body weight. Additional studies should also endeavor to control the fatty acid composition of the diets by standardizing the source of dietary fat in all experimental groups. Furthermore, future studies should determine if the improvements in body composition observed in the present studies can be duplicated when dietary fat is supplied as polyunsaturated or monounsaturated long chain fatty acids.

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II. Effect of Dietary Carbohydrate Source on the Development of Obesity in aP274-*Agouti* Transgenic Mice

Abstract

We evaluated the effects of *ad libitum* or energy restricted (70% of *ad libitum*) high-fat diets (basal) varying in carbohydrate source on adiposity in aP2-agouti transgenic mice. In meal response tests, the diet containing mung bean noodles (MUNG) as the sole source of carbohydrate evoked a significantly lower glucose response ($p<0.05$) compared to all other foods. Moreover, the response to the sweetened instant oatmeal (IO-S) was approximately 23% greater ($p<0.05$) than the response to the flavored instant oatmeal (IO-F), which was similar to the rolled oats (ROLL).

Weight gain in animals consuming the ROLL diet *ad libitum* for 6 weeks was 50-66% ($p<0.05$) lower compared to all other diets consumed *ad libitum*. While energy restriction during weeks 6-12, irrespective of diet, reduced body weight by 21-28% compared with animals continued on the basal diet *ad libitum*, final body weight was 17% higher in the basal-R compared with the ROLL diet, despite equivalent energy restriction.

Subscapular fat pad mass was significantly higher ($p<0.05$) in the energy restricted basal and IO-S groups compared with the ROLL diet, at the same level of energy intake. Despite equivalent energy restriction, mass of both the retroperitoneal and perirenal fat pads were significantly lower in animals fed the ROLL and MUNG diets ($p<0.05$ for both) compared with the basal and IO-S diets.

Plasma leptin levels were significantly lower ($p<0.05$) in animals fed the energy restricted MUNG diet compared with similarly energy restricted basal and IO-S fed

animals. Consumption of the basal diet, either *ad libitum* or with energy restriction, led to larger adipocytes ($p<0.05$) compared with both ROLL and MUNG diets.

In *ad libitum* fed animals, the level of expression of fatty acid synthase in the liver was significantly lower in animals consuming the ROLL and MUNG diets compared with all other groups. Expression of fatty acid synthase and PPAR-α in retroperitoneal adipose tissue was significantly higher following unrestricted consumption of the basal diet for 6 weeks relative to all other diets consumed *ad libitum* for the same length of time. Expression of UCP-2 in retroperitoneal adipose tissue was enhanced by the basal diet ($p<0.05$) consumed *ad libitum* for 6 weeks and was reduced in ROLL fed ($p<0.05$) animals compared to animals consuming the IO-S and IO-F diets *ad libitum*. Similarly, expression of PPAR-γ in the retroperitoneal depot was enhanced by the basal diet relative to all other diets ($p<0.05$) and was lower in ROLL-fed ($p<0.05$) animals compared with animals fed the IO-S and IO-F diets *ad libitum* for 6 weeks.

Expression of gastrocnemius UCP-3 was 57% ($p<0.05$) greater in animals fed the energy-restricted MUNG vs. basal diets, while PPAR-α in soleus of MUNG animals was increased by 70% ($p<0.05$) vs. the basal diet at the same level of energy intake. These data suggest that dietary carbohydrate source modulates adipose tissue accumulation and body weight by shifting substrate utilization in favor of lipid oxidation in skeletal muscle as well as reducing lipogenesis in visceral adipose tissue.

Introduction

There is considerable controversy surrounding the role of dietary carbohydrate in the development and/or prevention of obesity and insulin resistance (1-4). In fact, most dietary prescriptions for the prevention and treatment of overweight and obesity focus on reducing dietary fat consumption (2,5-7). However, weight loss induced by fat-reduced diets in generally modest and short-lived (8,9).

There is no doubt that different carbohydrates vary considerably in their rate of absorption. Furthermore, blood glucose and insulin responses are influenced by the amount and type of carbohydrate in the diet (10). The Western diet typically contains large quantities of soft drinks, corn syrup, potatoes, and processed grain products; all of which contain readily digestible, refined carbohydrates (11,12). The metabolic response to these foods is characterized by rapid increases in blood glucose and insulin levels (13-15). However, some forms of dietary starch, often referred to as complex carbohydrates, elicit similar responses and consequently, the American Diabetes Association (1) has concluded that the chemical form of dietary carbohydrate has little clinical relevance.

The metabolic benefits of low glycemic index foods stems from the slower rate of glucose absorption from the small intestine, occurring following their consumption (10). The slower rate of glucose absorption from the small intestine is associated with a reduction in the postprandial rise in gut hormones and insulin (10). Overtime, these hormonal changes serve to maintain lower free fatty acid and counter-regulatory hormone levels, in association with lower blood glucose levels, and may alter the pattern of substrate utilization over the long term. Higher postprandial increases in blood glucose and insulin levels preferentially promote carbohydrate oxidation, with a

reduction in the capacity for fatty acid oxidation (16,17). Reduced capacity for fat oxidation has been shown to promote weight gain in rodent models, as well as humans (18,19).

Byrnes, et al. (20) have shown that insulin resistance can be induced by feeding rats a high-glycemic index starch diet. After 9 weeks of consuming a diet rich in rapidly digested, high-glycemic index amylopectin starch, Sprague-Dawley rats demonstrate slower clearance of an intravenous glucose bolus and secrete twice as much insulin to clear the glucose load than did rats fed a diet containing more slowly digested, amylose starch. Rat strain appear to exert a passive influence on the development of insulin resistance induced by amylopectin feeding since type of dietary starch had no effect on clearance of a glucose load in male Wistar rats. Nonetheless, plasma insulin response to the glucose challenge began to diverge in Wistar rats after 8 weeks of dietary intervention, such that after 12 weeks of consuming the high-amylopectin diet, the plasma insulin response to a glucose challenge was 100% greater than in amylose-fed rats. Moreover, basal plasma insulin levels in rats fed the high- amylopectin diet were almost double those seen in rats fed the high-amylose diet. These findings suggest that high-glycemic index diets contribute to the deterioration of insulin sensitivity and glucose tolerance (20). Perhaps more importantly, Wiseman, et al. (21) have shown that insulin resistance induced by amylopectin feeding could be neither reversed nor prevented by a period of amylose feeding in normal rats.

Despite the evidence supporting a role for dietary carbohydrate source *per se* in the development of insulin resistance in rodents, many professional organizations have concluded that the chemical form of dietary carbohydrate source has little clinical

relevance (1,22). These conclusions are largely based on studies which demonstrate similar postprandial blood glucose and insulin responses to dietary sucrose and starch or the lack of a relationship between discretionary sucrose intake and weight loss (23-27). Furthermore, prevailing dietary recommendations (1,22,28) emphasize increasing consumption of complex carbohydrates by including among other things, high-glycemic index foods such as rice and potatoes (13,29). This may complicate the interpretation of studies designed to assess the metabolic effects of increasing dietary carbohydrate intake since many starchy foods are rapidly digested and produce higher glycemic and insulinemic responses than sucrose (13-15,26,29). West, et al. (27) found no difference in the amount of weight lost by subjects consuming hypocaloric, reduced-fat (33 en%) diets at two levels of sucrose intake. Nonetheless, epidemiological studies consistently demonstrate that replacing refined grains and potatoes with whole-grain, minimally processed foods, along with increasing consumption of fruits and vegetables, lowers dietary glycemic load and reduces insulin demand, which may influence risk of obesity and associated comorbidities (30-33).

The relationship between whole-grain intake and metabolic risk factors for type 2 diabetes and cardiovascular disease was examined using data gathered during the Framingham Offspring Study (33). After adjusting for potential confounding dietary factors (intake of polyunsaturated fatty acids, fish, meat, fruit and vegetables), whole-grain intake was found to be inversely associated with body mass index ($p<0.05$), waist-to-hip ratio ($p<0.05$), LDL-cholesterol ($p<0.02$), and fasting insulin levels ($p=0.03$) (33). Consistent with this, data from the Nurses' Health Study (30) found a significant positive association between dietary glycemic load and risk of coronary heart disease, particularly

in women with above-average body weights. In addition, glycemic index was a stronger predictor of coronary heart disease risk than was the usual classification of simple versus complex carbohydrates (30).

The clinical utility of glycemic index may depend on the ability of health professionals to properly define glycemic index and distinguishing between complex carbohydrates and low glycemic index foods. This obstacle may have indirectly promoted the use of low-carbohydrate diets (34,35), as health professionals and consumers attempt to mimic the physiological benefits of low glucose and insulin response diets at the expense of dietary carbohydrate consumption. The popularity of the Atkins' Diet (34) and the Zone Diet (35) may be attributable to the simplicity of advocating carbohydrate restriction, as opposed to the consumption of carbohydrates, which would result in postprandial responses similar to low carbohydrate diets (31,33).

Short-term, uncontrolled studies indicate that the Atkins' diet results in significant weight loss after 8 weeks, with maximum sustained weight loss of 10.3% of initial body weight after 24 weeks (36,37). However, until recently there were no long-term studies of the efficacy of low-carbohydrate, high-protein, high-fat diets for weight loss and reduced risk factors for coronary artery disease. The first long-term (one-year), randomized, controlled trial of such a diet was conducted by Foster, et al. (38). While subjects on the low-carbohydrate diet had lost more weight than subjects on a conventional (low-fat) diet at 3 ($p=0.001$) and 6 ($p=0.02$) months, there was no significant difference in the amount of weight lost between diets at 12 months. The difference in weight loss in the first six months was related to an overall greater energy deficit in the low-carbohydrate groups, despite unrestricted protein and fat consumption

by these subjects and instructions to restrict energy intake in the conventional diet group. Both diets improved insulin sensitivity, as determined by an oral glucose tolerance test; progressively less insulin was secreted to maintain euglycemia (39). Thus, these data do not demonstrate an effect of dietary macronutrient composition, independent of weight loss, on insulin sensitivity. Nonetheless, the low-carbohydrate diet was associated with greater improvements in serum levels of HDL-cholesterol ($p<0.05$) and triglyceride ($p<0.05$) after 12 months of dietary intervention compared with subjects consuming the conventional diet. The magnitude of reduction in serum triglycerides and increase in HDL-cholesterol levels with the low-carbohydrate diet was similar to that obtained by pharmacological treatments, such as fibrates and niacin (40). With respect to the improvements in serum lipids, the benefit of the low-carbohydrate diet (38) was greater than that expected by moderate weight loss alone (41), suggesting the reduction in dietary carbohydrate was at least partially responsible. This is consistent with previous studies demonstrating that high-carbohydrate, low-fat diets decrease HDL levels and increase serum triglyceride concentrations, whereas low-carbohydrate, high-fat diets reduce serum triglycerides and increase HDL levels (37,42-45).

Using the USDA's Continuing Survey of Food Intakes by Individuals 1994-1996 (CSFII 1994-1996), Bowman, et al. (46) evaluated the relationships between carbohydrate consumption, dietary energy content, nutrient quality, and body mass index. Analysis of subjects consuming a wide range of carbohydrate intake, indicated those deriving more than 55% of total daily energy needs from dietary carbohydrate consumed 200 grams more total food per 1000 kcals but the lower energy density of the diet reduced total caloric intake by 200-300 calories per day compared with subjects in

whom dietary carbohydrate accounted for a smaller percentage of total daily energy intake. Across a range of carbohydrate intake, it was found that as dietary fiber and carbohydrate intake increased, consumption of protein, total fat and saturated, polyunsaturated and monounsaturated decreased. Adults consuming a high-carbohydrate diet had the lowest total fat intake per 100 grams of food compared to subjects consuming lesser amounts of carbohydrate. Women in whom dietary carbohydrate provided at least 55% of total daily energy had lower mean BMI levels ($p<0.05$) compared to women consuming less carbohydrate; there was a nonsignificant trend for lower BMI in men with higher carbohydrate intake. Overall, adults who obtained more than 55% of total dietary energy from carbohydrates consumed energy-restricted diets, rich in whole-grain foods and fruits and vegetables. Importantly, diets containing less than 30% of total energy from carbohydrates were not necessarily energy-restrictive or nutritious compared with high-carbohydrate diets. This finding is particularly important when considering the popularity of carbohydrate-restricted diets (34,35), most of which recommend carbohydrate intake at levels lower than in subjects classified as having very low carbohydrate intake in the study by Bowman, et al. (46).

In a study by Wolever, et al. (47), subjects with impaired glucose tolerance were given dietary counseling to encourage consumption of a weight maintaining, high-carbohydrate, low-glycemic index diet for four months (47). As a result, these subjects experienced a significant reduction in total daily energy intake and an increase in dietary fiber consumption. Moreover, subjects increased their consumption of low-glycemic index starchy foods by 11%, while there was an 8% reduction in intake of high-glycemic index starchy foods. In contrast, a separate group of subjects counseled on the

consumption of a high-carbohydrate, high-glycemic index diet consumed more energy, and increased their consumption of high-glycemic index starchy foods by 8%, while reducing intake of low-glycemic index starchy foods by 4%. Fasting glucose levels tended to rise less on the low-glycemic index versus the high-glycemic index diet. The low-glycemic index diet was associated with increased β-cell responsiveness, relative to baseline measures, as indicated by a reduction in the amount of insulin secreted following an intravenous glucose bolus, which occurred in the absence of changes in insulin sensitivity. The glucose disposition index (DI), calculated based on estimates of insulin sensitivity and β-cell responsiveness to intravenous glucose, is used as an index of the ability of the β-cell to compensate for changes in insulin sensitivity by increasing insulin secretion (48). Consumption of the low-glycemic index diet for four months significantly improved DI (+56%, $p<0.05$ vs. baseline), whereas the high-glycemic index diet failed to improve β-cell responsiveness or glucose disposition (47). This represents an important physiological effect of the low-glycemic index diet since the critical factor associated with the deterioration of impaired glucose tolerance to diabetes is decreased insulin secretion in response to a glucose challenge (49).

Advocates of carbohydrate-restricted diets (34,35) often argue that high-carbohydrate diets increase serum triglycerides and reduce HDL levels, thereby enhancing risk of cardiovascular disease (30,31). However, these changes may be a function of an increase in dietary glycemic load occurring in response to increased carbohydrate consumption. While dietary glycemic load can be altered by changing carbohydrate intake or reducing dietary glycemic index (50), data from Wolever, et al. (47) suggest these manipulations have different effects in subjects with impaired glucose

tolerance. Specifically, reducing dietary glycemic index improved HbA_{1c}, diastolic blood pressure and glucose disposition, while reducing dietary carbohydrate intake in the context of a high-monounsaturated diet failed to improve these parameters (47).

Based on the controversy surrounding the practical utility of the glycemic index and the popularity of carbohydrate-restricted diets, we designed studies to assess the metabolic response to high-fat diets varying in dietary carbohydrate source. The influence of processing and added sucrose was studied by comparison of instant oatmeal with added sugar to rolled oats. Furthermore, we have attempted to ascertain the importance of endogenous dietary fiber in the metabolic benefits of low glycemic index foods by comparing two foods (rolled oats and mung bean noodles) previously shown to elicit low blood glucose responses but which differ in content of endogenous dietary fiber.

Materials and Methods

Animal Model

The mouse *agouti* gene encodes a 131 amino acid secreted protein that is transiently expressed in neonatal skin to regulate coat color pigmentation in a paracine manner (51,52). Placing the *agouti* gene under the control of the β-actin promoter in mice or dominant mutations at the agouti locus, results in ubiquitous *agouti* expression and the development of "yellow obese syndrome", a phenotype characterized by yellow coat color, obesity, hyperinsulinemia, insulin resistance, increased body length and susceptibility to neoplasia (53,54). In white adipose tissue of *agouti* mutant mice, elevated rates of lipogenesis occur concomitantly with decreased rates of lipolysis (54).

Furthermore, recombinant *agouti* protein has been shown to increase fatty acid synthase activity and triglyceride accumulation in adipocytes in vitro (55).

The human homologue of the *agouti* protein is 132 amino acids long and functionally similar to the mouse protein (56). However, the human gene is normally expressed in adipose tissue, in obese and nonobese individuals (56). This adipose tissue specific expression pattern is mimicked in aP2-*agouti* transgenic mice, in whom the *agouti* gene is placed under the influence of the adipocyte fatty acid binding protein (aP2) promoter (57). These animals express recombinant *agouti* protein specifically in adipose tissue and have a uniformly yellow/brown coat color (57). Unlike animals overexpressing normal *agouti* protein, aP2-*agouti* transgenic mice become overweight only when hyperinsulinemia is induced by daily subcutaneous insulin injections (57) or by chronic ingestion of a high-sucrose diet (58). This study was approved by the University of Tennessee Institutional Animal Care and Use Committee.

Comparison of US17 and High Fat, High Sucrose Diet

We compared the effects of two diets on weight gain and adiposity in aP274-*agouti* transgenic mice fed ad libitum for four weeks to determine if these diets differed in their ability to promote the development of obesity. Table 6 summarizes the macronutrient composition and energy content of these diets. The basal diet is a high-fat, high-sucrose diet (Research Diets, New Brunswick, NJ) previously used in this laboratory to induce obesity in aP2-agouti transgenic mice (59), and was compared to the US17 diet (Monsanto, St. Louis, MO), which has a fatty acid profile similar to the typical Western diet, with the following energy distribution: 44.4 en% carbohydrate, 35.4 en% fat, and

Table 6

Macronutrient Composition of US17 and High-Fat, High-Sucrose Diet

	US17	High-Fat, High Sucrose
Nutrient	**g/kg diet**	
Casein	229	160
Sucrose	114	637.9
Cornstarch	86	-----
Cellulose	57	50
Maltodextrin 10	86	-----
Soybean Oil	-----	100
Lard	-----	10
Cocoa Butter	43	-----
Linseed Oil	5	-----
Palm Oil	60	-----
Safflower Oil	33	-----
Sunflower Oil	31	-----

21.2 en% protein (60). The high-fat, high-sucrose (HFS) diet contained 62 en% carbohydrate (as sucrose), 24 en% fat (soybean oil and lard) and 14 en% protein (59).

Animals were given free access to either the US17 or high-fat, high-sucrose basal diet ad libitum for 4 weeks. At the end of the feeding period, animals were sacrificed by cardiac exsanguination under anesthesia. Blood was collected into an EDTA containing vacutainer blood collection tube and stored on ice prior to centrifugation at 1000 rpm for 10 minutes to collect plasma. The liver, representative fat pads (retroperitoneal, subscapular, epididymal) were dissected, weighed and immediately snap frozen.

Meal Tolerance Tests

The blood glucose response to test foods was determined in fasted aP274-*agouti* transgenic mice. Food was removed from the animal's cages at 07:00 and animals fasted for 8 hours. Blood glucose levels were determined using the One Touch Ultra hand held

glucometer (Johnson and Johnson, Malpitas, CA). A small amount of blood was obtained by tail stick using a 1 cc standard insulin syringe. After determining fasting blood glucose levels, animals were given access to a standard amount (1 gram/kg body weight) of one of four test diets: mung bean (MUNG), sweetened instant oatmeal (IO-S), flavored instant oatmeal (IO-F), or rolled oats (ROLL). Blood glucose levels were determined by tail stick as previously described at 30, 45, 60, and 120 minutes after food ingestion for calculation of the area under the blood glucose curve ($AUC_{glucose}$) (61). The $AUC_{glucose}$ was determined as follows: Σ((0.5*(blood glucose concentration at 30 min + baseline blood glucose concentration)*(30 min-0 min))+(0.5*(blood glucose concentration at 45 min + blood glucose concentration at 30 min)*(45 min-30 min))+(0.5*(blood glucose concentration at 60 min + blood glucose concentration at 45 min)*(60 min-45 min))+(0.5*(blood glucose concentration at 120 min + blood glucose concentration at 60 min)*(120 min-60 min)))*grams of carbohydrate consumed.

Phase I: Ad Libitum Energy Intake

Animals and Diets

Sixty aP274-*agouti* transgenic mice of 6 to 8 weeks in age were adapted to handling for use in this study. Animals were housed in pairs or individually in standard shoe-box rodent cages in a climate-controlled (37° C) environment with a 12-h light-dark cycle. Prior to this study, animals had been maintained on standard rodent chow. Animals were randomly assigned by body weight (27.34±0.46 g, Mean±SEM) to one of the five dietary treatment groups (n=12 per treatment). All diets (Table 7) were AIN-93 based (62), and were custom prepared by Research Diets, Inc. (New Brunswick, NJ). The basal

Table 7

Composition of Test Diets Varying in Carbohydrate Source

Nutrient	Basal	Mung Bean Noodle	Instant Oatmeal, Sweetened	Instant Oatmeal, Flavored	Rolled Oatmeal
	grams per kilogram diet				
Casein	160	158.6	68.2	68.6	14.4
DL-Methionine	3	3	3	3	3
Sucrose	637.9	------	------	------	------
Cornstarch	------	------	------	------	------
Dextrinized Cornstarch	------	------	------	------	------
Mung Bean Noodles	------	741	------	------	------
Instant Oatmeal, Sweetened	------	------	842.1	------	------
Instant Oats			581		
Sucrose			235.8		
Vitamin-Mineral Pre-Mix #42			25.4		
Instant Oatmeal, Flavored	------	------	------	847.5	------
Instant Oats				584.8	
Sucrose				237.3	
Vitamin-Mineral Pre-Mix #44				25.4	
Rolled Oats	------	------	------	------	935
Cellulose	50	46.3	0	0	0
Soybean Oil	100	100	59.4	59.7	35.5
Lard	10	10	10	10	10
TBHQ	0.014	0.014	0.014	0.014	0.014
Mineral Mix, (AIN-93G-MX)	7	------	------	------	------
Mineral Mix, (special formulation)	------	3.5	3.5	3.5	3.5
Calcium Carbonate	8.3	7.84	1.36	2.7	11.38
Calcium Phosphate, Dibasic	5.7	5.7	4.82	4.82	0
Potassium Phosphate, Monobasic	8	7	0	0	0
Potassium Citrate	3.8	4.4	4.28	4.36	0.91
Sodium Chloride	13.75	13.55	8.63	0	13.7
Vitamin Mix	10	10	10	10	10
Choline Bitartrate	2.5	2.5	2.5	2.5	2.5
Macronutrient Composition, g/kg diet					
Protein, grams	143.8	143.8	143.8	143.8	143.8
Carbohydrate, grams	647.9	648	648.1	648	647.7
Fat, grams	110	110.4	110.2	110.2	110
Fiber, grams	50	50	56.6	57.9	87.9
Energy Distribution, % per kcal					
Protein, % per kcal	14	14	14	14	14
Carbohydrate, % per kcal	62	62	62	62	62
Fat, % per kcal	24	24	24	24	24

diet (Basal) was a high-fat, high-sucrose diet which has been previously used in our laboratory to induce increased adiposity, hyperinsulinemia, and weight gain in this animal model (59). In the MUNG and ROLL diets, dietary carbohydrate was derived from a single source: mung bean noodles and rolled oats, respectively. The remaining diets were designed to mimic commercially available instant oatmeal with added sugar (IO-S) and flavored instant oatmeal with added sugar (IO-F). Because these diets were formulated to be comparable to commercially available products, there were some differences in the vitamin and mineral content, as well as energy content. These differences were attributable to the pre-mixed vitamin and mineral supplements supplied by our sponsor (The Quaker Oats Company), the composition of which are shown in Table 8. All diets were designed to provide 14 en% protein, 62 en% carbohydrate, and 24 en% fat and contained 4.16 kcal/g. With the exception of potassium, the vitamin and mineral content of the diets was standardized to the content provided by standard rodent chow (62). The rolled oatmeal diet contained a significantly greater amount of potassium and therefore, the potassium content of all other diets was standardized to this level. The fiber content of the basal, mung bean, and both instant oatmeal diets was raised to be equivalent to standard rodent chow (1.6% fiber per kcal) (62). The rolled oatmeal diet contained a significantly greater amount of fiber (2.1% fiber per kcal) than all other diets. However, since fiber content influences glycemic index, the fiber content of this diet was not adjusted (63).

All animals had *ad libitum* access to food and water during the six week feeding period. Food intake and water were monitored and replaced daily.

Table 8

Nutrient Composition of Vitamin-Mineral Pre-Mixes Provided by Quaker Oats Company

Nutrient	Pre-Mix #42 for Instant Quaker Oats Sweetened Variety	Pre-Mix #44, for Instant Quaker Oats Flavored Variety
	Amount in 100 grams of Pre-Mix	
Sodium	7890 mg	21200 mg
Potassium	184 mg	137 mg
Vitamin A	122000 IU	95000 IU
Thiamin	30 mg	27 mg
Riboflavin	37 mg	33 mg
Niacin	540 mg	427 mg
Calcium	10900 mg	8770 mg
Iron	813 mg	323 mg
Vitamin E	0.3947 IU	0.0924 IU
Vitamin B_6	61.30 mg	57.2 mg
Folic Acid	8560 µg	7600 µg
Phosphorus	364 mg	272 mg
Magnesium	97.50 mg	48.9 mg
Zinc	1.27 mg	0.32 mg
Copper	2 mg	2 mg
Pantothenic Acid	0.45 mg	0.47 mg
Manganese	5.05 mg	2.01 mg
Salt	20.79 g	56.48 g
Carbohydrate, total	4.84 g	7.86 g
Sugars, total	1.14 g	1.01 g
Protein	4.84 g	1.23 g
Fat, total	2.78 g	0.675 g
Saturated Fat	0.5113 g	0.1119 g
Polyunsaturated Fat	1.09 g	0.259 g
Monounsaturated Fat	1.06 g	0.258 g
Total Dietary Fiber	15.66 g	7.878 g
Insoluble Fiber	1.50 g	0.4238 g
Soluble Fiber	14.16 g	7.4538 g
β-glucan	0.70 g	0.1445 g
Total Energy	138.75 kcal	42.4219 kcal

Phase II: Restricted Energy Intake

Animals and Diets

This study was divided into two 6-week stages. In the first stage (6 weeks), forty-eight male aP274-*agouti* transgenic mice were given *ad libitum* access to the basal (high-fat, high-sucrose) diet. Body weight was monitored every five days for 6 weeks. Following this first stage, animals were randomly assigned (by body weight, 34.57± 0.68 g) to one of seven treatment groups (n=8 per treatment group). One group was continued *ad libitum* on the basal diet (basal). The other groups were maintained with energy restriction (-R; 70 en% ad lib) by using a pair-fed protocol, as follows. The mice in the basal-R group were continued on the basal diet with energy restriction. The remaining groups received either the MUNG, ROLL, IO-S, or IO-F diet with energy restriction. At the end of this stage, animals were sacrificed and tissues collected as described below.

Determination of Weekly Fasting Blood Glucose Levels

Food was removed from the animal's cages at 07:00 and animals fasted for 8 hours. Fasting blood glucose levels were monitored weekly. A minimal amount of blood was obtained by sticking the animal's tail with a standard 1cc insulin syringe and blood glucose level determined using the One Touch Ultra hand held glucometer (Johnson and Johnson, Malpitas, CA). The glucometer was calibrated according to the manufactor's instructions prior to testing each week.

Collection of Fasting Blood Samples and Preparation of Plasma

Two days prior to sacrifice, 1 cc of whole blood was obtained by tail stick using a 1 cc insulin syringe from fasted animals. The blood was transferred to an EDTA-treated vacutainer blood collection tube and stored on ice until centrifuged at 1000 rpm for 10 minutes to obtain plasma. Plasma was transferred to 0.5 ml eppendorf tubes and stored at -20° C until time of analysis.

Sacrifice of Animals

Animals were sedated in a sealed plexiglass box saturated with CO_2 prior to cardiac exsanguination. Blood was collected from unfasted animals by cardiac puncture using a heparinzed 5 cc syringe and a 20 gauge 1-inch needle which was then transferred to an EDTA-treated vacutainer blood collection tube. All samples were stored on ice until centrifuged at 1000 rpm for 15 minutes to collect plasma. Plasma was stored at -20 °C until time of analysis.

Dissection and Processing of Tissues

Following cardiac exsanguination, the liver, selected fat pads (epididymal, subscapular, retroperitoneal, perirenal), and selected contralateral muscles (soleus, gastrocnemius) were immediately dissected and weighed. The liver, epididymal fat pad, subscapular fat pad, soleus muscle and gastrocnemius muscles were snap frozen in liquid nitrogen and stored at -80° C until time of analysis.

Collection and Treatment of Perirenal Fat Pads for Basal and Isoproterenol-Stimulated Lipolysis and Determination of Glycerol Release

Immediately upon dissection, the perirenal fat pad was divided into two sections of approximately equal mass and placed into appropriately labeled six-well cell culture plates containing freshly prepared 1X Dulbecco's Modified Eagle Medium (DMEM; with high glucose, L-glutamine, 25 mM HEPES buffer, pyridoxine chloride, without sodium pyruvate; Life Technologies, Carlsbad, CA) supplemented with 1% fetal bovine serum (FBS) and 1% bovine serum albumin fraction V (BSA; Life Technologies, Carlsbad, CA). All samples were allowed to equilibrate at 37°C, prior to beginning the assay. To determine basal glycerol release, one section of the perirenal fat pad was transferred to an appropriately labeled well of a 24-well cell culture plate containing 1 ml of Kreb's Ringer Buffer (KRB; 129 mM NaCl, 5 mM $NaHCO_3$, 4.8 mM KCl, 1.2 mM KH_2PO_4, 1.0 mM $CaCl_2$, 1.2 mM $MgSO_4$, 5 mM glucose, 20 mM HEPES, 1% BSA, pH 7.3). For stimulated lipolysis, 1 µl of 100 µM isoproterenol was added to KRB prior to transferring the remaining perirenal fat pad section to the cell culture plate. The plates were then incubated at 37° C, 5% CO_2 with 90% humidity for 2 hours. Following incubation, both the media and fat pads were transferred to appropriately labeled eppendorf tubes and stored at -20° C for further analysis.

Following the procedure described by Boobis, et al. (64), lipolysis was assessed by glycerol release, since free fatty acids can be reesterified by adipocytes and therefore, may underestimate lipolysis. Conversely, glycerol reesterification by adipocytes is negligible. The method used for determination of glycerol in the lipolysis medium is based on the conversion of glycerol to dihydroxyacetone in the presence of NAD catalyzed by

glycerol dehydrogenase. Prior to beginning the assay, the media aliquots collected following incubation of fat pads with lipolysis medium were allowed to thaw on ice.

To establish a standard curve, 100 mM glycerol was prepared by diluting 100 µl of 13.6 M glycerol (Sigma-Aldrich, St. Louis, MO) in 13.5 ml of freshly prepared KRB with 1% BSA, as previously described. The working solution of 10 mM glycerol was prepared by dilution of 500 µl 100 mM glycerol in 4.5 ml KRB. The standard curve was generated by serial dilution of 10 mM glycerol to achieve a range of 0 to 10 mM glycerol. Subsequently, 25 µl of each standard (0, 1, 2.5, 5, 7.5, and 10 mM glycerol) and sample was pipetted into appropriately labeled wells of a 96-well Packard black bottom plate (Hewlett Packard, Palo Alto, CA).

After samples had been pipetted into 96-well plates, the reaction mixture was prepared as follows. Hydrazine buffer containing 0.2 M 100% hydrazine hydrate (a ketone-trapping agent), 0.1 M 2-amino-2-methyl-1-propanol, and 1 mM EDTA in distilled water was prepared and the pH adjusted to 9.9 with 12.4 N HCl. For each standard and sample assayed, 100 µl of reagent mixture containing 4 µl β-NAD (500 mM), 200 µl hydrazine buffer and 1 µl glycerol dehydrogenase (from *Enterobacter aerogenes*, Roche Applied Sciences, Indianapolis, IN) was prepared and added to each well of the 96-well plate containing the standards and samples. The samples were allowed to incubate for 90 minutes at room temperature with gentle shaking. In the presence of hydrazine and at pH 9.9, the reaction proceeds to completion and NADH is formed in stoichiometric quantities relative to glycerol in the medium. The quantity of NADH formed is then determined by dual wavelength spectrophotometry with excitation at 380 nm and emission at 520 nm.

Quantitation for each sample was normalized for the protein content of each section of perirenal fat pads for each animal. Each fat pad section was homogenized in 1 ml KRB, which was prepared as previously described with one exception. Since these samples were to be assayed for protein content, BSA was not added to the KRB. Following homogenization, the protein content of each sample was determined as described by Bradford (65) and modified for use with Coomassie® Plus Protein Assay Reagent (Pierce, Rockford, IL) by Spector (66).

A fresh set of protein standards was prepared in glass culture tubes by serial dilution of BSA stock standards (2.0 mg/ml, Pierce, Rockford, IL) in KRB to achieve a range of 125-2000 μg/ml. Subsequently, 50 μl of each standard and sample was transferred to appropriately labeled glass culture tubes and 1.5 ml of Coomassie® Plus reagent added to each. The absorbance of each standard was determined by measuring the absorbance at 595 nm against a water reference sample. The standard curve was prepared by plotting the absorbance of each standard against its respective concentration in μg/ml. The concentration of protein in each sample was then determined using the standard curve. Subsequently, the concentration of glycerol in each sample was normalized for the protein content of the sample.

Cell Sizing

Cell sizing was determined following the 12 week protocol. Due to the fact that energy restriction was employed during the final six weeks of this study, the size of the retroperitoneal fat pad limited our analysis to cell sizing, without analysis of gene expression in this depot. Upon dissection, the abdominal fat pad was placed into

appropriately labeled wells of a 6-well culture plate containing DMEM (Life Technologies, Carlsbad, CA) supplemented with 1% FBA and 1% BSA. The fat was then transferred to a culture dish containing 3 ml of 0.8 mg/ml collagenase (Sigma-Aldrich, St. Louis, MO) in Hank's Balanced Salt Solution (8 g/L NaCl, 400 mg/L KCL, 60 mg/L KH_2PO_4, 47.86 mg/l Na_2HPO_4, 1 g/L glucose). The sample was digested in this medium for 30 minutes at room temperature with gentle shaking. Following digestion, the digested sample was passed through 400 micron nylon mesh into a 50 ml centrifuge tube. The sample was allowed to sit for 1-2 minutes to permit the adipocytes to separate from the digestion medium. Once the cells had formed a discrete layer, the lower aqueous layer was removed with a transfer pipet being careful not to disturb the upper cell layer. The cells were then rinsed twice in DMEM supplemented with 1% FBS and 1% BSA. Following removal of DMEM, cells were rinsed twice in Isoton II Diluent (7.93 g/l NaCl, 0.38 g/l Na_2EDTA, 0.4 g/l KCl, 1.95 g HNa_2PO_4, 0.3 g/l NaF; Beckman-Coulter, Providence, RI). Following the final rinse, 500 µl of cell suspension was carefully pipeted from the upper layer and transferred to a vessel containing 150 ml of Isoton II diluent and measured by the Coulter Counter principle (67) using the MultiSizer 3 System (Beckman-Coulter, Providence, RI).

Using the MultiSizer 3, adipocytes are suspended in Isoton II diluent, a weak electrolyte solution, and subsequently, are drawn through a small aperture separated by two electrodes between which an electric current flows. Voltage is applied across the aperture, creating a sensing zone. As particles pass through the sensing zone, an equal volume of conducting diluent is displaced, momentarily increasing the impedance of the aperture. This change in impedance produces a small but proportional current. The

current fluctuation is amplified and converted into a voltage pulse large enough to be accurately quantified and measured. The Coulter principle states that the amplitude of the voltage pulse is directly proportional to the volume of the particle that produced the fluctuation. Scaling of the height of the voltage pulses in volume enables a size distribution to be acquired and displayed (67).

Determination of Final Plasma Glucose Levels

Final plasma glucose levels were determined using the glucose oxidase method as first described by Washko, et al. (68) and modified by Marks (69). This procedure is based on the following coupled enzymatic reactions: In the presence of water and oxygen, glucose oxidase catalyzes the conversion of glucose to gluconic acid and hydrogen peroxide. With hydrogen peroxide and peroxidase available, the otherwise colorless o-diansidine dihydrochloride is oxidized and the product of the reaction is brown in color. The intensity of the brown color measured spectrophotometrically at 475 nm is proportional to the original glucose concentration of the sample. To quantify the glucose content of the samples, the absorbance of a glucose standard (100 mg/dL, Sigma-Aldrich Diagnostics, St. Louis, MO) was determined. The absorbance of the sample was divided by the absorbance of the standard and this ratio multiplied by the concentration of the glucose standard.

Reagents for determination of plasma glucose levels were obtained from Sigma-Aldrich Diagnostics (St. Louis, MO). The enzyme solution was prepared by adding 1 capsule of PGO enzymes to 100 ml distilled water in an amber bottle. Each capsule of PGO enzymes contains 500 units of glucose oxidase (*Aspergillus niger*) and 100 U/L

horseradish peroxidase. The color reagent solution was prepared by reconstituting one vial (50 mg) of o-dianisidine dihydrochloride with 20 ml of distilled water. To prepare the combined enzyme-color reagent solution, 1.6 ml of color reagent solution was added to 100 ml of enzyme solution and mixed by inversion.

For each sample, 25 µl of plasma was transferred to an appropriately labeled borosilicate glass culture tube containing 5 ml of combined enzyme-color reagent solution. A standard was similarly prepared using a known glucose standard solution (100 mg/dL, Sigma-Aldrich Diagnostics, St. Louis, MO). The tubes were vortexed, covered in foil to protect them from exposure to ambient light, and allowed to incubate at room temperature for 45 minutes. At the end of the incubation period, the absorbance was read at 475 nm against a water blank. The concentration of glucose in each sample was calculated by dividing the absorbance of the samples by the absorbance of the glucose standard, and multiplying by the concentration of the glucose standard.

Determination of Fasting Plasma Triglyceride Levels

Plasma triglyceride levels were determined according the technique described by Buccolo and David (70). Using this method, triglycerides are first hydrolyzed by lipoprotein lipase to yield glycerol and free fatty acids. Glycerol is then phosphorylated in an ATP-dependent reaction by glycerol kinase yielding glycerol-1-phosphate. The glycerol-1-phosphate is oxidized to dihydroxyacetone phosphate, with the concomitant reduction of NAD to NADH in a reaction catalyzed by glycerol-1-phosphate dehydrogenase. The resulting NADH is then oxidized, coupled to reduction of 2-(p-iodophenyl)-3-p-nitrophenyl-5-phenyltetrazolium chloride (INT) to formazan, which is

pink in color, in the presence of diaphorase. The intensity of the color produced is directly proportional to the triglyceride concentration in plasma.

The reagents used in this assay were obtained from Sigma-Aldrich Diagnostics (St. Louis, MO). Plasma (10 μl) was transferred to a borosilicate glass culture tube containing 1 ml reconstituted triglyceride reagent (2.0 mM ATP, 2.0 mM NAD, 3.0 M Mg^{2+}, 1.0 mM 2-(p-iodophenyl)-3-p-nitrophenyl-5-phenyltetrazolium chloride, 200 U/L glycerol kinase, 4000 U/L glycerol-1-phosphate dehydrogenase, 455 U/L diaphorase, pH 7.8). A standard was similarly prepared using a known triglyceride standard (250 mg/dL, Sigma-Aldrich Diagnostics, St. Louis, MO). The samples were vortexed and allowed to sit at room temperature for 15 minutes. Following this period, the absorbance of each sample was measured at 500 nm against a water blank. The concentration of triglycerides in each sample was calculated by dividing the absorbance of the samples by the absorbance of the triglyceride standard, and multiplying by the concentration of the triglyceride standard.

Determination of Fasting Plasma Glycerol Levels

Final plasma glycerol levels were determined using direct colorimetric kit obtained from Randox Laboratories (San Diego, CA). This procedure is based on the following reactions: glycerol kinase acts on glycerol in the presence of ATP to yield glycerol-3-phosphate (64). Subsequently, the action of glycerol phosphate oxidase in the presence of oxygen yields dihydroxyacetone phosphate and hydrogen peroxide. In the presence of hydrogen peroxide, 3,5-dichloro-2-hydroxybenzene sulphonic acid and 4-aminophenazone, the action of peroxidase generates n-(4-antipyryl)-3-chloro-5-

sulphonate-p-benezoquinoneimine (ACSB), which is pink in color. The intensity of the pink color measured at 520 nm is proportional to the original glycerol concentration.

The reagent mixture containing 0.4 mM 4-aminophenazone, 1 mM ATP, 0.4 U/ml glycerol kinase, 1.5 U/ml glycerol-3-phosphate oxidase, and 0.5 U/ml perioxidase was reconstituted with 15 ml of Pipes Buffer (40 mM 1,4-piperazinediethanesulfonic acid, 1.5 mM 3,5-dichloro-2-hydroxybenzene sulphonic acid, 17.5 mM Mg^{2+}, pH 7.6). In glass culture tubes, 30 µl of plasma was combined with 1 ml of the reagent mixture, mixed and allowed to sit at room temperature for 10 minutes. A standard of known glycerol concentration (100 µmol/L) was similarly prepared. Following the incubation period, the absorbance of the standard and samples were determined by spectrophotometry at 520 nm. To quantify the concentration of glycerol in each sample, the absorbance of a glycerol standard (100 µmol/l) was determined. The ratio of the absorbance of the sample to the absorbance of the standard was multiplied by the concentration of the glycerol standard to calculate the concentration of glycerol in each sample.

Determination of Fasting Plasma Insulin Levels

This assay is based on the double antibody technique for determination of plasma insulin levels in rats as described by Morgan, et al. (71) using the rat radioimmunoassay kit obtained from Linco Research (St. Charles, MO). A standard curve was prepared using purified rat insulin in insulin standard buffer at the following concentrations: 0.1, 0.2, 0.5, 1.0, 2.0, 5.0, and 10.0 ng/ml. For each standard and sample, 50 µl of standard/sample, 50µl of ^{125}I-labeled insulin, and 50 µl guinea pig anti-rat insulin serum

in assay buffer were transferred to appropriately labeled glass culture tubes. All tubes were vortexed, covered with foil and allowed to incubate for 20 hours at 4°C.

Following incubation, 500 µl of precipitating reagent containing goat anti-guinea pig IgG serum was added to each standard and sample. All tubes were vortexed and allowed to incubate for 30 minutes at 4° C. Subsequently, all tubes were centrifuged at 3000 rpm for 30 minutes to separate antibody-bound from free label. The supernatant was decanted by inverting the tubes for 45 seconds and the percentage of total binding determined for each sample by counting in a gamma counter for 5 minutes. The calculations necessary for determination of plasma insulin concentration were performed automatically using the data reduction capabilities of the gamma counter, which involved plotting the percentage of total binding for each sample on the y-axis and the known concentrations of the standards on the x-axis (72).

Determination of Fasting Plasma Leptin Levels

This assay is based on the double antibody technique for determination of plasma leptin levels in human plasma described by Ma, et al. (73) using the mouse leptin radioimmunoassay kit obtained from Linco Research (St. Charles, MO). A standard curve was prepared using purified mouse leptin in leptin standard buffer at the following concentrations: 0.2, 0.5, 1.0, 2.0, 5.0, 10.0 and 20.0 ng/ml. For each standard and sample, 50 µl of standard/sample and 50 µl rabbit anti-mouse leptin serum in assay buffer were transferred to appropriately labeled glass culture tubes. All tubes were vortexed, covered with foil and allowed to incubate for 20 hours at 4°C. Following incubation, 50 µl of ^{125}I-labelled leptin was added to each standard/sample. The tubes

were vortexed, covered with foil and allowed to incubate for an additional 20 hours at 4° C. Following this period, 500 µl of precipitating reagent containing goat anti-rabbit IgG serum was added to each standard and sample. All tubes were vortexed and allowed to incubate for 30 minutes at 4° C. Subsequently, all tubes were centrifuged at 3000 rpm for 30 minutes to separate antibody-bound from free label. The supernatant was decanted by inverting the tubes for 45 seconds and the percentage of total binding determined for each sample by counting in a gamma counter for 5 minutes. The calculations necessary for determination of plasma leptin concentration were performed automatically using the data reduction capabilities of the gamma counter, which involved plotting the percentage of total binding for each sample on the y-axis and the known concentrations of the standards on the x-axis (72).

RNA Isolation

Total RNA was extracted from the medial lobe of the liver, adipose tissue, and muscle using the guanidine-HCL method (74) and phenol-chloroform extraction techniques (75). Tissue samples (0.5 to 2 g) were ground in liquid nitrogen and subsequently, homogenized using a polytron homogenizer in 10 ml of 4 M guanidine isothiocynate (GTC) with β-mercaptoethanol (14 µl per ml of GTC). The homogenate was then transferred to a polypropylene centrifuge tube and one starting volume of phenol:choloroform:isoamyl alcohol (25:24:1, pH 7.9) added prior to vortexing. The homogenate was placed on ice for 15 minutes prior to centrifugation at 10,000 x g for 15 minutes at 4° C. Following centrifugation, the aqueous layer was transferred to a clean polypropylene centrifuge tube and 1 ml of 3 M sodium acetate added. The samples were

mixed by inversion. Subsequently, 5 ml of acid phenol:chloroform:isoamyl alcohol (125:24:1, pH 4.5) was added to each sample. The samples were vortexed and allowed to sit on ice for 15 minutes prior to centrifugation at 10,000 x g for 20 minutes at 4° C. Following centrifugation, the aqueous layer was transferred to a 15 ml centrifuge tube. Samples were allowed to precipitate overnight in 100% isopropanol at -20° C.

Samples were transferred to clean polypropylene centrifuge tubes prior to centrifugation at 10,000 x g for 20 minutes at 4° C. Following centrifugation, the supernatant was decanted and the pellet allowed to air dry for 15 minutes at room temperature. Subsequently, the pellet was resuspended in nuclease-free water containing 0.1 mM EDTA. The samples were allowed to precipitate for 30 minutes at -20° C prior to centrifugation at 10,000 x g for 30 minutes at 4° C. The supernatant was decanted and the pellet resuspended in 100% ethanol. The samples were allowed to incubate overnight at -20° C.

Samples were transferred to a clean polypropylene centrifuge tube and centrifuged at 10,000 x g for 20 minutes at 4° C. The supernatant was decanted and the pellet resuspended in formamide prior to quantification using dual wave length (260/280 nm) spectrophotometry (76).

Gene Expression

Expression of murine fatty acid synthase (FAS), peroxisomal proliferator activated receptor (PPAR) gamma (PPAR-γ), PPAR-α, uncoupling protein (UCP) 2 (UCP-2), and UCP-3 were quantitated by real-time reverse transcriptase polymerase chain reaction (RT-PCR) (77) using the Smart Cycler Real Time PCR System (Cepheid, Sunnyvale, CA)

with a TaqMan 1000 Reaction Core Reagent Kit (Applied Biosystems, Branchburg, NJ). The sequences for probes and forward and reverse primer sequences are provided in Table 9.

To establish a standard curve for each gene, 1 μl of total RNA (concentration: 1 ng/μl) from each sample was pooled and subsequently, serial-diluted in the range of 1.5625-25 ng to establish a standard curve in each tissue for each gene of interest. The PCR reagent mixture contained 50-200 μg RNA, 6.85 μl DEPC-treated water, 2.5 μl 10X PCR buffer (500 mM KCl, 100 mM Tris-HCL, pH 8.3), 5.5 μl 25 mM $MgCl_2$, 0.75 μl each of 75 mM deoxynucleotide triphosphates (dATP, dGTP, dCTP, dTTP), 0.50 μl of 10 μM forward primer, 1.0 μl of 10μM reverse primer, 0.125 μl of MuLV reverse transcriptase (50 U/μl), 0.125 μl RNase inhibitor, 0.15 μl AmpliTaq DNA polymerase, and 0.25 μl of 10 μM probe. The polymerase chain reaction was performed according to the instructions for the Smart Cycler System (Cephid) and TaqMan Real Time PCR Core Kit (Applied Biosystems, Foster City, CA) using 50-200 ng total RNA and the following 3-stage cycling protocol: stage 1 (reverse transcription) at 48.0° C for 1800 seconds, stage 2 (denaturation and inactivation of reverse transcriptase) at 95.0° C for 600 seconds, and stage 3 (40 two-temperature cycles), denaturation at 95°C for 15 seconds, annealing and extension at 60°C for 60 seconds. Quantitation for each sample was normalized using the corresponding 18s expression in each tissue for each animals in the same manner as the other genes (77).

Table 9

Sequences of Primers and Probes Used for Determination of Gene Expression by Real Time RT-PCR

	Primer Sequences (5' to 3')		Probe
Gene	**Forward**	**Reverse**	**Sequence (5' to 3')**
FAS	CCCAGAGGCTTGTGCTGACT	CGAATGTGCTTGGCTTGGT	TET-CCGATCTGGAATCCGCACCGG-TAMRA
UCP-3	CCTCTACGACTCTGTCAAGCAGTTCTAC	CAGCCTGCCAGAATCCTGAT	Cal Red-CGCTGGAGTGGTCCGCTCCCT-BHQ
UCP-2	GCGTTCTGGGTACCATCCTAAC	GCGACCAGCCCATTGTAGA	Cal Red-CGCACTGAGGGTCCACGCAGC-BHQ
PPAR-α	CGATGCTGTCCTCCTTGATGA	CGCGTGTGATAAAGCCATTG	Cal Red-ACGCGATCAGCATCCCGTCTTTG-BHQ
PPAR-γ	TGCGAGTGGTCTTCCATCAC	GCCTATGAGCACTTCACAAGAAATT	Cal Red-TCTGGCCCACCAACTTCGGAATCAG-BHQ
18S	AGTCCCTGCCCTTTGTACACA	GATCCGAGGGCCTCACTAAAC	FAM-CGCCCGTCGCTACTACCGATTGG-BHQ

Statistical Analysis

Data were analyzed by analysis of variance (ANOVA) to compare overall group means with subsequent analysis via least significant difference test to identify significantly different group means using the SPSS Statistics Package (78). Variables which did not follow a normal distribution were transformed according to accepted statistical practices (log base 10, square, square root, inverse, inverse square root) to achieve normal distribution prior to ANOVA (79). When normality could not be achieved, significantly different group means were identified by non-parametric tests (78,79).

Results

Comparison of US17 and Basal Diets

Table 10 summarizes the data on body composition and plasma metabolites in animals consuming either the US17 or Basal diet for 4 weeks. Total food and energy intake were similar between diets. There were no significant differences in body weight or fat pad mass between diets, although animals consuming the basal diet tended to be heavier and have larger fat pads. Non-fasting plasma glucose and insulin levels were similar between diets. Based on these findings and our previous success using the Basal diet to induce obesity in this animal model, the composition of all experimental diets was based on the Basal diet.

Table 10

Comparison of the Effects of 4 Weeks *Ad Libitum* Consumption of US17 Diet and Basal Diets in aP274-*Agouti* Transgenic Mice

A. Effects of Diet on Body Composition

	Body Weight (g)		Liver (g)	Selected Fat Pads (g)			Sum of 3 Fat Pads (g)
	Final	Gain		Epididymal	Abdominal	Subscapular	
Basal	31.28 ± 1.20[a]	6.34 ± 0.89[a]	1.69 ± 0.053[a]	.073 ± 0.009[a]	.83 ± 0.10[a]	.76 ± 0.11[a]	1.67 ± 0.22[a]
US17	31.18 ± 0.74[a]	5.60 ± 0.71[a]	1.80 ± 0.11[a]	.090 ± 0.007[a]	.74 ± 0.05[a]	.60 ± 0.06[a]	1.44 ± 0.12[a]

Values are Mean±SEM; Matching superscripts indicate statistically equivalent values.

B. Effects of Diet on Energy Intake, Energy Efficiency, and Plasma Metabolites

	Diet Consumption		Energy Efficiency Ratio*	Non-Fasting Plasma Metabolites	
	Food Intake (g)	Energy Intake (kcal)		Glucose (mg/dL)	Insulin (ng/ml)
Basal	141.90 ± 12.81[a]	744.96 ± 67.24[a]	.87 ± 0.12[a]	238.03 ± 8.50[a]	4.62 ± .42[a]
US17	141.27 ± 13.87[a]	820.33 ± 80.45[a]	.77 ± 0.11[a]	234.36 ± 15.89[a]	4.32 ± .67[a]

Values are Mean ±SEM; Energy Efficiency ratio: (total energy intake/grams body weight gained)*100; Matching superscripts indicate statistically equivalent values.

Meal Tolerance Tests

Figure 4 summarizes the data regarding the blood glucose response to test foods. The response ($AUC_{glucose}$) to MUNG was significantly lower ($p<0.05$) than all other foods. Somewhat unexpectedly, we found the response to IO-S to be approximately 23% greater ($p<0.05$) than the response to IO-F, which was similar to ROLL.

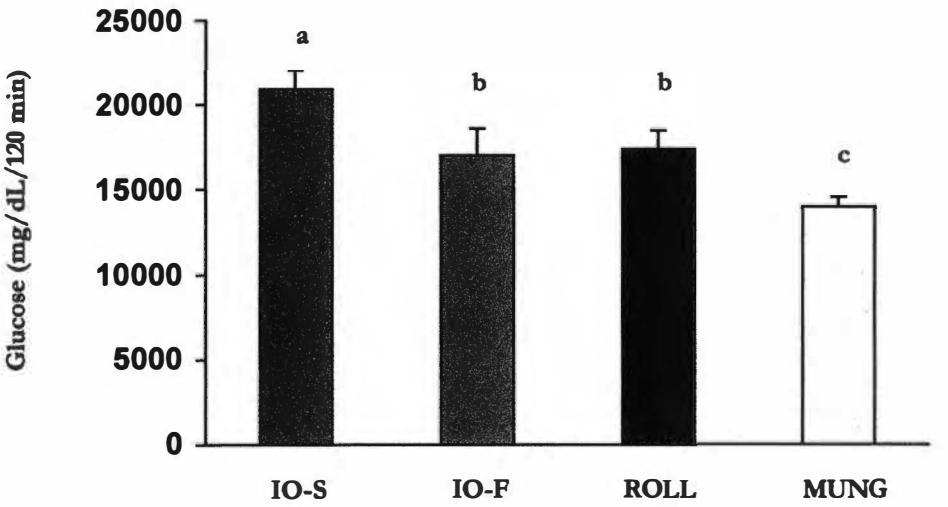


Figure 4. Area under the blood glucose response curve for test foods in aP274-*Agouti* transgenic mice. Data is expressed as mean±SEM. Non-similar superscripts indicate significant differences at $p<0.05$.

Weight Gain

The patterns of body weight change by week for *ad libitum* fed and energy restricted animals are illustrated in Figures 5 and 6, respectively. While all diets increased body weight when fed *ad libitum* for six weeks, weight gain (Figure 7A) was 50-66% lower ($p<0.05$) in the ROLL group compared to all others. Consequently, final body weight was significantly lower (by 8-13%, $p<0.05$; Figure 7B) in the ROLL group compared with all other groups.

Prior to being assigned to treatment groups for the energy restricted phase of the study, animals were fed the obesity-promoting basal diet *ad libitum* for 6 weeks. During this period body weight increased from 25.43±3.92 g to 34.59±4.42 g ($p<0.05$ vs. baseline body weight), with mean weight gain of 9.15±0.48 g. Following this period, animals were separated into treatment groups (n=8 per treatment group) by body weight, such that there were no significant differences in body weight by group before beginning the energy restricted phase.

As illustrated in Figure 6, body weight stabilized in animals continued on the basal diet *ad libitum* during weeks 6-12, resulting in final body weight following 12 weeks of *ad libitum* feeding of 36.37±1.32 g. Body weight of animals following 12 weeks of *ad libitum* of animals following 12 weeks of *ad libitum* consumption of the basal diet was only 8% higher than for animals fed the same diet *ad libitum* for 6 weeks; nonetheless, representative fat pad mass was 35% greater in animals fed the basal diet *ad libitum* for 12 weeks compared to animals consuming the same diet for only 6 weeks. Thus, adipose tissue accumulation was not accompanied by parallel increases in body weight during weeks 6-12.

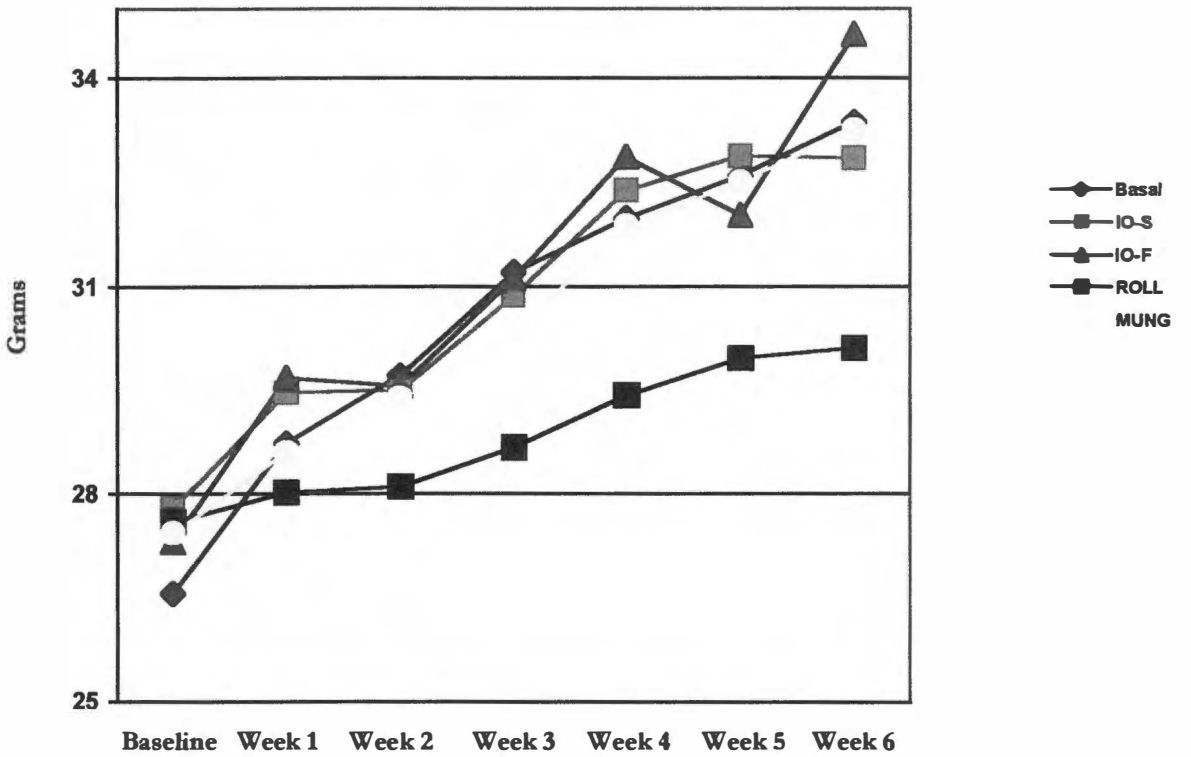


Figure 5. Pattern of change in body weight over 6 weeks by dietary treatment in *ad libitum* fed aP274-*Agouti* transgenic mice. Data is expressed as mean±SEM.

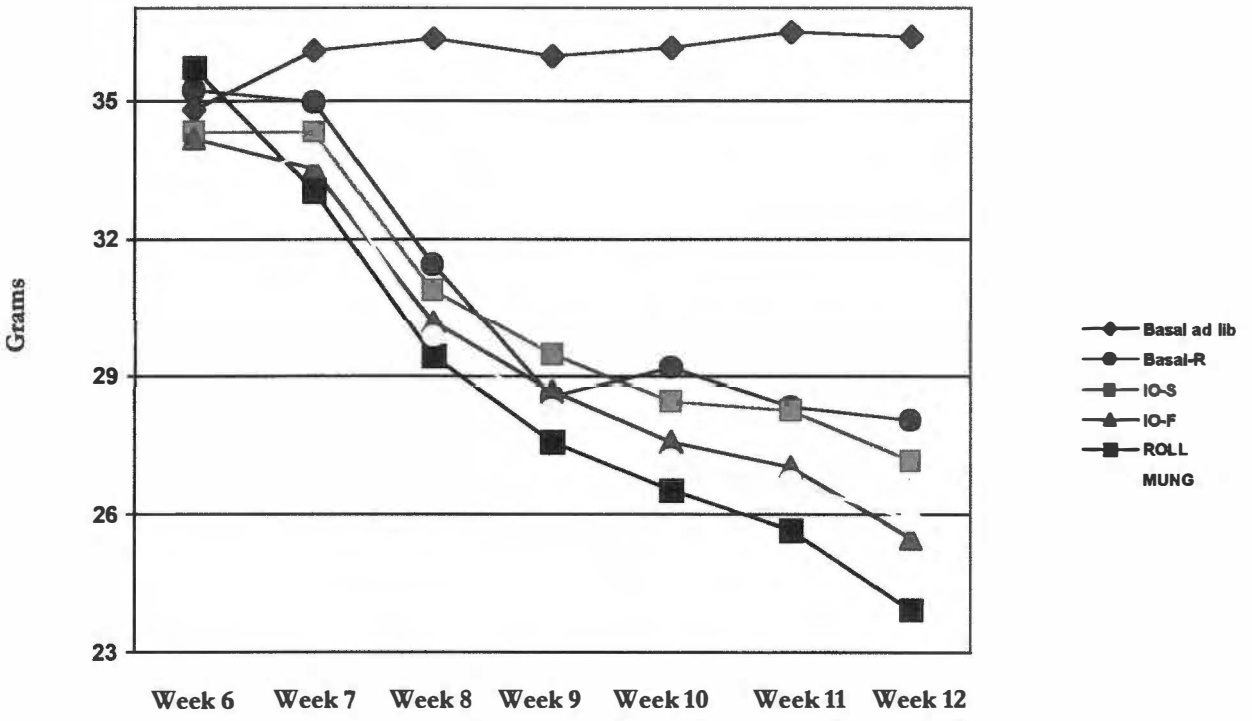
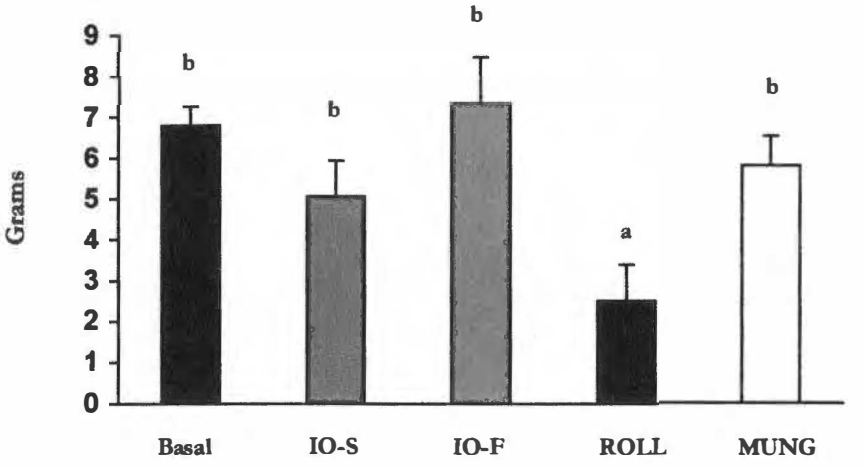


Figure 6. Pattern of change in body weight by dietary treatment during weeks 6-12 in energy restricted aP274-*Agouti* transgenic mice. Data is expressed as mean±SEM.

A



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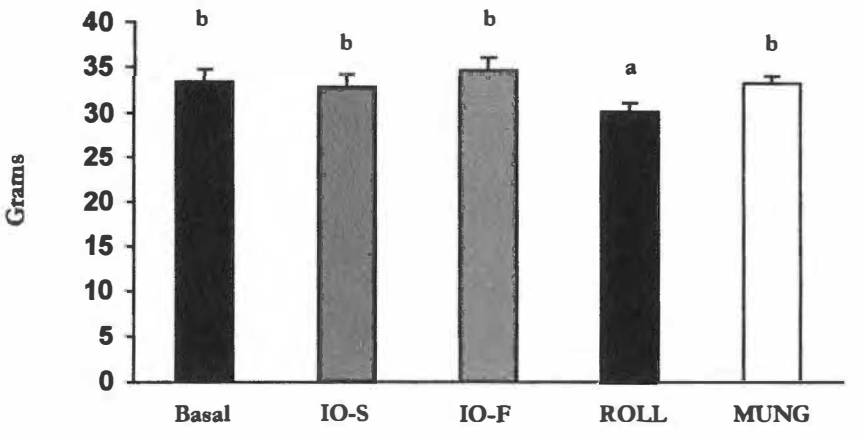


Figure 7. Effect of dietary treatment on (A) weight gain and (B) final body weight in *ad libitum* fed aP274-*Agouti* transgenic mice. Data is expressed as mean±SEM for each group. Non-similar superscripts indicate significant differences at $p < 0.05$.

During the final six weeks of the study, energy restriction, irrespective of diet, reduced body weight by ~21-28% compared with animals continued on the basal diet *ad libitum* ($p<0.05$; Figure 8A). Despite equivalent energy restriction, final body weight in the Basal-R group was 17% higher ($p<0.05$) than in ROLL-fed animals (Figure 8B).

Liver Weight

Following 6 weeks of *ad libitum* intake, the livers of animals consuming the basal and IO-S diets were ~12% heavier ($p<0.05$) than in animals consuming the ROLL and MUNG diets. Liver weight of IO-F animals differed only from the ROLL group ($p<0.05$).

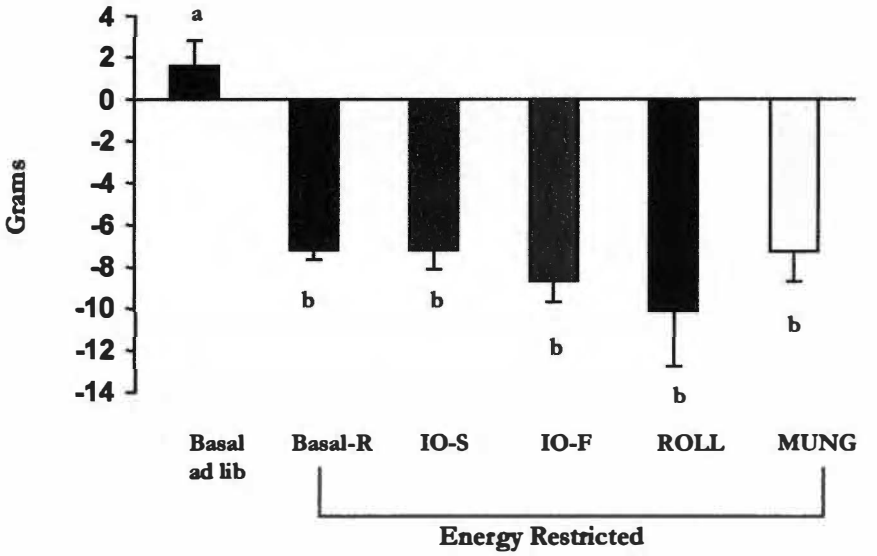
Regardless of diet, energy restriction during weeks 6-12 reduced liver weight compared with unrestricted consumption of the basal diet for 12 weeks ($p<0.05$). Liver mass of animals consuming the energy restricted ROLL and MUNG diets was significantly lower ($p<0.05$) than in Basal-R.

Adiposity

Changes in body weight are reflected in adipose tissue mass. The subscapular and epididymal fat depots were chosen to assess the effects of diet on subcutaneous adipose tissue accumulation. Diets were largely without influence on epididymal fat pad mass, regardless of energy intake; therefore, differences in subcutaneous adipose tissue mass are largely accounted for by the subscapular depot.

When energy intake was unrestricted for 6 weeks, subcutaneous fat pad mass was 38-52% lower ($p<0.05$) in the ROLL group compared with the other diets consumed

A



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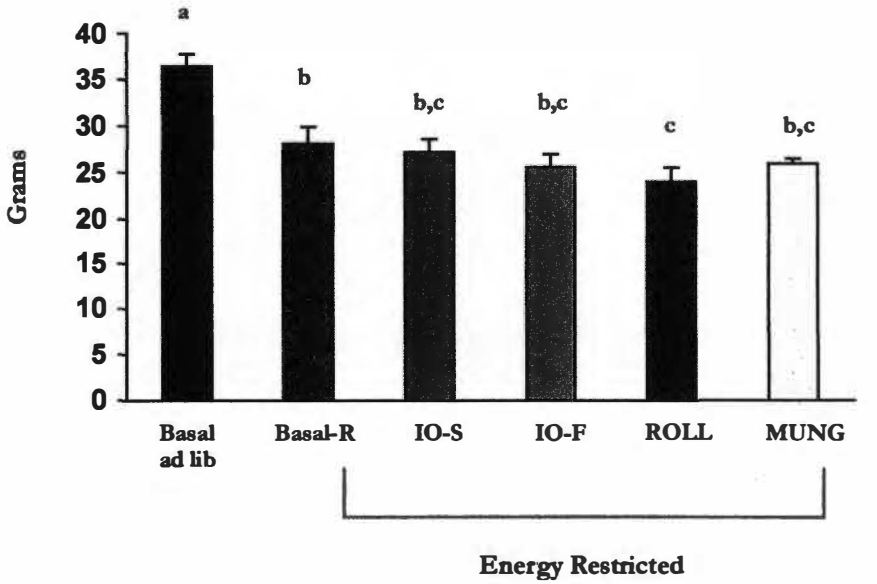


Figure 8. Effect of dietary treatment on (A) weight gain and (B) final body weight in energy restricted aP274-*Agouti* transgenic mice. Data is expressed as mean ± SEM for each group. Non-similar superscripts indicate significant differences at $p<0.05$.

ad libitum (Figure 9A).

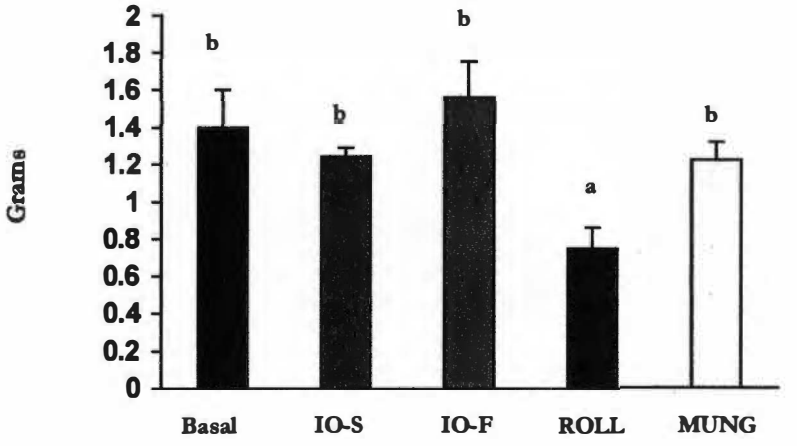
Regardless of diet, energy restriction during weeks 6-12 reduced adiposity relative to animals consuming the basal diet *ad libitum*. Subscapular fat pad mass was significantly higher (by 85-98%, $p<0.05$) in the Basal-R and IO-S groups compared with the ROLL group at the identical level of energy restriction. Consequently, subcutaneous fat pad mass was significantly reduced in the ROLL (by 43%, $p<0.05$) compared with the Basal-R and IO-S groups, which suggests that subcutaneous adipose tissue deposition is sensitive to both quantitative (e.g. energy) and qualitative (e.g. carbohydrate) changes in diet.

Despite the fact that only negligible increases in body weight were observed in animals continued on the Basal diet *ad libitum* during weeks 6-12, subcutaneous adipose tissue deposition appears to have continued; consequently, the combined mass of selected subcutaneous fat pads was ~39% higher ($p<0.05$; Figure 9B) in animals consuming the basal diet for 12 weeks compared with animals consuming the same diet for only 6 weeks.

Despite identical levels of energy restriction, subcutaneous adipose tissue mass was 43% lower ($p<0.05$) in animals consuming the ROLL compared with basal and IO-S diets during the final 6 weeks of the study (Figure 9B). This suggests the ROLL diet reduces subcutaneous adipose tissue deposition independently of energy intake.

The retroperitoneal and perirenal fat depots were chosen to represent visceral adipose tissue. Retroperitoneal fat mass was 39-43% lower ($p<0.05$) in the ROLL group compared with all other diets consumed *ad libitum*. Similarly, perirenal fat pad mass was 26-37% lower ($p<0.05$) following 6 weeks of *ad libitum* consumption of the ROLL diet

A



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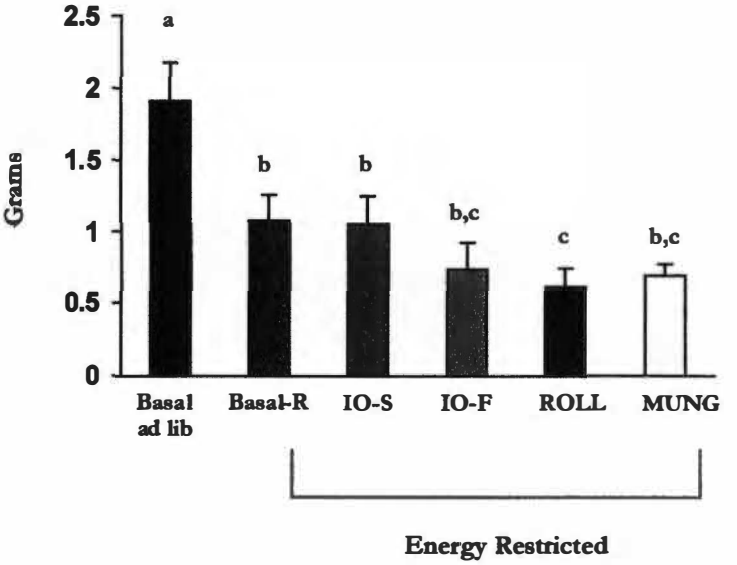


Figure 9. Effect of dietary treatment on subcutaneous (subscapular & epididymal) fat pad mass in (A) *ad libitum* fed and (B) energy restricted aP274-*Agouti* transgenic mice. Data is expressed as mean ± SEM for each group. Non-similar superscripts indicate significant differences at $p<0.05$.

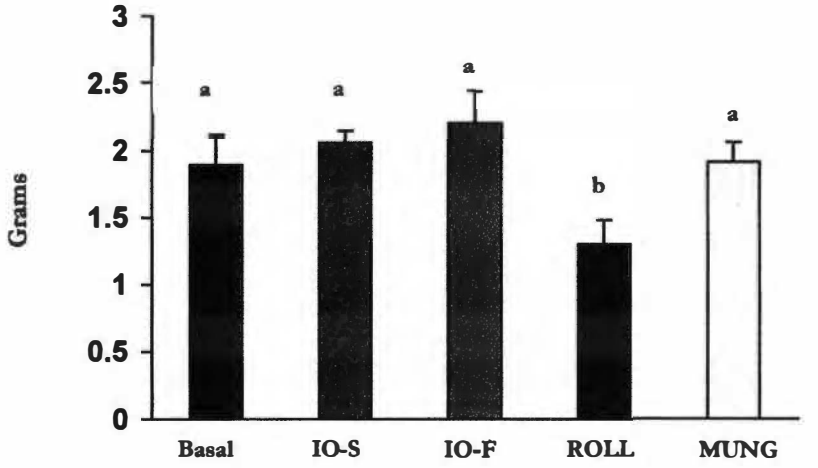
compared with all others. Collectively, these visceral depots were 32-41% lower ($p<0.05$) following 6 weeks of unrestricted consumption of the ROLL diet compared with all others (Figure 10A). Furthermore, twelve weeks of unrestricted consumption of the basal diet resulted in a 33% increase in visceral fat pad mass relative to animals consuming the same diet for only 6 weeks.

Compared with *ad libitum* intake of the basal diet during weeks 6-12, energy restriction reduced the mass of the retroperitoneal (by 43-75%, $p<0.05$) and perirenal (by 43-80%, $p<0.05$) fat pads. Despite identical energy restriction, retroperitoneal fat pad mass was significantly lower (by 43-57%, $p<0.05$) in ROLL and MUNG compared with the HFS and IO-S groups. Similarly, the mass of the perirenal depot was significantly lower (by 60-65%, $p<0.05$) in ROLL and MUNG compared with IO-S and basal at the same level of energy intake. Thus, visceral adipose tissue accumulation was significantly reduced in animals consuming the ROLL (by 59-86%, $p<0.05$) and MUNG (48-60%, $p<0.05$) compared with the basal-R and IO-S despite identical energy intake (Figure 10B).

As depicted in Figure 11A, the combined mass of the four selected fat pads was 34-45% lower in the *ad libitum* fed ROLL ($p<0.05$) group compared with all other diets. Consequently, these fat depots made a smaller contribution to overall body weight in the ROLL ($p<0.05$) animals compared with basal, IO-S, IO-F, and MUNG.

Ad libitum consumption of the basal diet for 12 weeks further increased combined visceral and subcutaneous fat pad mass by ~35% relative to the mass of these depots in animals consuming the same diet for only 6 weeks. Energy restriction reduced the individual mass of these fat pads by 44-73% ($p<0.05$) compared with animals continued on the basal diet for the final six weeks of the study (Figure 11B). Consistent with

A



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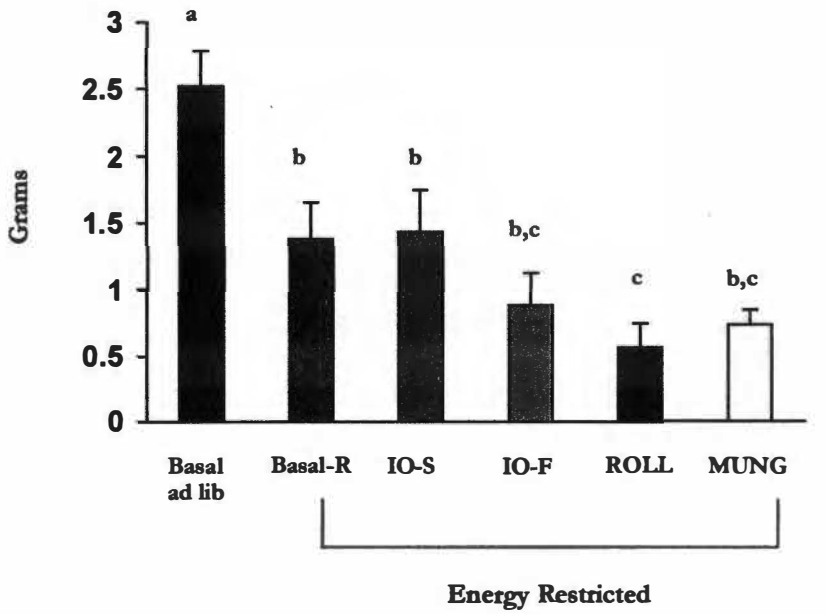
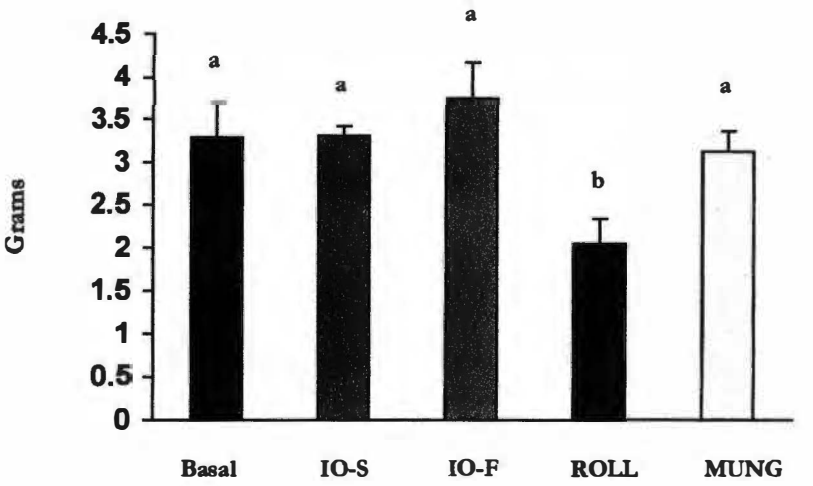


Figure 10. Effect of dietary treatment on visceral fat pad (retroperitoneal & perirenal) mass in (A) *ad libitum* fed and (B) energy restricted aP274-*Agouti* transgenic mice. Data is expressed as mean ± SEM for each group. Non-similar superscripts indicate significant differences at $p<0.05$.

A



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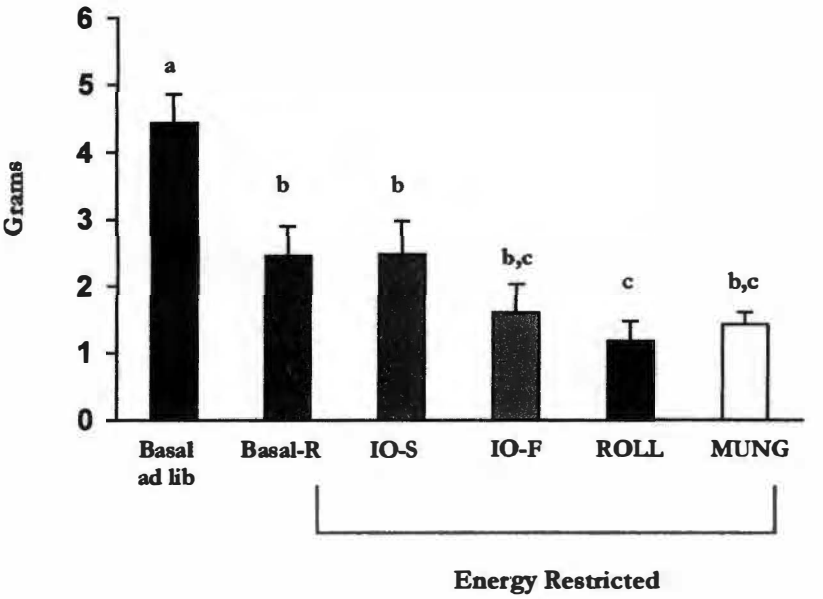


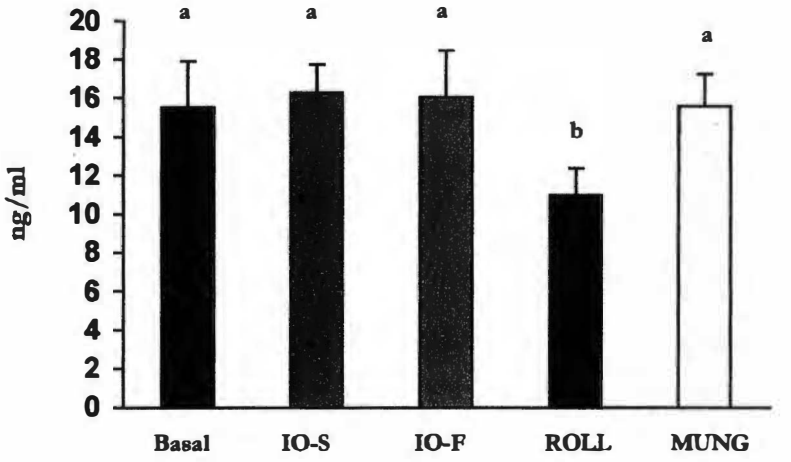
Figure 11. Effect of dietary treatment on combined mass of representative fat pads (epididymal, subscapular, retroperitoneal, perirenal) in (A) *ad libitum* fed and (B) energy restricted aP274-*Agouti* transgenic mice. Data is expressed as mean ± SEM for each group. Non-similar superscripts indicate significant differences at $p<0.05$.

changes in individual depots, combined fat pad mass was significantly lower ($p<0.05$) in ROLL and MUNG fed animals compared with animals fed the basal-R and IO-S, despite identical levels of energy intake. As a result of these changes, the combined mass of these depots accounted for ~12% of overall body weight in animals fed the basal diet *ad libitum* for 12 weeks ($p<0.05$ vs. all energy restricted diets). However, despite identical energy restriction, these fat pads made a significantly greater ($p<0.05$) contribution to total body weight in basal-R and IO-S than in ROLL and MUNG fed animals.

Fasting Plasma Leptin Levels

Plasma leptin levels were 29-33% lower ($p<0.05$) in animals fed the ROLL diet *ad libitum* for 6 weeks compared with all other groups, which is consistent the observed differences in fat pad mass (Figure 12A). Furthermore, fasting plasma leptin levels in animals fed the basal diet *ad libitum* for 12 weeks were 90% higher ($p<0.05$) than leptin levels fed the basal diet for only 6 weeks. This supports the hypothesis that leptin levels are reflective of changes in fat mass, since body weight did not increase appreciably in animals continued on the basal diet during weeks 6-12.

Plasma leptin levels were 29-61% lower ($p<0.05$) in all energy restricted groups compared with animals continued on the basal diet *ad libitum* during weeks 6-12 (Figure 12B). As can be seen in Figure 12B, the MUNG diet appeared to exert the greatest influence, independent of energy restriction, leading to fasting leptin levels that were significantly lower (by 40-45%, $p<0.05$) than both basal-R and IO-S. Plasma leptin levels were strongly correlated with final body weight ($r=0.632$, $p<0.01$), and mass of individual fat pads ($r=0.692$-0.744, $p<0.001$).



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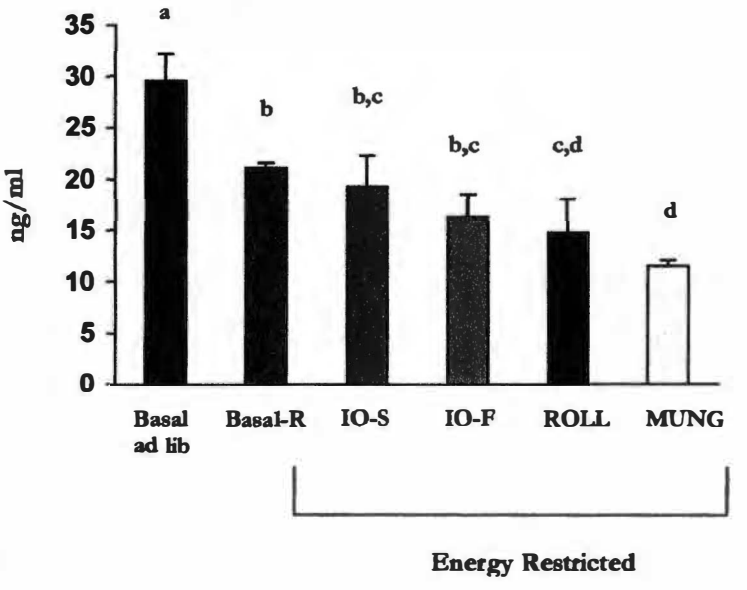


Figure 12. Effect of dietary treatment on fasting plasma leptin levels in (A) *ad libitum* fed and (B) energy restricted aP274-*Agouti* transgenic mice. Data is expressed as mean±SEM for each group. Non-similar superscripts indicate significant differences at $p<0.05$.

Fasting Plasma Insulin Levels

Fasting plasma insulin levels were significantly lower in animals consuming the IO-S, ROLL, and MUNG diets relative to the basal ($p<0.05$) and IO-F ($p<0.05$) diets consumed *ad libitum* for six weeks (Figure 13A).

Plasma insulin levels were significantly higher in animals consuming the basal diet, either *ad libitum* for 12 weeks or with energy restriction during weeks 6-12 (Figure 13B), compared to the energy restricted IO-S, IO-F, ROLL, and MUNG diets. Fasting plasma insulin levels were positively correlated with final body weight ($r=0.411$, $p<0.01$) in all animals.

Fasting Blood Glucose Levels

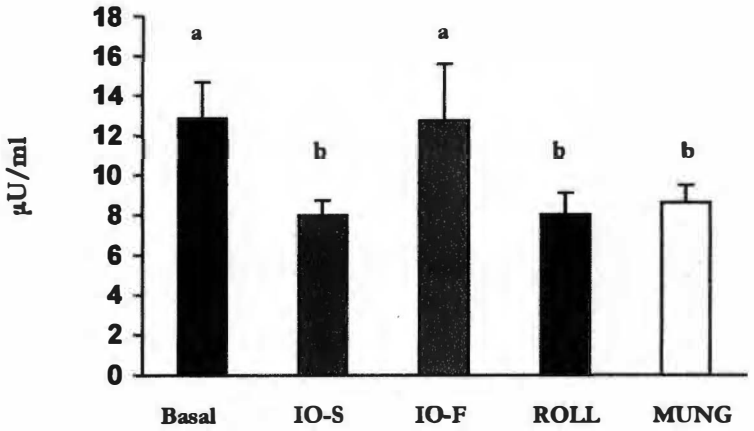
In animals fed *ad libitum*, non-fasting blood glucose levels were significantly greater (by 7-25%, $p<0.05$; Figure 14A) in ROLL-fed animals compared with all other groups.

As illustrated in Figure 14B, fasting blood glucose levels in animals consuming the energy restricted ROLL and MUNG were significantly lower ($p<0.05$ for both) than animals consuming the basal diet either *ad libitum* or with energy restriction.

Plasma Triglyceride Levels

Figure 15A summarizes non-fasting plasma triglyceride levels in ad libitum fed mice. The energy restricted ROLL and MUNG diets lowered fasting plasma triglyceride levels compared with animals continued on the basal diet ad libitum ($p<0.05$ for both; Figure 15B).

A



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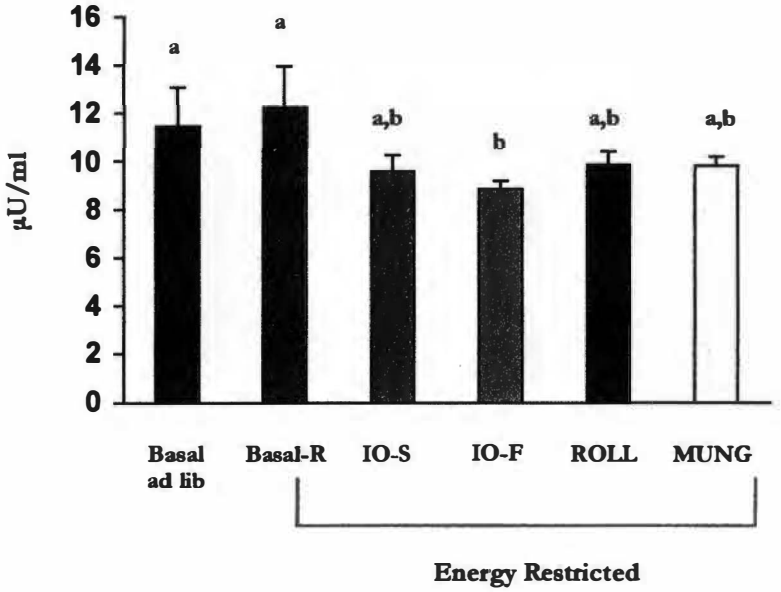
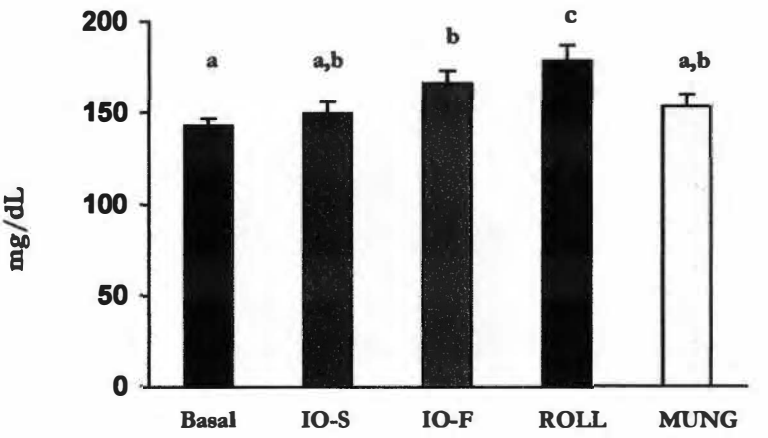


Figure 13. Effect of dietary treatment on fasting plasma insulin levels in (A) *ad libitum* fed and (B) energy restricted aP274-*Agouti* transgenic mice. Data is expressed as mean ± SEM for each group. Non-similar superscripts indicate significant differences at $p<0.05$.

A



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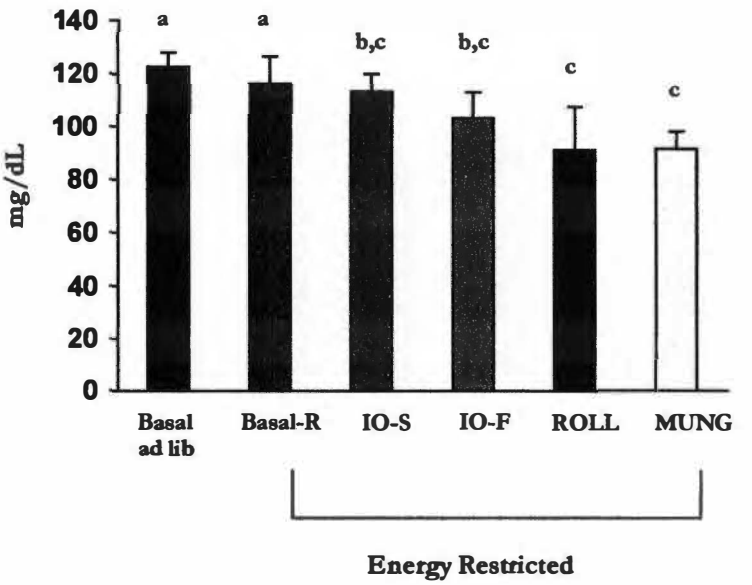
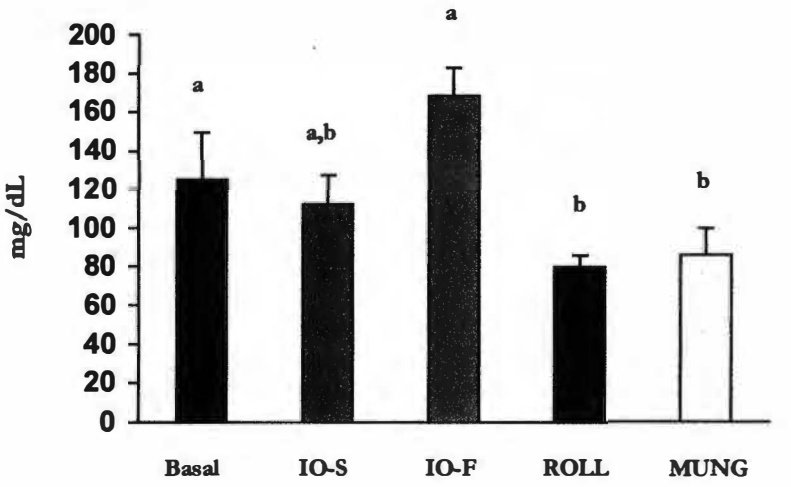


Figure 14. Effect of dietary treatment on plasma glucose levels in (A) non-fasted *ad libitum* and (B) fasted, energy restricted aP274-*Agouti* transgenic mice. Data is expressed as mean ± SEM for each group. Non-similar superscripts indicate significant differences at $p<0.05$.

A



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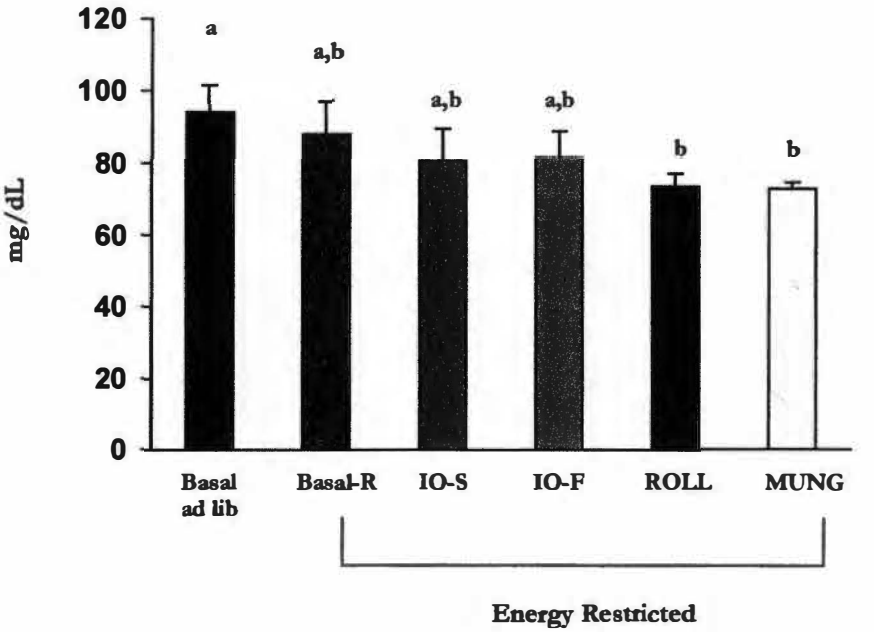


Figure 15. Effect of dietary treatment on plasma triglyceride levels in (A) non-fasted *ad libitum* fed and (B) fasted, energy restricted aP274-*Agouti* transgenic mice. Data is expressed as mean±SEM for each group. Non-similar superscripts indicate significant differences at $p<0.05$.

Adipocyte Size

Adipocyte size was determined in animals undergoing the 12 week feeding protocol (Figure 16). Adipocytes were of similar size in animals consuming the basal diet *ad libitum* or with energy restriction, with both being significantly larger (by 20-42%, $p<0.05$ for both) than adipocytes from ROLL- and MUNG-fed animals. Animals continued on the basal diet *ad libitum* for the final 6 weeks of the study were significantly larger ($p<0.05$) than those isolated from the energy restricted IO-S, IO-F groups. Retroperitoneal adipocyte size was strongly correlated with the mass of this fat pad ($r=0.833$, $p<0.001$). Adipocyte size was also correlated with fasting plasma levels of leptin ($r=0.537$, $p<0.01$), glucose ($r=0.384$, $p<0.01$) and insulin ($r=0.306$, $p<0.05$). Adipocyte size was also positively correlated with final body weight ($r=0.674$, $p<0.001$).

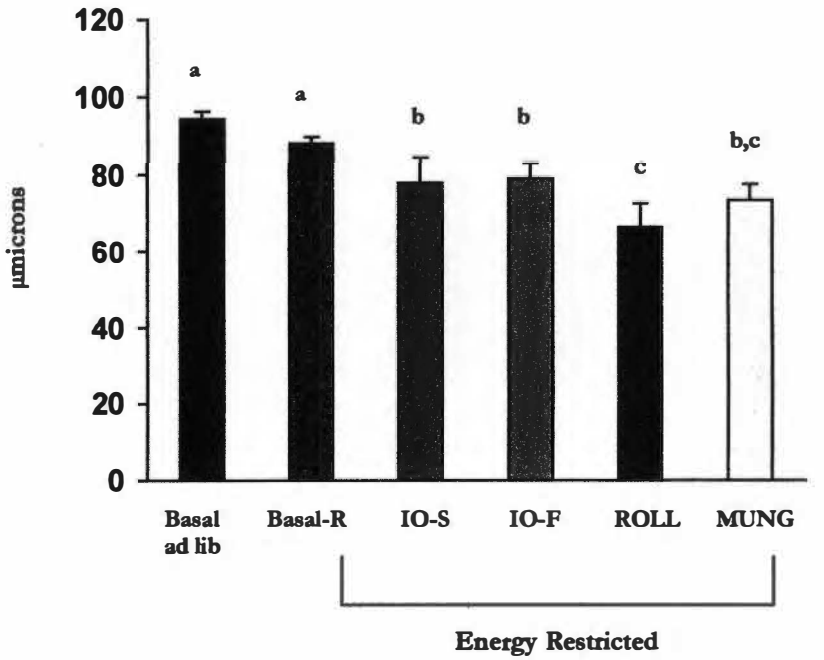


Figure 16. Effect of dietary treatment on retroperitoneal adipocyte size in energy restricted aP274-*Agouti* transgenic mice. Data is expressed as mean ± SEM for each group. Non-similar superscripts indicate significant differences at $p<0.05$.

Adipocyte Lipolysis

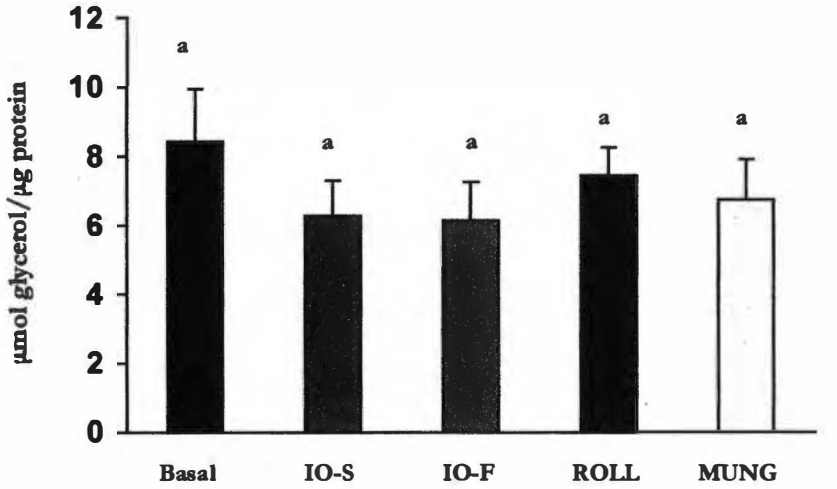
In animals fed *ad libitum* for 6 weeks, diet had no effect on basal lipolysis as assessed by glycerol release (Figure 17A). While isoproterenol significantly increased glycerol release above basal in all groups, glycerol release in response to isoproterenol was significantly greater ($p<0.05$) in *ad libitum* fed animals consuming the ROLL and MUNG compared to the basal and IO-S (Figure 17B). Isoproterenol-stimulated glycerol release (expressed as % of basal) was significantly greater in IO-F, ROLL and MUNG ($p<0.05$ for all) compared with the basal and IO-S groups *ad libitum* fed animals.

Basal glycerol release was similar in animals consuming the basal diet *ad libitum*, regardless of duration of feeding. In addition, basal glycerol release was similar in animals consuming the basal diet *ad libitum* for 12 weeks and in those consuming the energy restricted basal diet during weeks 6-12 (Figure 18A). Basal glycerol release was significantly greater in animals consuming the basal diet either *ad libitum* (by 78-92%, $p<0.05$) or with energy restriction (by 45-56%, $p<0.05$) compared with the energy restricted IO-S, IO-F, ROLL, and MUNG diets.

Isoproterenol failed to increase glycerol release (expressed as percent stimulation above basal) to an appreciable degree in animals consuming the basal diet *ad libitum* for 12 weeks (Figure 18B). In contrast, isoproterenol increased lipolysis (expressed as percent stimulation above basal) by 35-107% ($p<0.05$) in all energy restricted groups, with the largest increase observed in adipocytes isolated from IO-F and MUNG.

In all animals, body weight was positively associated with both basal ($r=0.645$, $p<0.001$) and isoproterenol-stimulated lipolysis ($r=0.509$, $p<0.01$). Regardless of energy intake, perirenal fat mass was positively associated with both basal ($r=0.565$, $p<0.001$)

A



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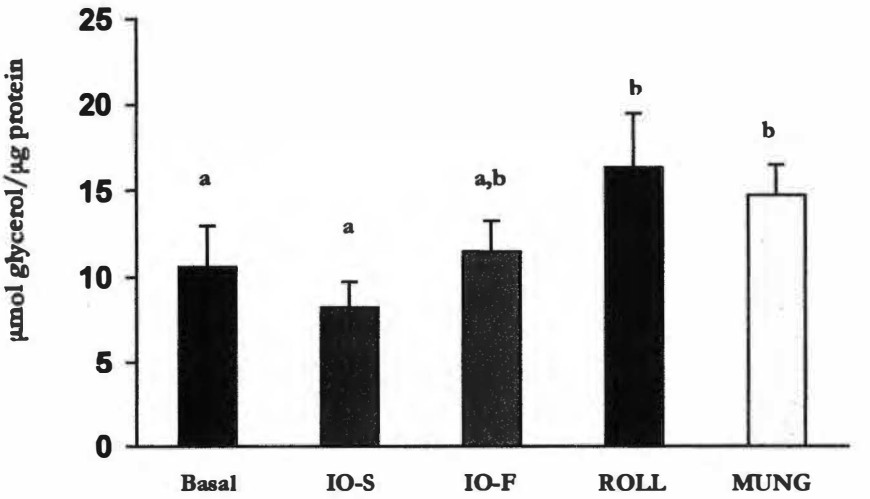
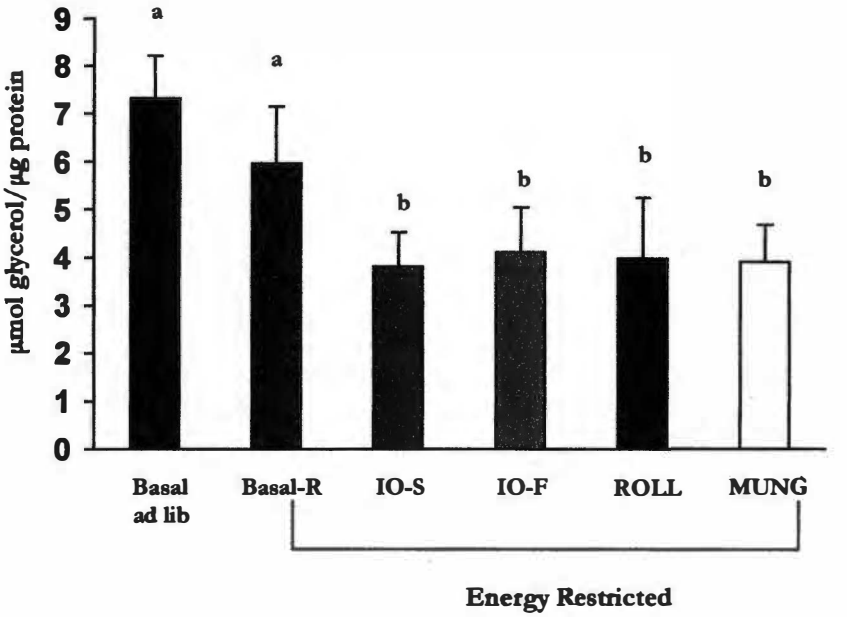


Figure 17. Effect of dietary treatment on (A) basal and (B) isoproterenol-stimulated lipolysis in *ad libitum* fed aP274-*Agouti* transgenic mice. Data is expressed as mean±SEM. A: There were no similar differences between groups. B: Non-similar superscripts indicate significant differences a $p<0.05$.

A



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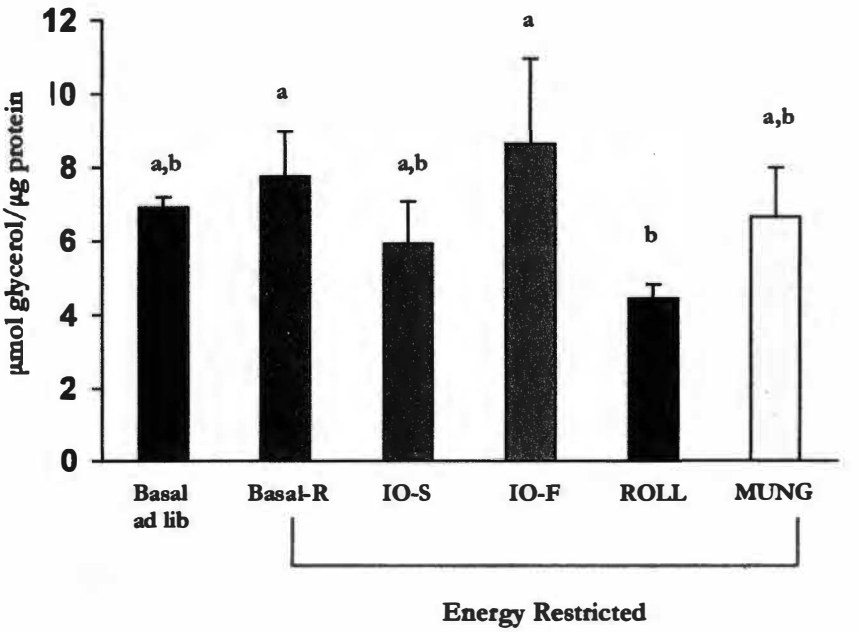


Figure 18. Effect of dietary treatment on (A) basal and (B) isoproterenol-stimulated lipolysis in energy restricted aP274-*Agouti* transgenic mice. Data is expressed as mean± SEM. Non-similar superscripts indicate significant differences at $p<0.05$.

and isoproterenol-stimulated lipolysis ($r=0.421$, $p<0.01$). In addition, fasting plasma leptin levels were positively associated with basal ($r=0.508$, $p<0.01$), but not isoproterenol-stimulated lipolysis. Basal lipolysis was positively correlated with adipocyte size ($r = 0.702$, $p < 0.01$) and was 68% higher ($p<0.05$) in animals fed the basal diet in either form compared with the remaining energy restricted groups.

Gene Expression

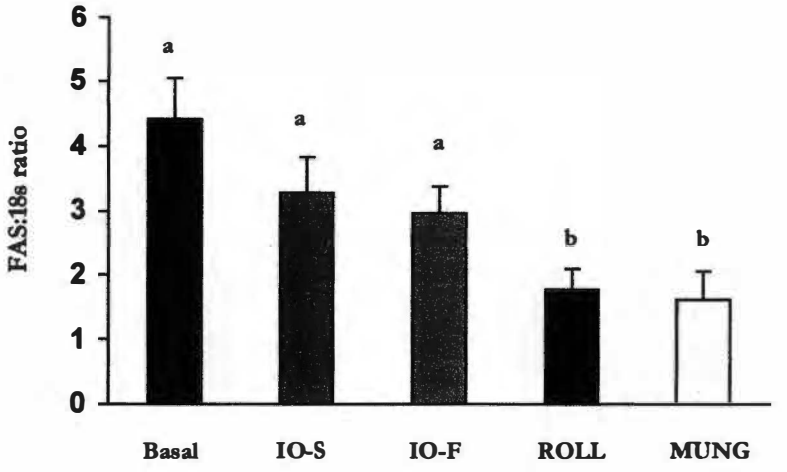
Liver

In animals fed *ad libitum* for 6 weeks, expression of fatty acid synthase in the liver was 43-62% lower ($p<0.05$) in animals consuming the ROLL and MUNG diets compared with the basal, IO-S, and IO-F groups (Figure 19A). Hepatic PPAR-α expression was approximately 42% lower ($p<0.05$) in ROLL-fed animals compared with those consuming the basal diet following six weeks of unrestricted energy intake (Figure 19B).

Energy restriction reduced the expression of fatty acid synthase (Figure 20A) and PPAR-α (Figure 20B) in the liver by ~52 and 41% ($p<0.05$ for both), respectively, compared with animals continued on the basal diet *ad libitum*. In energy restricted animals, expression of PPAR-α was reduced (by 28%, $p<0.05$) in animals consuming the IO-S, IO-F, and ROLL compared with the basal-R and MUNG, suggesting an independent influence of endogenous dietary fiber in suppression of PPAR-α expression.

In animals fed *ad libitum* for 6 weeks, there was a positive relationship between the expression of PPAR-α and FAS ($r=0.407$, $p< 0.05$) in the liver. Plasma leptin levels were also significantly associated with hepatic PPAR-α ($r=0.457$, $p<0.01$) and FAS ($r=0.382$, $p<0.01$) expression.

A



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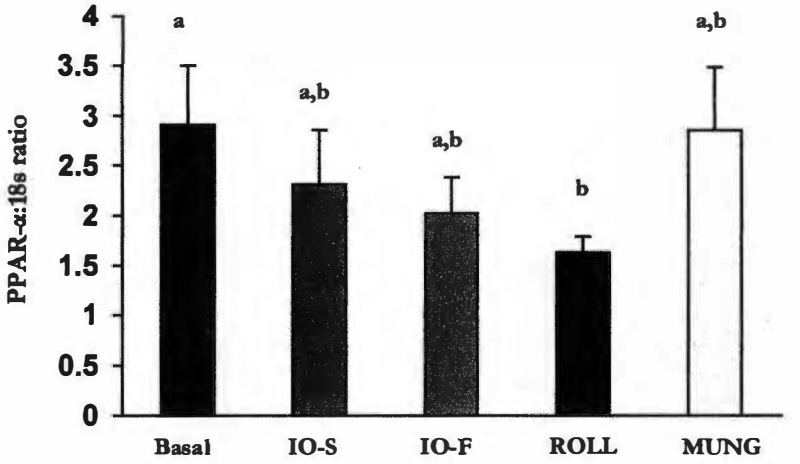
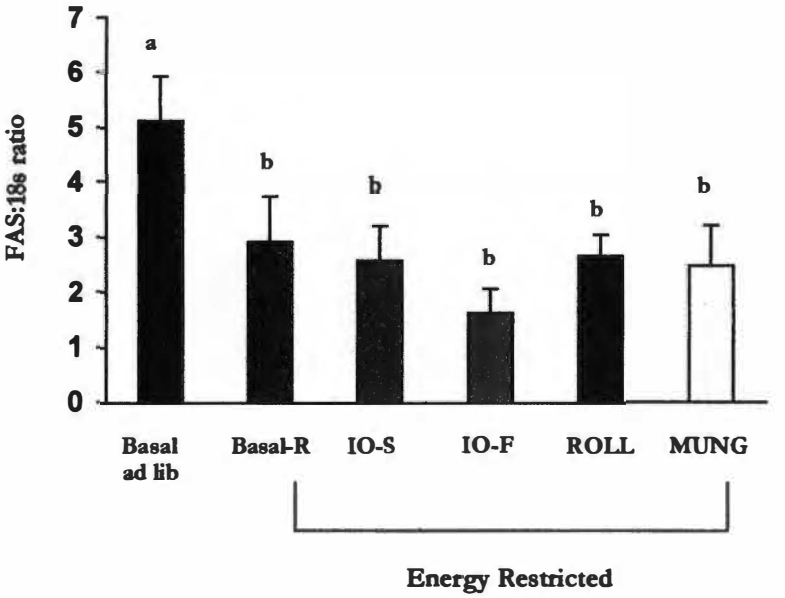


Figure 19. Effect of dietary treatment on hepatic expression of (A) fatty acid synthase and (B) PPAR-α in *ad libitum* fed aP274-*Agouti* transgenic mice. Non-similar superscripts indicate significant differences at $p<0.05$.

A



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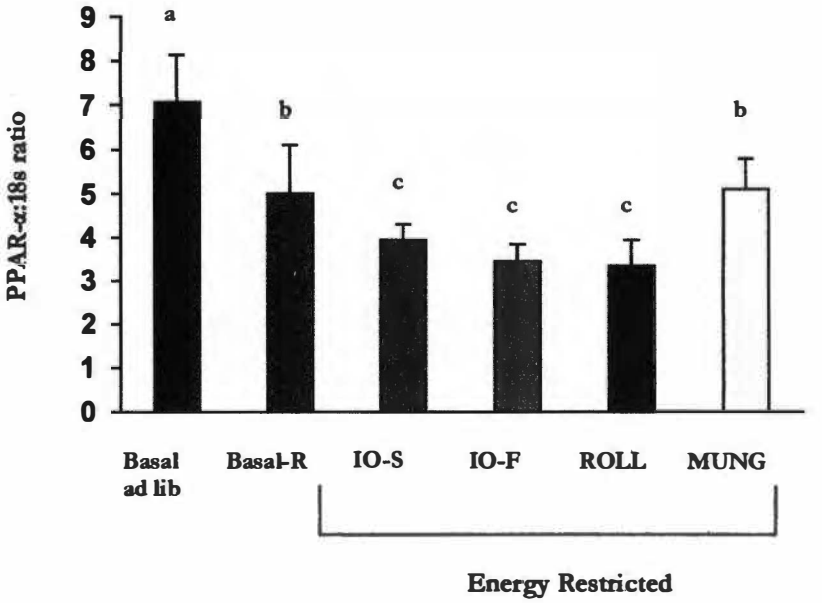


Figure 20. Effect of dietary treatment on hepatic expression of (A) fatty acid synthase and (B) PPAR-α in energy restricted aP274-*Agouti* transgenic mice. Data is expressed as mean±SEM. Non-similar superscripts indicate significant differences at $p<0.05$.

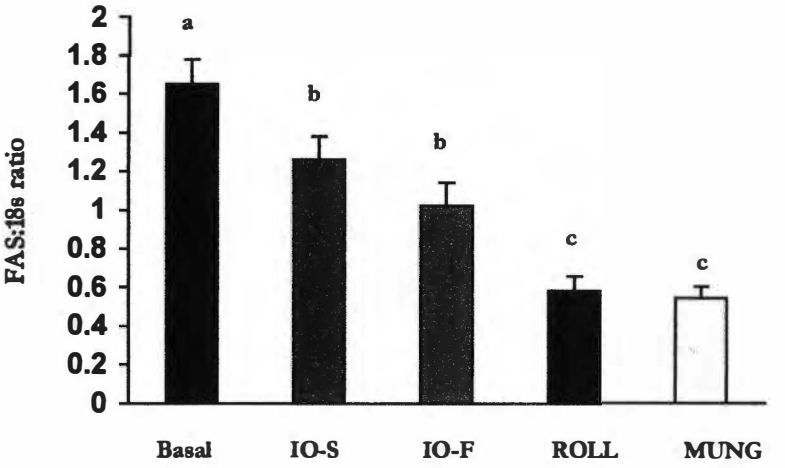
Expression of FAS and PPAR-α in the liver were negatively correlated with grams of body weight lost during the period of energy restriction ($r=-0.445$ and -0.499, respectively, $p<0.01$ for both). During the 12 week study, hepatic FAS expression was positively correlated with fasting plasma glucose levels ($r=0.558$, $p<0.001$) and final body weight ($r=0.346$, $p<0.05$).

Retroperitoneal Adipose Tissue

The expression of lipid-related genes in the retroperitoneal fat depot was examined following *ad libitum* energy intake for 6 weeks. Expression of fatty acid synthase in the retroperitoneal fat pad was greater in basal animals ($p<0.05$) compared with all other groups. Furthermore, FAS expression in this depot was 50% lower ($p<0.05$) in animals consuming either the ROLL or MUNG diets compared with IO-S and IO-F (Figure 21A). In all animals, the increase in body weight occurring in response to unrestricted energy intake for six weeks was positively associated retroperitoneal FAS expression ($r=0.372$, $p<0.05$). A positive relationship was observed between FAS expression and PPAR-α ($r=0.590$, $p < 0.05$), UCP-2 ($r=0.503$, $p<0.05$), and PPAR-γ ($r=0.567$, $p<0.001$) expression in retroperitoneal adipose tissue. Retroperitoneal FAS expression was also positively correlated with FAS expression in the liver ($r=0.440$, $p<0.05$).

Expression of PPAR-α was enhanced by consumption of the basal diet ($p<0.05$ vs. all other groups), but there was no significant differences in PPAR-α expression between other diets (Figures 21B).

A



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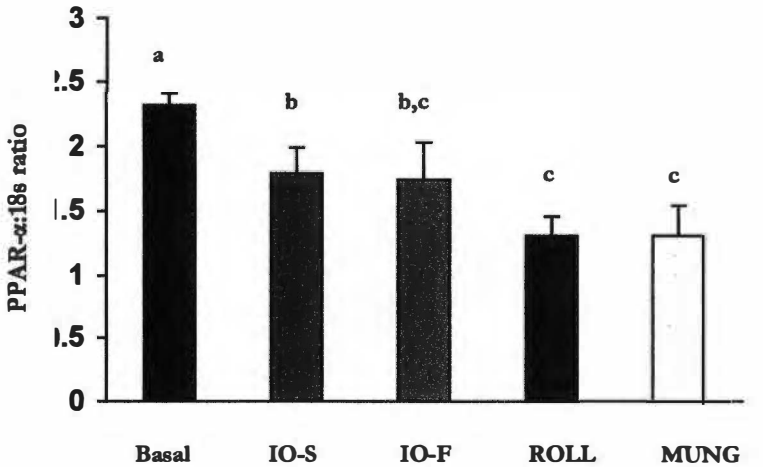


Figure 21. Effect of dietary treatment on expression of lipid related genes in retroperitoneal adipose tissue of *ad libitum* fed aP274-*Agouti* transgenic mice. Data is expressed as mean ± SEM for each group. A: Fatty acid synthase, Non-similar superscripts indicate significant differences at $p<0.05$. B: PPAR-α, Non-similar superscripts indicate significant differences at $p<0.05$.

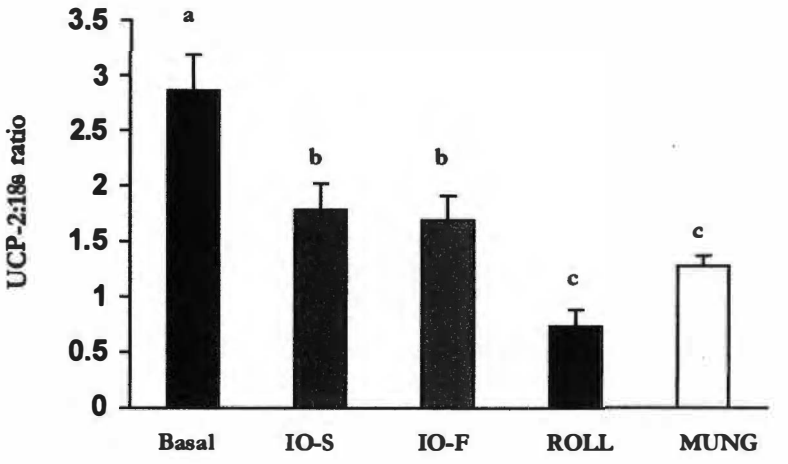
UCP-2 expression was reduced by roughly 60% ($p<0.05$) in ROLL compared to IO-S and IO-F fed animals. Expression of UCP-2 in animals consuming the MUNG diet differed only from the level of expression in HFS animals. Retroperitoneal UCP-2 expression was negatively associated with fasting glucose levels ($r=-0.407$, $p<0.05$), which is consistent with the role of this gene in lipid metabolism.

Expression of PPAR-γ was markedly increased (100-500%) in animals consuming the basal diet ($p<0.05$) compared with all other groups (Figure 22B). Furthermore, PPAR-γ expression was reduced by 61% in animals consuming the ROLL diet ($p<0.05$) compared with both the IO-S and IO-F groups. In MUNG animals, induction of PPAR-γ expression was intermediate between ROLL and the instant oatmeal diets. Consistent with role of PPAR-γ in the induction of adipogenesis, a positive association was observed between PPAR-γ and FAS expression ($r=0.567$, $p<0.01$), fasting insulin levels ($r=0.315$, $p<0.05$) and weight gain ($r=0.321$, $p<0.05$) in this depot.

Subscapular Adipose Tissue

In *ad libitum* fed animals, expression of FAS in this depot was lower ($p<0.05$) in animals consuming the IO-F diet compared with those in the basal and ROLL groups (Figure 23A). Energy restriction increased expression of fatty acid synthase in subscapular adipose tissue by nearly 200% compared with *ad libitum* intake of the basal diet ($p<0.05$) (Figure 23B). Expression of FAS in this depot was negatively associated with final body weight ($r=-0.499$, $p<0.05$) and adiposity ($r=-0.555$, $p<0.05$).

A



B

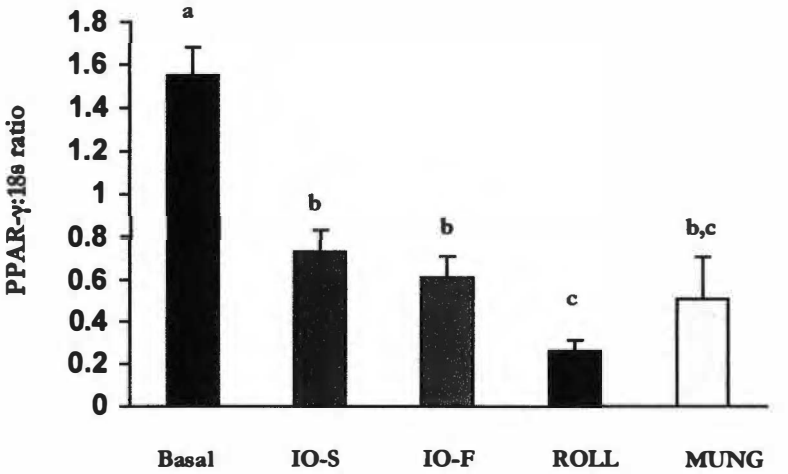
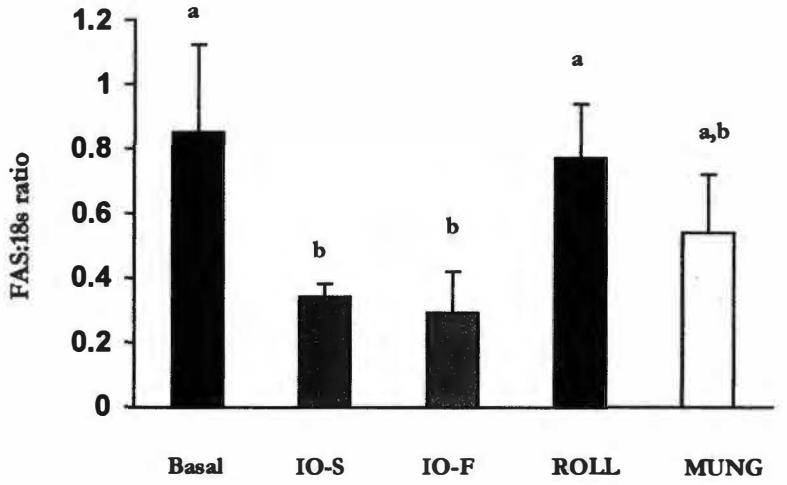


Figure 22. Effect of dietary treatment on expression of lipid related genes in retroperitoneal adipose tissue of *ad libitum* fed aP274-*Agouti* transgenic mice. Data is expressed as mean ± SEM for each group. A: UCP-2, Non-similar superscripts indicate significant differences at $p<0.05$. B: PPAR-γ, Non-similar superscripts indicate significant differences at $p<0.05$.

A



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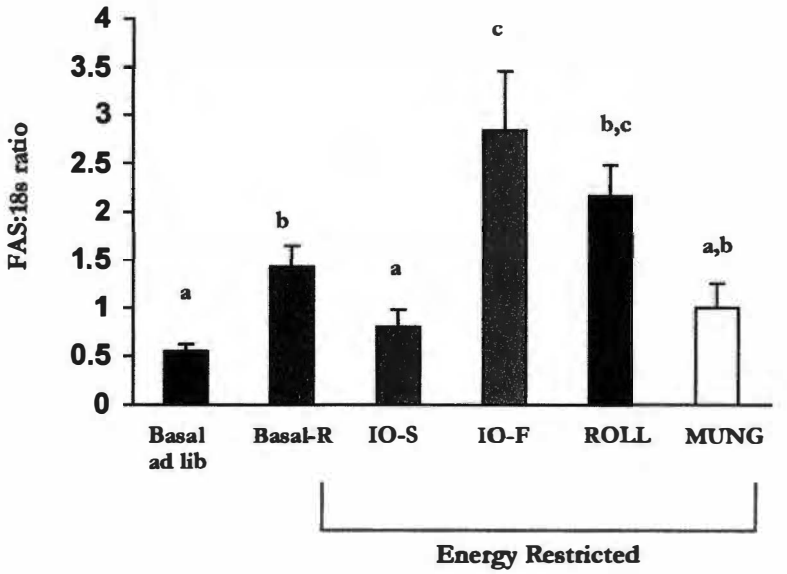


Figure 23. Effect of dietary treatment on subscapular adipose tissue FAS expression in (A) *ad libitum* fed and (B) energy restricted aP274-*Agouti* transgenic mice. Data is expressed as mean ± SEM for each group. Non-similar superscripts indicate significant differences at $p<0.05$.

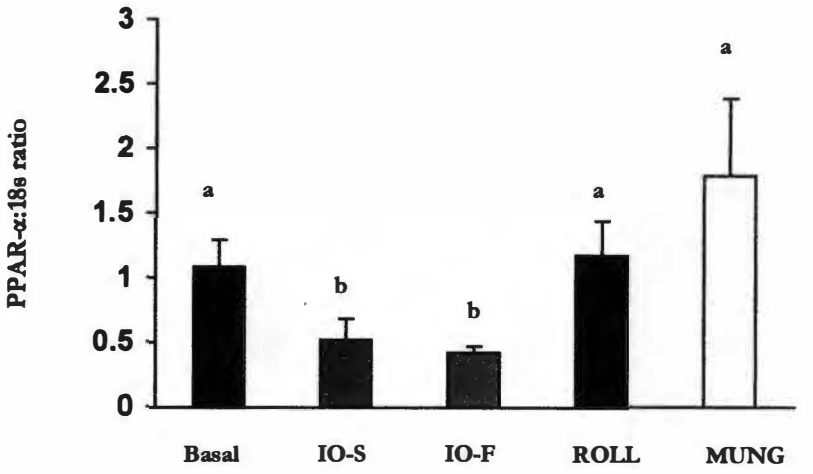
In the absence of energy restriction, expression of PPAR-α in subscapular adipose tissue was reduced by 70% ($p<0.05$) in animals consuming IO-S and IO-F compared with basal, ROLL and MUNG (Figure 24A).

There was a trend for higher levels of PPAR-α in energy restricted ROLL and MUNG animals, but these did not reach significance (Figure 24B). Nonetheless, fasting insulin levels were significantly correlated with expression of PPAR-α ($r=0.631$, $p<0.001$) in this depot. While there were no differences in the level of UCP-2 expression between groups in *ad libitum* fed or energy restricted animals, a positive relationship was noted between the level of UCP-2 and PPAR-α expression in subscapular fat ($r=0.595$, $p<0.001$).

When consumed *ad libitum*, these diets failed to significantly influence the level of PPAR-γ expression in subscapular fat (Figure 25A). Somewhat unexpectedly, a negative association was observed between subscapular fat pad mass and FAS expression ($r=-0.348$, $p<0.05$) in *ad libitum* fed animals. Similarly, final body weight of *ad libitum* fed animals was inversely associated with the level of subscapular FAS ($r=-0.377$, $p<0.05$) expression.

Expression of PPAR-γ was nearly two-fold higher in energy restricted ROLL compared with MUNG ($p<0.05$) fed animals (Figure 25B), suggesting that consumption of the ROLL diet is associated with the emergence of smaller adipocytes in this region. There was a positive association between subscapular FAS and PPAR-γ expression ($r=0.571$, $p<0.05$) in all animals.

A



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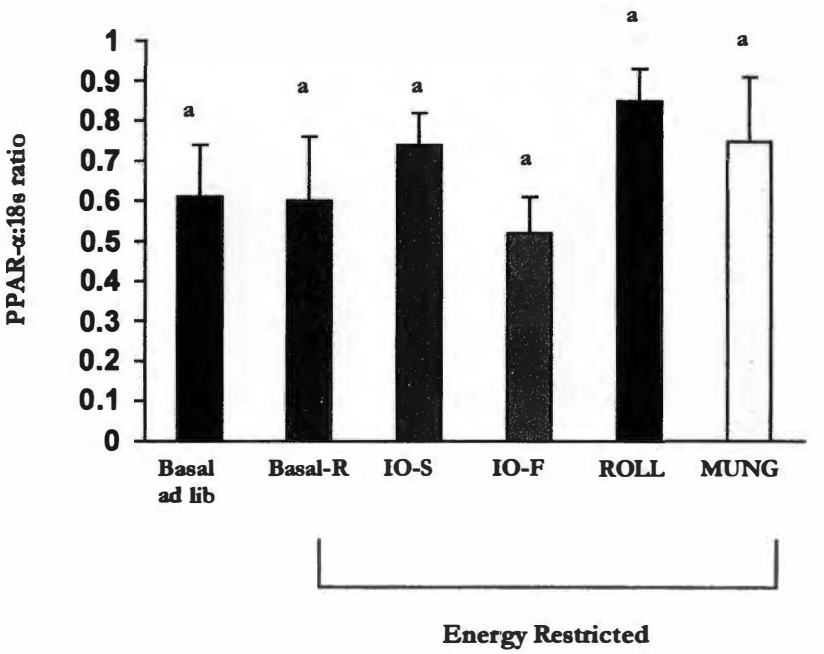
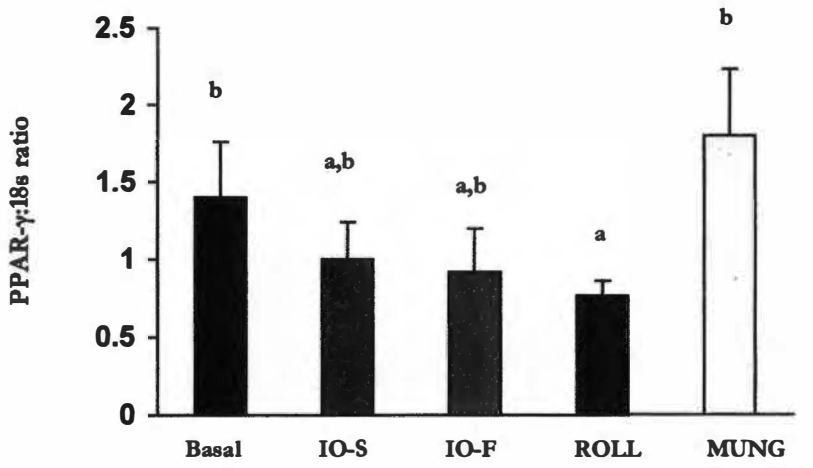


Figure 24. Effect of dietary treatment on subscapular adipose tissue PPAR-α expression in (A) *ad libitum* fed and (B) energy restricted aP274-*Agouti* transgenic mice. Data is expressed as mean ± SEM for each group. A: Non-similar superscripts indicate significant differences at $p<0.05$. B: Matching superscripts indicate no significant differences between groups.

A



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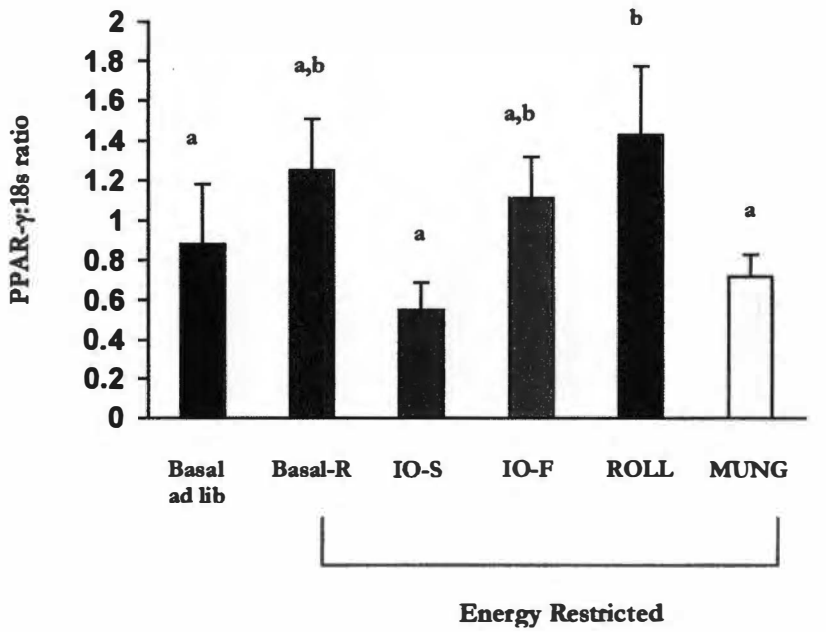


Figure 25. Effect of dietary treatment on subscapular adipose tissue PPAR-γ expression in (A) *ad libitum* fed and (B) energy restricted aP274-*Agouti* transgenic mice. Data is expressed as mean±SEM. Non-similar superscripts indicate significant differences at $p<0.05$.

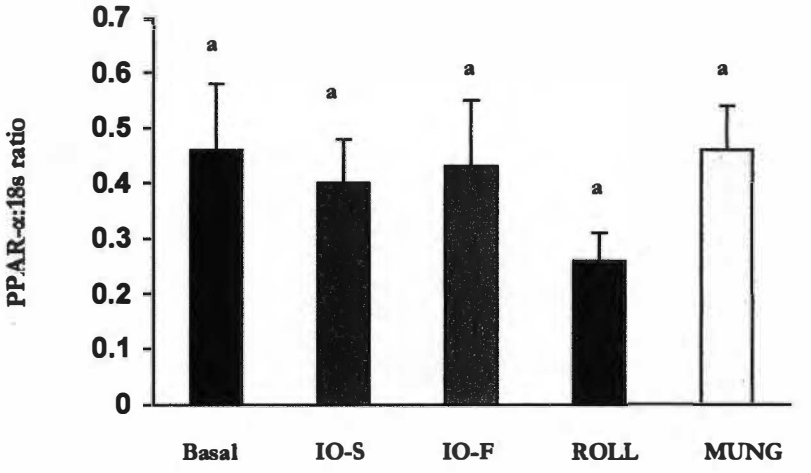
Skeletal Muscle

Diet had no effect on expression of PPAR-α or UCP-3 in gastrocnemius (Figures 26A and 26B) or soleus (Figures 27A and 27B) muscle of *ad libitum* fed animals. A significant negative association was observed between gastrocnemius PPAR-α expression and fasting plasma insulin levels ($r=-0.322$, $p<0.05$) in all animals. This is consistent with studies demonstrating an induction of gastrocnemius PPAR-α expression by conditions which promote enhanced lipid utilization in association with reduced circulating insulin levels. Subcutaneous fat pad mass was inversely correlated with the level of PPAR-α ($r=-0.412$, $p<0.05$) and UCP-3 ($r = -0.616$, $p<0.05$) in gastrocnemius muscle, which suggests that enhanced lipid oxidation in skeletal muscle redirects available lipids away from storage in adipose tissue in favor of oxidation in skeletal muscle .

In energy restricted animals, expression of PPAR-α in gastrocnemius muscle was 2-3-fold ($p<0.05$) higher in basal-R, IO-S, ROLL and MUNG-fed animals compared with the basal *ad libitum* group (Figure 28A). Energy restriction resulted in a 98% increase ($p<0.05$) in UCP-3 expression in gastrocnemius muscle (Figure 28B). Furthermore, expression was significantly greater in MUNG and IO-F compared with basal-R animals.

Expression of PPAR-α in gastrocnemius muscle was negatively correlated with final body weight ($r=-0.489$, $p<0.05$) and the combined mass of selected fat pads ($r=-0.441$, $p<0.05$). Likewise, PPAR-α expression was negatively correlated with plasma leptin levels ($r=-0.516$, $p<0.05$). Consistent with this, a positive association between gastrocnemius PPAR-α expression and grams of body weight lost ($r=0.448$, $p=0.05$) was observed.

A



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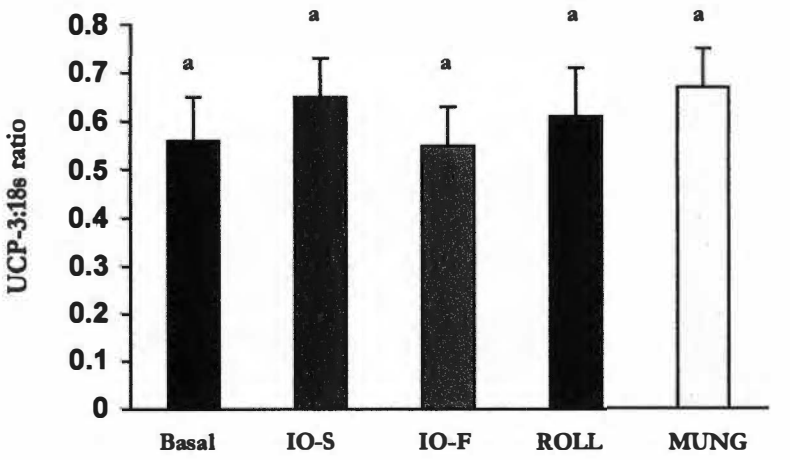
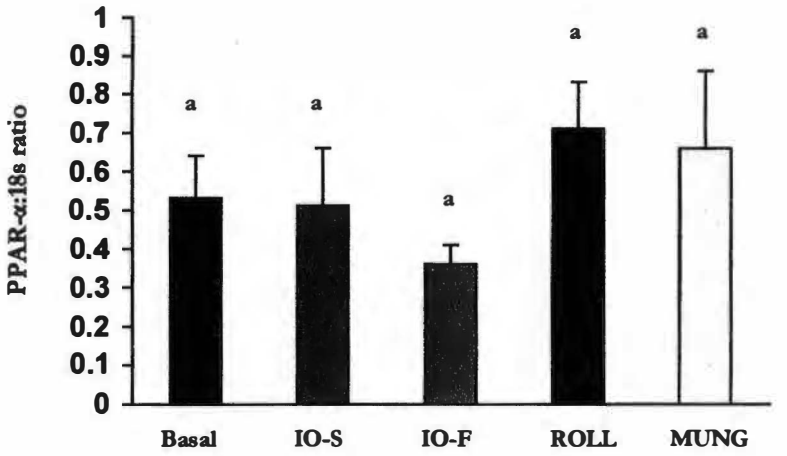


Figure 26. Effect of dietary treatment on expression of (A) PPAR-α and (B) UCP-3 in gastrocnemius muscle of *ad libitum* fed aP274-*Agouti* transgenic mice. Data is expressed as mean±SEM. Matching superscripts indicate no significant differences between groups.

A



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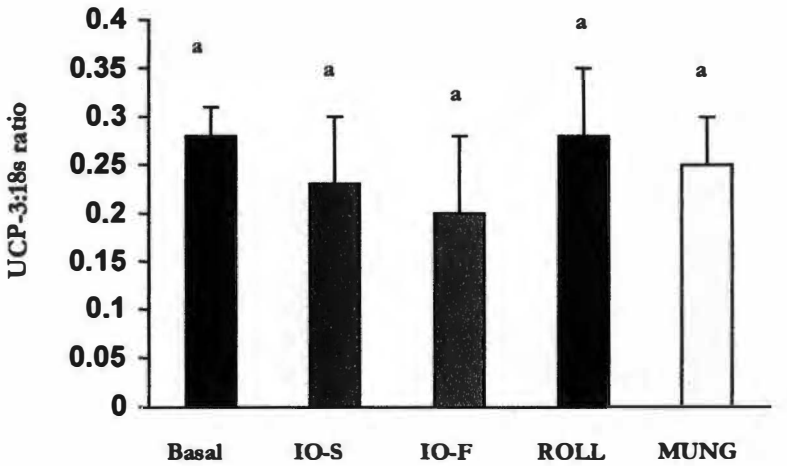
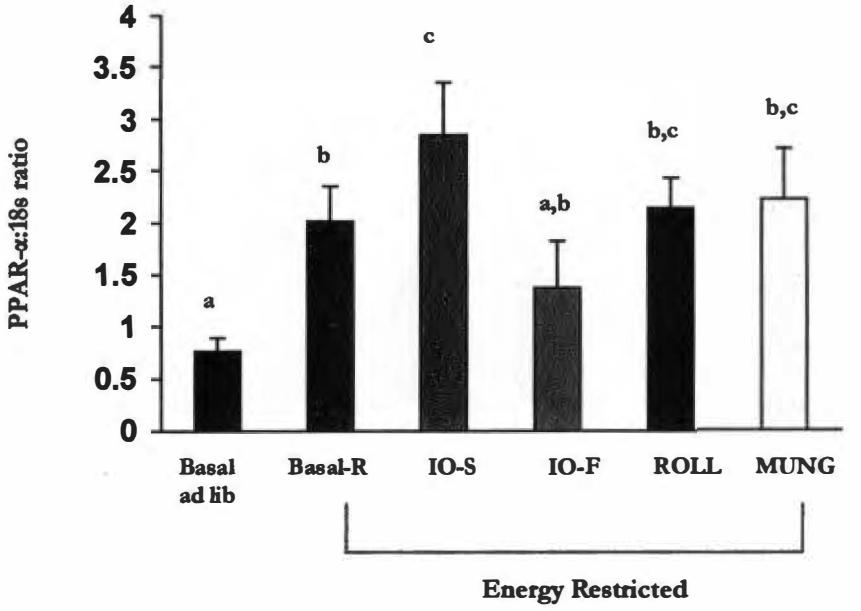


Figure 27. Effect of dietary treatment on expression of (A) PPAR-α and (B) UCP-3 in soleus muscle of *ad libitum* fed aP274-*Agouti* transgenic mice. Data is expressed as mean±SEM. Matching superscripts indicate no significant differences between groups.

A



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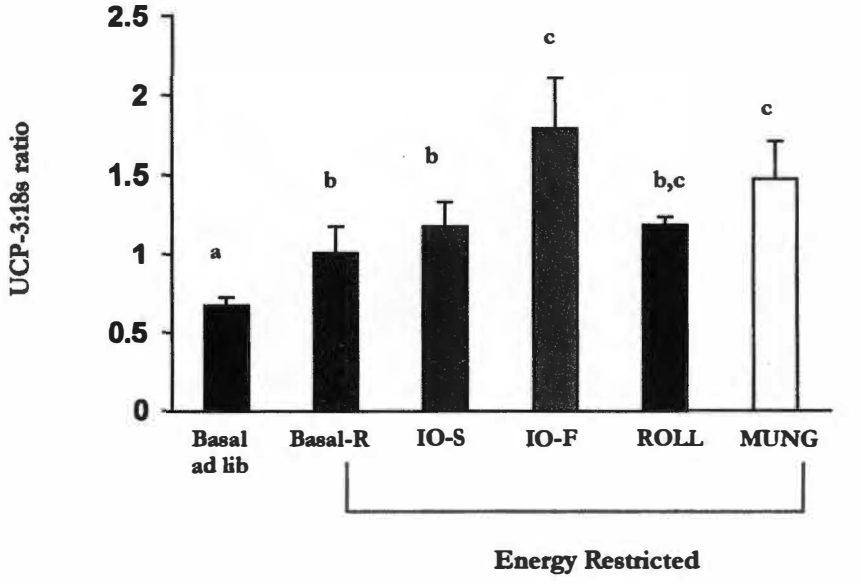
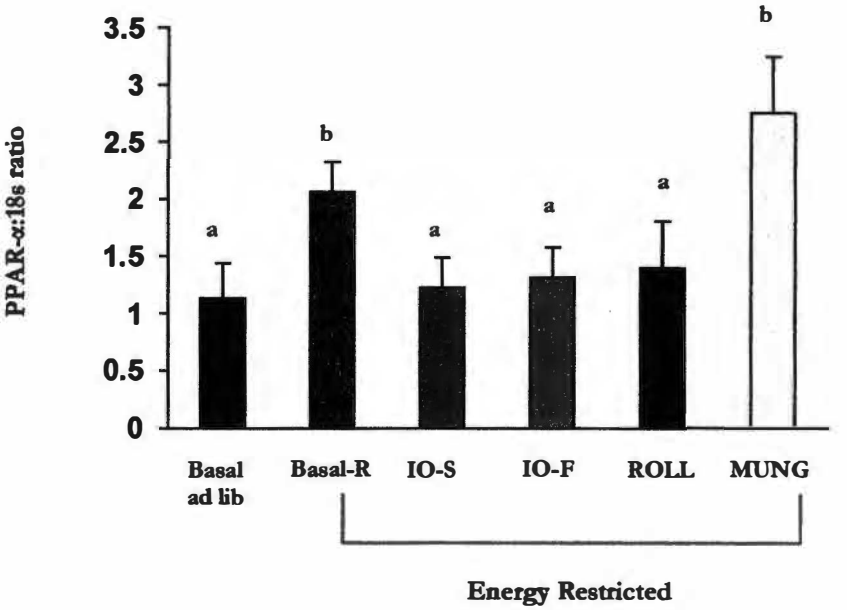


Figure 28. Effect of dietary treatment on expression of (A) PPAR-α and (B) UCP-3 in gastrocnemius muscle of energy restricted aP274-*Agouti* transgenic mice. Data is expressed as mean±SEM. Non-similar superscripts indicate significant differences at $p<0.05$.

A



B

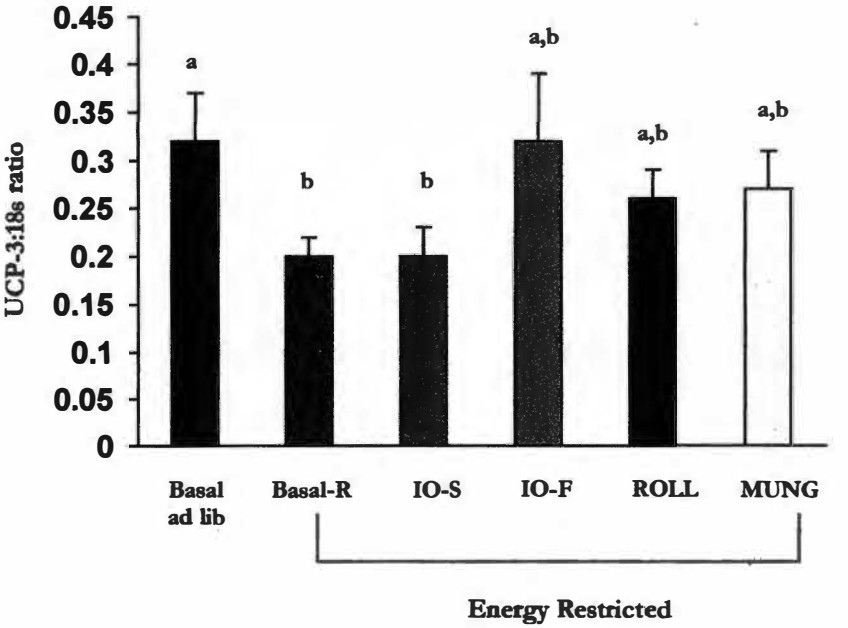


Figure 29. Effect of dietary treatment on expression of (A) PPAR-α and (B) UCP-3 in soleus muscle of energy restricted aP274-*Agouti* transgenic mice. Data is expressed as mean±SEM. Non-similar superscripts indicate significant differences at $p<0.05$.

Expression of UCP-3 in gastrocnemius muscle was inversely associated with final body weight ($r=-0.537$, $p<0.05$), combined mass of selected fat pads ($r=-0.683$, $p<0.05$), and fasting leptin levels ($r=-0.555$, $p<0.05$). Furthermore, UCP-3 expression in this tissue was negatively correlated with liver FAS ($r=-0.509$, $p<0.05$), while a positive relationship was noted with liver PPAR-α ($r=0.359$, $p<0.05$) and intrascapular FAS ($r=0.406$, $p<0.05$) expression.

Discussion

In these studies, we demonstrate that a qualitative change in diet composition, specifically carbohydrate source, influences weight gain, adiposity and gene expression in animals fed energy restricted diets. To our knowledge this is the first study to evaluate the effects of commonly consumed carbohydrate-containing foods on the development of obesity in this model of diet-induced obesity (53,57,80). Our data demonstrates that adipose tissue deposition is reduced in animals fed a diet containing carbohydrates which elicit lower blood glucose response compared with sucrose-rich diets of identical macronutrient composition. The pattern of gene expression in liver, adipose tissue and skeletal muscle suggests that within a mixed diet, carbohydrate containing foods which result in lower blood glucose responses, may alter the pattern of substrate utilization and establish an environment which enhances lipid oxidation in skeletal muscle, limiting lipid flux to adipose tissue.

In fasting aP274-*agouti* transgenic mice the blood glucose response to mung bean noodles was significantly lower than the response to rolled oats or instant oatmeal. The lower blood glucose response to mung bean noodles is attributable to the

physiochemical structure of mung bean starch (81,82). Mung bean starch is reportedly the best raw material for preparation of starch noodles as a result of its amylose content (83,84). Straight chain amylose represents 40-47% of total starch in mung beans (81,82,85), which is considerably more than the other legumes (86) and most cereal starches (87). Furthermore, mung bean starch granules are smaller and more densely packed than starch granules of other legumes, which makes them more resistant to swelling and digestion by α-amylase (81,88,89). In normal and diabetic rats, Kabir, et al. (90) have determined the glycemic index of mung bean starch is 67, relative to white bread. Furthermore, rats consuming mung bean starch (32% amylose) had lower blood glucose and insulin responses ($p<0.05$) compared to rats consuming waxy cornstarch (5% amylose) (90,91). This is consistent with the greater susceptibility of high-amylopectin starch to hydrolysis by α-amylase *in vitro* (92).

Oats are rich in soluble fiber and readily available in a number of highly palatable varieties, including rolled oats, instant oatmeal, and oat bran (93,94). Studies consistently report that diets rich in soluble fiber improve postprandial blood glucose, insulin, and lipid levels in lean and obese subjects (95-98). The component of oat bran presumed responsible for the benefits of diets rich in soluble fiber is the linear polysaccharide, β-glucan (93). Viscous fibers, like β-glucan, impede glucose absorption from the lumen of the small intestine and consequently, prolong absorption time (93,96,9). Wood, et al. (96) report that 79-96% of the variation in plasma glucose and insulin responses following a 50 g glucose load supplemented with various doses of β-glucan can be explained by the increase in the viscosity of the digesta in normal women. However, Tappy, et al. (95) have reported that the β-glucan content of normal rolled oats (4% by

weight) is insufficient to significantly reduce blood glucose and insulin responses in subjects with NIDDM.

The differences in the blood glucose response to the IO-S and IO-F in aP274-agouti transgenic mice are somewhat puzzling. Like rolled oats, instant oatmeal is considered a whole grain food since the bran, endosperm and germ are retained during processing (100). The greater blood glucose response in IO-S compared with ROLL was not surprising; instant oats are thinner than rolled oats. The thinner particle size in the instant oatmeal increases the surface area over which digestive enzymes can act (101) and Jenkins, et al. (102) have demonstrated that the *in vitro* rate of digestion for grains increases as particle size decreases (101). Particle size similarly affects the postprandial response to other cereals and legumes (101,103). However, particle size cannot be the sole determinant of the *in vivo* response, since the $AUC_{glucose}$ for IO-S was significantly greater than IO-F. Although β-glucan influences viscosity, and the properties of β-glucan are related to its water soluble nature (93), this would not have been exploited in our study using uncooked foods (104). Prior to adding the vitamin-mineral premix the nutrient composition of IO-S and IO-F should have been identical. Thus, we can only conclude that differences in the micronutrient composition of the premixes are responsible for the variations in the blood glucose response between the IO-S and IO-F foods.

Careful examination of the nutrient composition of the vitamin-mineral premixes (Table 6) reveals some differences which may have influenced the blood glucose response. The most glaring discrepancy between the two formulations is the energy content. The formulation used for IO-S provides a significantly greater amount of

protein and fat (g per 100 g), resulting in total energy content of the premix for IO-S that is 2-fold higher than the premix used for IO-F. Although we would have expected differences in the formulation of the pre-mixes to have little, if any, impact on the *in vivo* response, this remains the only factor distinguishing the IO-S from IO-F.

These studies suggest that the dietary carbohydrate composition of a mixed diet influences body weight and adiposity in aP274-*agouti* transgenic mice. We demonstrate that a qualitative change in the dietary carbohydrate composition influences body weight, independently of energy intake. While the macronutrient energy distribution (as % kcal) was identical for all diets (14% protein, 62% carbohydrate, and 24% fat), the ROLL diet contained significantly more fiber than all other diets. Thus, it is likely that the lower body weight in the ROLL animals is related to the fiber content of the dietary carbohydrate.

Body weight of animals fed the basal diet for 12 weeks was only modestly greater than animals consuming the same diet ad libitum for 6 weeks. Nonetheless, mass of representative fat pads was 35% greater in animals fed the basal diet for 12 weeks compared to those animals fed the basal diet for only six weeks. Thus, adipose tissue accumulation continued during weeks 6-12 without a parallel increase in body weight. This represents an important physiological effect of prolonged consumption of a high-fat, sucrose-rich diet, and suggests that such a diet influences adipocyte morphology and metabolism without detectable changes in body weight. In an examination of the influence of diet composition on adipocyte morphology and metabolism, Soria, et al. (105) document some important physiological consequences of prolonged consumption of a sucrose-rich diet in rats. Epididymal fat mass, adipocyte volume, adipocyte

triglyceride content, and adipocyte number were similar in rats fed a control or sucrose-rich diet for three weeks. Furthermore, changes in body weight in the short term paralleled changes in adipose tissue mass. Following 15 weeks of consumption of the control diet, epididymal fat pad mass increased in parallel with body weight, associated with an increase in adipocyte volume and reduction in adipocyte number. However, in rats fed the sucrose-rich diet for 15 weeks, there was an acceleration of adipose tissue deposition in the epididymal depot and a notable dissociation of the changes in body weight and fat mass. Increased adiposity in rats fed the sucrose-rich diet for 15 weeks was the result of adipocyte hypertrophy, as adipocyte volume was 76% higher than in animals fed the control diet for 15 weeks. Adipocyte hyperplasia could not account for the increased adipose tissue deposition, as suggested by a reduction in adipocyte number to 61% of control fed animals. Although we analyzed cell size only in animals consuming the basal diet for 12 weeks, it is plausible to suggest that adipocyte hypertrophy was an important factor contributing to the increased adiposity, but similar body weight, in animals fed the basal diet for 12 versus 6 weeks.

With energy restriction, we begin to see an independent influence of dietary carbohydrate source on adiposity. Subscapular fat pad mass was significantly higher in the energy restricted basal and IO-S groups compared with ROLL, which was similar to IO-F and MUNG. However, mass of the retroperitoneal and perirenal depots was reduced in both the ROLL and MUNG groups compared with the basal and IO-S groups, despite identical energy restriction. Thus, consumption of the MUNG and ROLL diets reduced visceral adipose tissue accumulation. Furthermore, this effect can not be assigned solely to dietary fiber since the fiber content of the MUNG diet, which

was less than the ROLL diet, was similar to the basal and IO-S diets. It is possible that the absence of sucrose in the ROLL and MUNG diets are responsible for the differences in adipose tissue accumulation as compared with the basal and IO-S diets, both of which contained sucrose.

Chicco, et al. (106) compared the effects of partial or complete substitution of dietary starch for sucrose on body weight and glucose and lipid metabolism in rats. Animals consuming a 63% w/w sucrose-rich diet (63% SRD) were markedly insulin resistant after only 15 weeks compared with animals consuming the control diet (0% SRD) in which sucrose was completely replaced by starch for the same length of time. Animals consuming the 63% SRD were hyperglycemic in the fasting state, yet the insulin secretory response to an intravenous glucose bolus was preserved. After the initial 15 weeks, animals consuming the 63% SRD were divided into three groups. A subset of these animals continued on the 63% SRD for an additional 15 weeks experienced a further deterioration in glucose tolerance, which was accompanied by a nonsignificant reduction in the amount of insulin secreted following an intravenous glucose bolus. A separate subset of the animals consuming the 63% SRD for 15 weeks were switched to a modification of the 63% SRD diet, in which 30% of sucrose was replaced with starch (33% SRD) for the final 15 weeks of the study. Although consumption of the 33% SRD during weeks 15-30 improved basal hyperglycemia, these animals remained glucose intolerant. However, there were no significant differences in body weight between groups. The final subset of animals were switched to the control (0% SRD) diet for weeks 15-30. The complete substitution of starch for sucrose reduced body weight, normalized basal glucose levels and improved glucose tolerance to levels comparable to

animals consuming the 0% SRD diet for the entire 30 week period. Neither fasting plasma insulin levels nor the insulin response to intravenous glucose were significantly affected by diet. Thus, it seems that the partial substitution of starch for sucrose improves body weight and glucose tolerance without affecting plasma insulin levels (106).

Based on the above studies, we suggest that in our studies, the favorable effects of the ROLL and MUNG diets on reducing adiposity compared with the basal and IO-S diets are due to the sucrose content of the latter. We also found lower fasting blood glucose levels in animals consuming the energy restricted ROLL and MUNG compared with basal and IO-S diets, despite comparable plasma insulin levels in all groups. However, in contrast to Chicco, et al. (106), our findings suggest that the partial substitution of starch for sucrose cannot fully explain these effects as demonstrated by the similarities between animals fed the energy restricted basal diet, which contains sucrose as the sole dietary carbohydrate, and IO-S, which contains both sucrose and starch.

The composition of the basal diet was chosen to mimic the typical Western diet, which contains significant amounts of dietary fat and refined sugars, particularly sucrose (11,12). In addition, sucrose was added to both instant oatmeal diets to make them similar to these foods as they would be consumed in the diet. Conversely, sucrose was not added to either the ROLL or MUNG diet. Consequently, we may attribute some of the differences to the sucrose content of the diet. It is conceivable that sucrose containing diets provoke greater postprandial blood glucose and insulin responses, which

may over time lead to changes in adiposity, adipocyte morphology, as well as altering expression of genes involved in carbohydrate and lipid metabolism.

An additional factor in the retroperitoneal depot is likely adipocyte diameter, which was greater in the basal diet regardless of energy intake, compared with all other diets. Again, the effect of diet on adipocyte diameter is only partially attributable to the endogenous dietary fiber content of the ROLL diet, since adipocytes isolated from ROLL and MUNG fed animals were of similar size. Thus, a qualitative change in the carbohydrate content of the diet influences adipocyte diameter and this effect is only partially explained by dietary fiber.

Adipocyte size is likely influenced by the sucrose content of the diets (105,107). Adipocyte hypertrophy has been previously demonstrated in animals fed a high-fat, sucrose-rich diet for 2 months (107). In our present studies, the greater mass of the retroperitoneal adipose tissue depot in animals fed the basal diet *ad libitum* for 12 weeks is at least partially attributable to adipocyte hypertrophy, as adipocytes in the basal *ad libitum* group were 75% larger than those from energy restricted animals consuming the same diet and 300% larger than animals consuming the energy restricted ROLL diet. This is consistent with Salans, et al. (108), who demonstrated that adipocyte hypertrophy develops in rats fed *ad libitum*. The emergence of larger adipocytes is the result of increased lipid accumulation, as protein content is relatively constant over a range of size (107,108). Furthermore, Salans, et al. (108) found that insulin responsiveness was reduced as fat cells enlarged, which contributes to reduced insulin sensitivity of adipose tissue in sucrose-fed animals.

In the present studies, energy restriction, irrespective of dietary carbohydrate source, resulted in smaller adipocytes relative to the *ad libitum* fed animals. Weight loss induced by energy restriction has been previously demonstrated to reduce adipocyte size in humans and rodents (109,110). Furthermore, Ostman, et al. (109) have demonstrated in normal rats that all parameters of lipid metabolism are improved in parallel with the reduction in adipocyte size occurring with energy restriction.

Adipocyte diameter appears to be influenced by diet composition since adipocytes from the energy restricted basal group were 77% and 130% larger than those from MUNG and ROLL, respectively, at the same level of energy restriction. This is a finding similar to that of Lerer-Metzger, et al. (111), who reported a marked decrease in adipocyte size in animals fed a diet containing mung bean compared with wheat starch.

In vitro studies have shown that large adipocytes fail to increase glucose oxidation to CO_2 to an appreciable degree when incubated with insulin and high concentrations of glucose (112). While insulin failed to stimulate the complete oxidation of glucose under these conditions, incorporation of labeled glucose into triacylglycerols was stimulated to a larger extent in large compared with small adipocytes (112). Despite an apparent reduction in the capacity to metabolize glucose once inside the adipocyte, insulin treatment results in a dramatic increase in glucose transport and accumulation of free glucose in larger cells, which may further impair the metabolic characteristics of enlarged adipocytes (110,112,113). Based on these findings and our data regarding the blood glucose response to the test diets, as well as the positive correlation between fasting plasma glucose and insulin levels, it is possible that consumption of the basal and IO-S

diets exposed these animals to higher plasma glucose and insulin levels, which resulted in metabolic abnormalities similar to those documented in the studies described (104-110).

Differences in retroperitoneal fat mass in our studies may be at least partially attributable to alterations in glucose and lipid metabolism in larger adipocytes (113,114). The stimulatory effects of insulin on glucose oxidation and lipid synthesis are blunted in large compared with small adipocytes (115-117). Thus, adipocyte size, and consequently, insulin sensitivity can be influenced by nutritional and hormonal cues, independent of energy consumption. Based on the blood glucose response to the test diets, and the positive correlation between adipocyte size and plasma glucose and insulin levels, the postprandial response to the experimental diets of varying carbohydrate source may have contributed to alterations in adipocyte size.

Metabolic abnormalities observed in rats consuming (14 days to 12 months) a high sucrose (33-70 en%) diet include elevated insulin and triglyceride levels, increased expression and activity of hepatic lipogenic and glycolytic enzymes, and hepatic and skeletal muscle insulin resistance (107-112). In the present studies, glucose homeostasis was assessed by evaluating fasting plasma glucose and insulin levels. While fasting plasma glucose levels were significantly lower in animals consuming the MUNG and ROLL diets compared with the basal diet (*ad libitum* or with energy restriction), fasting plasma insulin levels did not differ by diet in the energy restricted groups. However, changes in insulin sensitivity versus insulin responsiveness cannot be determined based on fasting insulin levels, and a single fasting insulin measurement is unable to identify the specific tissues contributing to changes in insulin action (118,119). Nonetheless, based on the glucose response to our test foods, it is not unreasonable to speculate that the

postprandial insulin response would have followed a similar pattern, and that the persistence of these changes would influence whole body glucose and lipid metabolism.

Basal glycerol release (μmol glycerol/μg protein) tended to be higher following 6 weeks *ad libitum* energy intake compared with consumption of the energy restricted diets. Since body weight was greater in animals fed *ad libitum* for 6 weeks compared with the animals fed energy restricted diets for weeks 6-12, one can speculate that the greater lipolysis is the result of the influence of body weight on basal lipolysis. In humans, basal lipolysis is higher in obese compared with lean subjects and this is related to larger adipocytes in obese subjects (120,121). In *ad libitum* fed animals, the stimulation of lipolysis above basal levels was greater in animals consuming the IO-F, ROLL, and MUNG diets compared with the basal and IO-S. Interestingly, the IO-F, ROLL, and MUNG diets resulted in lower postprandial blood glucose response compared with IO-S, which suggests that the postprandial hormonal milieu may influence lipolysis in the long term. This hypothesis is consistent with Berger, et al. (107) who report that accelerated lipolytic rates occurring in animals made hyperinsulinemic by feeding a high-sucrose diet is apparent only after 2 months of diet consumption. Furthermore, we can speculate that long-term consumption of the high-fat, high-sucrose basal diet impaired adipocyte metabolism based on the inability of isoproterenol to stimulate glycerol release above the basal rate in adipose tissue isolated from animals consuming the basal diet ad libitum for 12 weeks. Unfortunately, we did not assess the antilipolytic actions of insulin on isoproterenol-stimulated lipolysis; consequently, we can not draw any conclusions regarding the insulin responsiveness of adipose tissue metabolism with respect to diet.

Animals consuming the basal diet *ad libitum* for six weeks had higher insulin levels compared with IO-S, ROLL, and MUNG. Energy restriction tended to reduce fasting insulin levels compared with animals continued on the basal diet for 12 weeks. There were no significant differences in plasma insulin levels in energy restricted rats consuming the IO-S, IO-F, ROLL, and MUNG diets, which is consistent with other reports (90,91,111). However, the influence of glycemic index on fasting insulin levels is debatable. Byrnes, et al. (20) have shown that feeding a high-amylopectin diet to rats for 9 weeks results in significantly higher fasting plasma glucose and insulin levels compared with rats fed a high-amylose diet. The high-amylopectin diet resulted in a greater insulin response during an intravenous glucose tolerance test, which increased with the duration of feeding and eventually led to fasting hyperinsulinemia (20). In contrast, Pawlak, et al. (122) did not find a hyperinsulinemic response in rats fed a high- glycemic index starch diet, despite an elevated insulin response during intravenous glucose tolerance tests. Thus, we can not exclude the possibility that postprandial insulin responses to the diets influenced body composition.

Depending on mouse strain and age, normal plasma leptin levels are in the range of 1-5 ng/ml, with hyperleptinemia generally defined as fasting plasma leptin levels exceeding 20 ng/ml (123,124). Consistent with the role of plasma leptin in regulation of adiposity (125), strong correlations were noted between fasting plasma leptin levels and fat pad mass in all animals, regardless of energy intake in the present studies. Furthermore, plasma leptin levels exceeded 20 ng/ml in all groups following 6 weeks of ad libitum feeding, which is consistent with the induction of leptin resistance by overfeeding a high-fat diet (126). However, leptin levels were significantly lower in

animals fed the ROLL diet ad libitum compared to all other groups, which suggests that leptin levels are influenced by qualitative changes in diet. Furthermore, leptin levels continued to increase when animals were continued on the basal diet for an additional six weeks. Thus, there was a physiological adaptation to the diet which appears to have blocked the normal response to increases in circulating leptin levels and may have contributed to the increase in adipose tissue mass.

In *ad libitum* fed animals, the normal physiological response to elevated plasma leptin levels (reduced energy intake, increase energy expenditure, adipose tissue depletion) is absent (127). However, there is some restoration of leptin responsiveness when energy intake is restricted. Nonetheless, while *ad libitum* energy intake led to significant hyperleptinemia, plasma leptin levels were not completely normalized following six weeks of energy restriction. Still the differential response of plasma leptin levels to energy restricted diets, suggests leptin levels are also related to qualitative changes in diet composition. While fasting and refeeding are known to regulate plasma leptin and *ob* gene expression in obese and lean humans and rodents (128,129), little other information regarding the regulation of leptin levels by dietary composition. The variations in plasma leptin levels following six weeks of energy restriction in the present studies may be related to dietary carbohydrate since all diets contained identical amounts of fat and protein and suggests that dietary glycemic index may have influenced plasma leptin levels in energy restricted animals. Despite identical energy intake and carbohydrate content of the diet, plasma leptin levels were lowest in MUNG animals. This is consistent with Hermann, et al. (130) who demonstrated that the normal diurnal peak in plasma leptin levels occurring at 22:00 hr can be altered by dietary carbohydrate. Feeding a high-

glycemic index carbohydrate resulted in an earlier rise in plasma leptin levels (at 13:00 hr) and higher AUC_{leptin} levels at 12:30 and 24:00 hr. Furthermore, these changes occurred despite the fact that diet did not influence fasting plasma glucose or insulin levels (131).

Our findings of reduced plasma leptin levels in animals consuming energy restricted diets containing carbohydrates shown to result in lower blood glucose responses, specifically MUNG and ROLL, are somewhat at odds with those of Kabir, et al. (131), who have demonstrated that a high-glycemic index starch diet decreased adipose tissue *ob* gene expression after 3 weeks and reduced serum leptin levels compared with a low-glycemic index starch fed for 12 weeks to normal rats. Despite lower plasma leptin levels in animals fed the high-glycemic index starch diet, animals did not increase their food intake, leading Kabir et al. (131) to hypothesize that the high glycemic index starch diet increased leptin sensitivity in advance of weight gain and increased adiposity. Our findings appear more consistent with the associations between adiposity and plasma leptin levels (125). However, neither the present studies nor those of Kabir, et al. (131) are inconsistent with the involvement of factors other than adipose tissue mass in regulation of plasma leptin levels. While Kabir, et al. (131) found no association between plasma leptin levels and circulating levels of glucose or insulin, a positive association was found between plasma leptin and free fatty acid levels. Similarly, we found no association between plasma leptin and insulin levels, however, leptin levels were positively correlated with fasting plasma glucose concentrations. Thus, it appears that regulation of plasma leptin levels in the present studies are dependent upon a hormonal and dietary factors.

Fatty acid synthase (FAS) is expressed in both the liver and adipose tissue and is a key lipogenic enzyme catalyzing the synthesis of the long chain saturated fatty acid, palmitate (132). FAS is subject to regulation by both nutrients and hormones in humans and rodents (132-140). In rodents and murine cell lines, FAS expression is reduced by fasting (133,134), polyunsaturated fatty acids (135), and diabetes (136), whereas its expression is enhanced by high-carbohydrate feeding (137,138), glucose (139), insulin (140,141), and obesity (135,142,143).

The present studies demonstrate that induction of FAS expression in the liver can be altered by dietary carbohydrate source *per se*. In the absence of energy restriction, FAS expression in the liver was significantly lower in animals consuming the MUNG and ROLL diets compared with those consuming the Basal, IO-S, and IO-F diets. It is important to note that each of these diets contains sucrose while neither the ROLL nor MUNG diets contain any added sucrose. Thus, the greater induction of FAS expression in the basal and instant oatmeal diets is consistent with more vigorous induction of FAS in the liver of animals fed diets containing simple sugars compared with complex carbohydrates (138). Furthermore, these findings suggest that when glucose is readily available, as in *ad libitum* fed animals, the rapidity with which carbohydrate digestion and absorption proceeds alters the expression of FAS. However, substrate availability is only one factor influencing FAS expression in *ad libitum* fed animals, since FAS expression was relatively low in the ROLL group despite higher plasma glucose levels. In addition, insulin levels were increased in the Basal and IO-F animals compared with IO-S, whereas FAS expression was similar in these groups, implying involvement of a hormonal or nutritional factor in addition to glucose and insulin in regulation of FAS

expression. Our findings in *ad libitum* fed animals are somewhat at odds with those of Kabir, et al. (90,91), who demonstrated that the substitution of a high-amylopectin (high glycemic index) starch for a low-amylopectin (low glycemic index) starch in a mixed diet increased FAS activity and mRNA levels in adipose tissue, but not in the liver, of normal rats. However, when energy intake was restricted, our results are in consistent with those of Kabir, et al. (90,91) as dietary carbohydrate source did not independently influence hepatic FAS expression.

FAS expression was significantly higher in animals consuming the basal diet *ad libitum* for 12 weeks compared with all energy restricted groups. This is consistent with studies documenting increased hepatic expression in obese animals and is consistent with the role of insulin in regulation of FAS expression (133-136).

Energy restriction during weeks 6-12 reduced hepatic FAS expression relative to animals consuming the basal diet *ad libitum* for 12 weeks, an effect consistent with the downregulation of FAS expression during fasting and is presumably due to lower insulin levels in fasting and energy restricted animals (133,136,140). In energy restricted animals, substrate availability appears to dominate over type of dietary carbohydrate in the regulation of FAS expression in the liver. Consequently, FAS mRNA levels were lower in all energy restricted groups compared with animals consuming the basal diet *ad libitum* for 12 weeks. This effect is consistent with the findings of Kabir, et al. (91), who demonstrated that dietary carbohydrate type did not influence the level of FAS expression in the liver of energy restricted animals.

Thus, when glucose is readily available as in *ad libitum* fed animals, expression of FAS in the liver is influenced by the rate of carbohydrate digestion and absorption.

Consequently, diets containing rapidly digestible sucrose result in higher levels of FAS mRNA, while diets containing more slowly digested carbohydrates are associated with lower FAS mRNA levels in the liver.

In the liver, PPAR-α levels were significantly greater in animals fed the basal diet *ad libitum* for 12 weeks compared with all energy restricted groups. While high-fat feeding and energy restriction represent opposing physiological conditions, both are characterized by greater fatty acid availability (145). Hepatic PPAR-α expression was increased with energy restriction relative to the level of expression observed in *ad libitum* fed animals consuming the same diet. This is consistent with the role of PPAR-α in mediating the response to fasting (146). Fasted PPAR-α deficient mice suffer from severe impairments in hepatic oxidation, resulting in hypoglycemia, hypothermia, hypoketonemia, elevated plasma free fatty acids, and fatty liver (146). The phenotype of PPAR-α null mice is due to the inability of elevated free fatty acids in the fasting state to stimulate the transcription of PPAR-α target genes in the liver, including genes involved in fatty acid transport and fatty acid oxidation (146). In animals consuming the basal diet *ad libitum* for 12 weeks, expression of PPAR-α in the liver was increased relative to energy restricted groups. The greater induction of hepatic PPAR-α expression in animals consuming the basal diet (14 en% fat) *ad libitum* may therefore, represent a compensatory mechanism induced to increase hepatic β-oxidation in the face of elevated circulating fatty acids resulting from chronic consumption of the basal diet. This would mimic the effect of PPAR-α activation by fibrates and would be expected to increase fatty acid flux from peripheral tissues to the liver, reduce fatty acid synthesis, and reduce the delivery of triacylglycerols to peripheral tissues (147,148). Interestingly, expression of PPAR-α in

the liver was significantly greater in animals consuming the energy restricted basal and MUNG diets compared with IO-S, IO-F, and ROLL. This is somewhat surprising due to the identical macronutrient composition of the diets. Based on the differences in the level of PPAR-α expression among energy restricted groups, we can speculate that these differences are related to the endogenous dietary fiber of the oatmeal based diets, which over the long term may have slowed glucose absorption and consequently, prevented plasma free fatty acid levels from rising to levels sufficient to induce PPAR-α expression to the same degree as the energy restricted basal and MUNG diets, which lacked endogenous dietary fiber.

In *ad libitum* fed animals, retroperitoneal fat pad mass was similar in the basal, IO-S, IO-F, and MUNG groups. However, the pattern of gene expression in this depot suggests that FAS and PPAR-γ responded differently to diet. If the expression of these genes is primarily regulated by dietary fat content, one would expect to the expression of these genes to respond similarly across diets. This is not the case. Despite similar retroperitoneal fat pad mass, expression of FAS was significantly reduced by consumption of the ROLL and MUNG compared with all other diets. Furthermore, expression of PPAR-γ was reduced in the ROLL group compared with basal, IO-S, and IO-F. Expression of UCP-2 in the retroperitoneal depot was significantly enhanced in animals consuming the basal, IO-S, and IO-F, relative to ROLL and MUNG diets. This is a response similar to the upregulation of UCP-2 gene expression in white adipose tissue of genetically obese (*ob/ob*, *db/db*) mice and rodents and humans with diet-induced obesity (149,150).

A plausible explanation for the induction of UCP-2 in retroperiteonal adipose tissue by 6 weeks *ad libitum* consumption of the basal diet may involve plasma leptin levels. With the exclusion of animals fed the ROLL diet *ad libitum*, retroperitoneal fat pad mass was similar in the basal, IO-S, IO-F, and MUNG groups. Nonetheless, expression of UCP-2 was greater in the basal, IO-S, and IO-F groups compared with MUNG. Leptin has been shown to upregulate UCP-2 expression in white adipocytes, while stimulating fatty acid degradation (151). The variations in UCP-2 expression in the retroperitoneal adipose tissue depot along with variable plasma leptin levels, may imply that UCP-2 expression was enhanced by leptin levels in an attempt to compensate for increased flux of free fatty acids and blunt adipose tissue deposition.

In animals fed *ad libitum* for six weeks, expression of UCP-3 and PPAR-α was similar across diets in both soleus and gastrocnemius muscle. However, energy restriction increased the expression of UCP-3 and PPAR-α in gastrocnemius muscle compared with ad libitum consumption of the basal diet for 12 weeks. This is consistent with the findings of Samec, et al. (152,153), who demonstrated that the metabolic differences between slow-twitch oxidative (soleus) and fast-twitch glycolytic (gastrocnemius) muscles are not observed during the fed state. Furthermore, while we demonstrate that energy restriction is associated with a marked increase in the expression of UCP-3 in gastrocnemius muscle compared with *ad libitum* fed animals, this effect is absent in soleus muscle. This profound upregulation of UCP-3 expression in gastrocnemius muscle of energy restricted animals compared with the lack of response in skeletal muscle is consistent with the greater metabolic plasticity of gastrocnemius muscle (154,155).

Soleus muscle is heavily reliant on lipids as an energy substrate in the basal state, while the gastrocnemius muscle prefers glucose as an energy substrate in the well fed state (154,155). Consequently, gastrocnemius muscle has the capacity to increase lipid utilization, and expression of genes involved in lipid oxidation, as glucose becomes limiting, while soleus muscle approaches maximal lipid use in the well fed state (154,155). Furthermore, the differences in expression of UCP-3 between soleus and gastrocnemius muscle parallel changes in lipid flux across the mitochondria (152,156). Consequently, the signal for enhanced UCP-3 expression in gastrocnemius muscle of energy restricted animals may be related to circulating levels of free fatty acids. However, other studies have demonstrated that the normal surge in UCP-3 expression occurs in fasting animals treated with the anti-lipolytic agent, nicotinic acid (152,153). This suggests that free fatty acid flux is not the only physiological important cue regulating UCP-3 expression. Using a regression model which included parameters of body composition, energy expenditure, fasting levels of free fatty acids, glucose and insulin, and postprandial glucose responses, Samec, et al. (152,153) have demonstrated that glucose tolerance is the only one of these factors which could predict the variability in skeletal muscle expression of UCP-3 and further suggests a link between UCP-3 and glucose homeostasis. Consistent with this, we observed an inverse relationship between fasting plasma insulin and glucose levels and gastrocnemius expression of UCP-3 and PPAR-α. Such a relationship is consistent with the preferential use of glucose by gastrocnemius muscle in the well fed state, and the metabolic switch to lipids when glucose is limiting.

Our findings also agree with Corbalan, et al. (156) who reported that high-fat fed rats had lower gastrocnemius muscle UCP-3 levels and mitochondrial O_2 consumption

compared to lean rats. Since skeletal muscle UCP-3 expression is upregulated in response to fasting (152), a downregulation may occur with overfeeding (156). Similarly, Gong, et al. (157) demonstrated a significant reduction in UCP-3 levels within 24 hours of refeeding. Decreased UCP3 levels are also found in denerved muscles, in which triglyceride accumulation is increased, suggesting that fatty acid oxidation may be reduced. Lower mitochondrial oxygen consumption is seen in high-fat fed rats and is indicative of lower lipid utilization in cafeteria fed rats (157,158).

In summary, these studies demonstrate that a qualitative change in diet, specifically carbohydrate source, influences weight gain and body composition in aP274-agouti transgenic mice. We hypothesize that diets containing low-glucose response dietary carbohydrate result in the sustained release of glucose into circulation, which may prevent an exaggerated postprandial response characterized by extreme hyperglycemia and an enhanced insulin secretory response. Over the long term, the altered postprandial response permits carbohydrate oxidation to continue throughout the postprandial period, at the expense of fat oxidation, whether an individual is sedentary or active. The chronic consumption of high glycemic index carbohydrates and the accompanying changes in the postprandial response may alter the pattern of substrate utilization and energy balance, such that lipid flux to adipose tissue is favored.

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PART 4

SUMMARY AND CONCLUSIONS

I. Summary and Conclusions

In summary, these studies demonstrate the importance of dietary carbohydrate in the development of obesity. Moreover, we demonstrate that body weight and adiposity are influenced by both quantitative and qualitative changes in dietary carbohydrate.

The consumption of a high-fat, carbohydrate-free diet significantly attenuated body fat accumulation and reduced energy efficiency in heterozygous (*fa*/+) Zucker rats, independently of energy intake. Moreover, the addition of a modest amount (10 en%) of highly refined carbohydrate (sucrose) to the high-fat diet reversed the effects of carbohydrate restriction, significantly enhancing adipose tissue deposition and energy efficiency. Consequently, animals consuming a high-fat diet containing a minimal amount of carbohydrate, weighed significantly more than animals consuming the high-fat, carbohydrate-free diet, despite greater energy intake by the latter. Although the exact mechanism for the effects of carbohydrate-restriction are unclear, the results of the present studies question the appropriateness of current dietary guidelines recommending the consumption of a low-fat, high-carbohydrate diet by all individuals, without regards to the prevailing metabolic state of the subject.

In addition, we have demonstrated that carbohydrates which evoke greater postprandial blood glucose excursions enhance the accumulation of adipose tissue, while low-glucose response diets have the opposite effect. There is considerable debate surrounding the importance of dietary carbohydrate source in the pathogenesis of obesity and related conditions. The current position of the American Diabetes Association is that carbohydrate quantity, not quality, is the critical factor influencing metabolic control. Based on our findings, we suggest additional research into this area.

Clearly, we have shown that dietary carbohydrate structure influences the postprandial response. Although the present studies were unable to determine the precise mechanism by which dietary carbohydrate source influences body weight and adiposity, we demonstrated a significant response to a qualitative change in the carbohydrate content of the diet. Additional research is necessary to determine the physiological mediators responsible. In addition, the time course over which the postprandial response to carbohydrate containing foods significantly alters metabolic processes leading to accelerated weight gain and adiposity are needed.

In conclusion, we demonstrate the importance of considering both the quantity and quality of dietary carbohydrate in the development of obesity. These studies demonstrate the complexity of the physiological mechanisms leading to obesity and further suggest that there is no "one size fits all" dietary prescription.

APPENDIX

SUMMARY OF EFFECTS OF DIETARY CARBOHYDRATE SOURCE IN *AD LIBITUM*-FED AND ENERGY RESTRICTED aP274-*AGOUTI* TRANSGENIC MICE

Table A1

Area Under the Blood Glucose Response Curve to Test Foods in aP274-*Agouti* Transgenic Mice

	$AUC_{glucose}$
IO-S	20891.79 ± 1139.21^{a}
IO-F	$17034.40 \pm 1579.99^{b,c}$
ROLL	17400.70 ± 1105.57^{b}
MUNG	14024.30 ± 555.98^{c}

Values are Mean±SEM. Non-similar superscripts indicate significant differences at $p<0.05$.

Table A2

Effects of Dietary Treatment on Body Weight in *Ad Libitum* Fed aP274-*Agouti* Transgenic Mice

A. Summary of Changes in Body Weight

	Baseline (g)	Final (g)	Grams Gained	% Change
Basal	26.57 ± 1.34^{a}	33.37 ± 1.34^{a}	$6.80 \pm 0.45^{b,c}$	$26.28 \pm 2.26^{b,c}$
IO-S	27.80 ± 0.80^{a}	32.85 ± 1.30^{a}	$5.05 \pm 0.87^{b,c}$	$18.98 \pm 3.54^{b,c}$
IO-F	27.31 ± 1.22^{a}	34.63 ± 1.41^{a}	7.32 ± 1.15^{c}	27.79 ± 5.04^{c}
ROLL	27.60 ± 0.92^{a}	30.11 ± 0.97^{b}	2.51 ± 0.89^{a}	9.61 ± 3.57^{a}
MUNG	27.44 ± 0.91^{a}	33.26 ± 0.79^{a}	$5.81 \pm 0.74^{b,c}$	$21.78 \pm 3.21^{b,c}$

Values are Mean±SEM; Matching superscripts indicate statistically equivalent values; ^{a,b,c}Non-similar superscripts indicate significant differences at $p < 0.05$

B. Weekly Changes in Body Weight

	Gross Body Weight (g)						
	Baseline	**End Week 1**	**End Week 2**	**End Week 3**	**End Week 4**	**End Week 5**	**End Week 6**
Basal	26.57 ± 1.34^{a}	28.74 ± 0.53^{a}	29.72 ± 1.63^{a}	31.21 ± 1.57^{a}	31.99 ± 1.54^{a}	32.60 ± 1.55^{a}	33.37 ± 1.34^{a}
IO-S	27.80 ± 0.80^{a}	29.47 ± 0.69^{a}	29.53 ± 0.53^{a}	30.88 ± 0.94^{a}	32.39 ± 0.37^{a}	32.88 ± 0.44^{a}	32.85 ± 1.30^{a}
IO-F	27.31 ± 1.22^{a}	29.69 ± 1.35^{a}	29.57 ± 1.17^{a}	31.30 ± 1.20^{a}	32.87 ± 1.25^{a}	34.03 ± 1.29^{a}	34.63 ± 1.41^{a}
ROLL	27.60 ± 0.92^{a}	28.02 ± 0.91^{b}	28.11 ± 0.92^{a}	28.68 ± 1.02^{b}	29.43 ± 0.98^{b}	29.96 ± 1.02^{b}	30.11 ± 0.97^{b}
MUNG	27.44 ± 0.91^{a}	28.67 ± 0.79^{a}	29.40 ± 0.66^{a}	30.33 ± 0.55^{a}	31.88 ± 0.74^{a}	32.51 ± 0.86^{a}	33.26 ± 0.79^{a}

Values are Mean±SEM; Matching superscripts indicate statistically equivalent values; ^{a,b}Non-similar superscripts indicate significant differences at $p < 0.05$.

Table A3

Effect of Dietary Treatment on Body Weight in Energy Restricted aP274-*Agouti* Transgenic Mice

A. Summary of Changes in Body Weight During Weeks 6-12

		Week 6 (g)	Week 12 (g)	Grams Gained/Lost	% Change
	Basal ad lib	34.81 ± 1.34[a]	36.37 ± 1.32[a]	1.56 ± 1.21[b]	4.85 ± 3.40[b]
Energy Restricted	Basal-R	35.23 ± 1.56[a]	28.04 ± 1.81^[b]	-7.19 ± 0.46[a]	-20.86 ± 2.18[a]
	IO-S	34.34 ± 0.69[a]	27.16 ± 1.36^[b,c]	-7.19 ± 0.93[a]	-21.11 ± 2.90[a]
	IO-F	34.19 ± 0.89[a]	25.50 ± 1.48^[b,c]	-8.69 ± 0.98[a]	-25.60 ± 3.04[a]
	ROLL	35.71 ± 3.03[a]	23.92 ± 1.51^[c]	-10.15 ± 2.56[a]	-28.16 ± 5.62[a]
	MUNG	33.16 ± 1.92[a]	25.87 ± 0.60^[b,c]	-7.29 ± 1.45[a]	-21.04 ± 3.00[a]

Values are Mean ± SEM; ^ Indicates a significant decrease in body weight from baseline at $p < 0.05$; Matching superscripts indicate statistically equivalent values; [a,b,c] Non-similar superscripts indicate significant differences at $p<0.05$.

B. Body Weight During Weeks 6-12

		Gross Body Weight (g)						
		Week 6	Week 7	Week 8	Week 9	Week 10	Week 11	Week 12
	Basal ad lib	34.81 ± 1.34[a]	36.09 ± 1.11[a]	36.34 ± 1.35[a]	35.97 ± 1.17[a]	34.72 ± 1.67[a]	36.49 ± 1.13[a]	36.37 ± 1.32[a]
Energy Restricted	Basal-R	35.23 ± 1.56[a]	34.98 ± 1.55[a]	31.46 ± 1.67[b]	28.56 ± 1.56[b]	29.20 ± 1.75[b]	28.34 ± 1.78[b]	28.04 ± 1.81[b]
	IO-S	34.34 ± 0.69[a]	34.33 ± 1.04[a]	30.91 ± 1.15[b]	29.49 ± 1.01[b]	28.46 ± 0.95[b]	28.26 ± 1.08[b]	27.16 ± 1.36[b,c]
	IO-F	34.19 ± 0.89[a]	33.54 ± 0.90[a]	30.21 ± 1.02[b]	28.68 ± 1.03[b]	27.58 ± 1.12[b]	27.03 ± 1.11[b]	25.50 ± 1.48[b,c]
	ROLL	35.71 ± 3.03[a]	33.06 ± 2.17[a]	29.44 ± 1.84[b]	27.58 ± 1.36[b]	26.53 ± 1.21[b]	25.66 ± 1.56[b]	23.92 ± 1.51[c]
	MUNG	33.16 ± 1.92[a]	33.85 ± 1.41[a]	29.88 ± 1.01[b]	28.26 ± 0.85[b]	27.19 ± 0.79[b]	26.72 ± 0.85[b]	25.87 ± 0.60[b,c]

Values are Mean ± SEM; Matching superscripts indicate statistically equivalent values; [a,b,c] Non-similar superscripts indicate significant differences at $p<0.05$.

Table A4

Effects of Dietary Treatment on Measures of Body Composition in aP274-*Agouti* Transgenic Mice

A. *Ad Libitum* Phase

	Liver		Selected Contralateral Muscles					
			Soleus		Gastrocnemius		Combined Muscles	
	Weight (g)	% Final Body Weight	Weight (g)	% Final Body Weight	Weight (g)	% Final Body Weight	Weight (g)	% Final Body Weight
Basal	1.72 ± 0.05[a]	5.21 ± 0.17[b]	0.016 ± 0.003	0.047 ± 0.01	0.087 ± 0.01	0.26 ± 0.02	0.10 ± 0.01	0.31 ± 0.02
IO-S	1.70 ± 0.09[a]	5.17 ± 0.22[b]	0.020 ± 0.004	0.060 ± 0.01	0.086 ± 0.01	0.26 ± 0.02	0.11 ± 0.01	0.32 ± 0.02
IO-F	1.73 ± 0.08[a]	5.00 ± 0.18[a,b]	0.016 ± 0.003	0.045 ± 0.01	0.084 ± 0.01	0.25 ± 0.04	0.10 ± 0.01	0.30 ± 0.04
ROLL	1.52 ± 0.04[b]	5.07 ± 0.18[a,b]	0.018 ± 0.004	0.062 ± 0.01	0.082 ± 0.01	0.28 ± 0.02	0.10 ± 0.01	0.34 ± 0.03
MUNG	1.55 ± 0.03[b]	4.67 ± 0.11[a]	0.018 ± 0.004	0.054 ± 0.01	0.074 ± 0.01	0.23 ± 0.02	0.09 ± 0.01	0.28 ± 0.02

Values are Mean±SEM; [a,b,c]Non-similar superscripts indicate significant differences at $p<0.05$.

B. Energy Restricted Phase

		Liver		Selected Contralateral Muscles					
				Soleus		Gastrocnemius		Combined Muscles	
		Weight (g)	% Final Body Weight	Weight (g)	% Final Body Weight	Weight (g)	% Final Body Weight	Weight (g)	% Final Body Weight
	Basal ad lib	1.93 ± 0.08[a]	5.36 ± 0.21	0.023 ± 0.003	0.06 ± 0.003	0.06 ± 0.01	0.17 ± 0.01[a]	0.09 ± 0.01	0.23 ± 0.02[a]
Energy Restricted	**Basal-R**	1.48 ± 0.08[b]	5.34 ± 0.25	0.029 ± 0.008	0.12 ± 0.05	0.07 ± 0.01	0.26 ± 0.06[b]	0.10 ± 0.02	0.38 ± 0.10[b]
	IO-S	1.37 ± 0.05[b,c]	5.10 ± 0.31	0.032 ± 0.011	0.13 ± 0.05	0.08 ± 0.01	0.31 ± 0.01[b]	0.12 ± 0.01	0.44 ± 0.06[b]
	IO-F	1.33 ± 0.07[b,c]	5.26 ± 0.28	0.024 ± 0.008	0.09 ± 0.03	0.08 ± 0.01	0.32 ± 0.05[b]	0.11 ± 0.02	0.41 ± 0.08[b]
	ROLL	1.30 ± 0.10[c]	5.43 ± 0.15	0.022 ± 0.001	0.09 ± 0.03	0.07 ± 0.01	0.28 ± 0.04[b]	0.09 ± 0.02	0.37 ± 0.06[b]
	MUNG	1.29 ± 0.07[c]	4.99 ± 0.27	0.031 ± 0.006	0.12 ± 0.03	0.08 ± 0.01	0.32 ± 0.04[b]	0.11 ± 0.02	0.44 ± 0.06[b]

Values are Mean±SEM; [a,b,c]Non-similar superscripts indicate significant differences at $p<0.05$.

Table A5

Effect of Dietary Treatment on Measures of Adiposity in *Ad Libitum* Fed aP274-*Agouti* Transgenic Mice

A. Mass of Selected Fat Pads

	Selected Subcutaneous Fat Pads (g)		Sum of Subcutaneous Fat Pads (g)	Selected Visceral Fat Pads (g)		Sum of Visceral Fat Pads (g)	Sum of Visceral & Subcutaneous Fat Pads (g)
	Subscapular	Epididymal		Retroperitoneal	Perirenal		
Basal	1.26 ± 0.19^{a}	0.13 ± 0.01^{a}	1.40 ± 0.20^{a}	1.16 ± 0.14^{a}	0.74 ± 0.09^{a}	1.89 ± 0.22^{a}	3.29 ± 0.41^{a}
IO-S	1.11 ± 0.05^{a}	0.13 ± 0.01^{a}	1.24 ± 0.05^{a}	1.28 ± 0.04^{a}	0.79 ± 0.06^{a}	2.06 ± 0.08^{a}	3.31 ± 0.11^{a}
IO-F	1.44 ± 0.18^{a}	0.12 ± 0.01^{a}	1.56 ± 0.19^{a}	1.33 ± 0.14^{a}	0.86 ± 0.10^{a}	2.20 ± 0.24^{a}	3.75 ± 0.42^{a}
ROLL	0.65 ± 0.10^{b}	0.11 ± 0.01^{a}	0.75 ± 0.11^{b}	0.81 ± 0.10^{b}	0.49 ± 0.08^{b}	1.30 ± 0.18^{b}	2.05 ± 0.29^{b}
MUNG	1.10 ± 0.10^{a}	0.12 ± 0.01^{a}	1.22 ± 0.10^{a}	1.24 ± 0.10^{a}	0.66 ± 0.05^{a}	1.91 ± 0.15^{a}	3.13 ± 0.24^{a}

Values are Mean ± SEM; ^{a,b}Non-similar superscripts indicate significant differences at $p<0.05$.

B. Contribution of Selected Fat Pads to Final Body Weight

	Percent Final Body Weight (%)						
	Combined Visceral & Subcutaneous Fat Pads	Visceral Fat Pads			Subcutaneous Fat Pads		
		Individual Tissues		Combined Visceral	Individual Tissues		Combined Subcutaneous
		Retroperitoneal	Perirenal		Epididymal	Subscapular	
Basal	9.58 ± 0.89^{a}	3.37 ± 0.31^{a}	2.18 ± 0.21^{b}	5.55 ± 0.49^{a}	0.40 ± 0.03^{a}	3.63 ± 0.44^{a}	4.03 ± 0.45^{a}
IO-S	10.04 ± 0.25^{a}	3.89 ± 0.08^{a}	2.38 ± 0.17^{b}	6.27 ± 0.19^{a}	0.39 ± 0.02^{a}	3.38 ± 0.14^{a}	3.77 ± 0.14^{a}
IO-F	10.55 ± 0.89^{a}	3.77 ± 0.31^{a}	2.43 ± 0.23^{b}	6.19 ± 0.53^{a}	0.34 ± 0.03^{a}	4.01 ± 0.40^{a}	4.35 ± 0.39^{a}
ROLL	6.60 ± 0.70^{b}	2.61 ± 0.25^{b}	1.58 ± 0.22^{a}	4.19 ± 0.44^{b}	0.33 ± 0.02^{a}	2.08 ± 0.26^{b}	2.41 ± 0.28^{b}
MUNG	9.34 ± 0.60^{a}	3.71 ± 0.30^{a}	$1.99 \pm 0.14^{a,b}$	5.70 ± 0.39^{a}	0.35 ± 0.02^{a}	3.29 ± 0.24^{a}	3.64 ± 0.24^{a}

Values are Mean ± SEM; ^{a,b}Non-similar superscripts indicate significant differences at $p<0.05$.

Table A6

Effect of Dietary Treatment on Measures of Adiposity in Energy Restricted aP274-*Agouti* Transgenic Mice

A. Mass of Selected Subcutaneous Fat Pads and Combined Mass of Visceral & Subcutaneous Fat Pads

		Selected Subcutaneous Fat Pads (g)		Sum of Selected Subcutaneous Fat Pads (g)	Sum of Selected Visceral & Subcutaneous Fat Pads (g)
		Subscapular	Epididymal		
	Basal ad lib	1.75 ± 0.25^{a}	0.16 ± 0.01^{a}	1.91 ± 0.26^{a}	4.43 ± 0.43^{a}
Energy Restricted	Basal-R	0.95 ± 0.18^{b}	0.13 ± 0.02^{a}	1.08 ± 0.18^{b}	2.45 ± 0.45^{b}
	IO-S	0.89 ± 0.19^{b}	0.15 ± 0.01^{a}	1.05 ± 0.20^{b}	2.47 ± 0.51^{b}
	IO-F	$0.62 \pm 0.18^{b,c}$	0.11 ± 0.01^{a}	$0.73 \pm 0.19^{b,c}$	$1.60 \pm 0.43^{b,c}$
	ROLL	0.48 ± 0.12^{c}	0.12 ± 0.02^{a}	0.61 ± 0.13^{c}	1.18 ± 0.30^{c}
	MUNG	$0.57 \pm 0.08^{b,c}$	0.13 ± 0.01^{a}	$0.69 \pm 0.08^{b,c}$	$1.43 \pm 0.18^{b,c}$

Values are Mean ± SEM; Matching superscripts indicate statistically equivalent values; ^{a,b,c}Non-similar superscripts indicate significant differences at $p < 0.05$.

B. Mass of Selected Visceral Fat Pads & Retroperitoneal Adipocyte Size

		Selected Visceral Fat Pads			Sum of Selected Visceral Fat Pads (g)
		Retroperitoneal Depot		Perirenal Weight (g)	
		Weight (g)	Adipocyte Size (μm)		
	Basal ad lib	1.61 ± 0.11^{a}	94.28 ± 2.00^{a}	0.91 ± 0.12^{a}	2.52 ± 0.21^{a}
Energy Restricted	Basal-R	0.92 ± 0.18^{b}	87.98 ± 1.66^{a}	0.46 ± 0.10^{b}	1.38 ± 0.27^{b}
	IO-S	0.91 ± 0.18^{b}	77.90 ± 6.57^{b}	0.52 ± 0.15^{b}	1.43 ± 0.31^{b}
	IO-F	$0.62 \pm 0.15^{b,c}$	78.67 ± 4.01^{b}	$0.26 \pm 0.10^{b,c}$	$0.88 \pm 0.24^{b,c}$
	ROLL	0.40 ± 0.12^{c}	66.39 ± 6.11^{c}	0.18 ± 0.06^{c}	0.57 ± 0.18^{c}
	MUNG	0.52 ± 0.07^{c}	$73.44 \pm 4.08^{b,c}$	0.21 ± 0.04^{c}	$0.74 \pm 0.11^{b,c}$

Values are Mean ± SEM; ^{a,b,c}Non-similar superscripts indicate significant differences at $p < 0.05$

Table A6 (continued)

C. Contribution of Selected Fat Pads to Final Body Weight

		Percent Final Body Weight (%)						
		Combined Visceral & Subcutaneous Fat Pads	Visceral Fat Pads			Subcutaneous Fat Pads		
			Individual Tissues		Combined Visceral	Individual Tissues		Combined Subcutaneous
			Retroperitoneal	Perirenal		Epididymal	Subscapular	
	Basal ad lib	12.04 ± 0.82[a]	4.41 ± 0.21[a]	2.47 ± 0.30[a]	6.89 ± 0.48[a]	0.44 ± 0.02[a]	4.71 ± 0.51[a]	5.16 ± 0.52[a]
Energy Restricted	Basal-R	8.28 ± 1.27[b]	3.11 ± 0.54[b]	1.52 ± 0.29[b,c]	4.63 ± 0.79[b]	0.46 ± 0.06[a]	3.19 ± 0.51[b]	3.66 ± 0.50[b]
	IO-S	8.69 ± 1.54[b]	3.19 ± 0.56[b]	1.81 ± 0.52[a,c]	5.00 ± 0.99[b]	0.55 ± 0.04[a]	3.13 ± 0.56[b]	3.69 ± 0.57[b]
	IO-F	5.84 ± 1.30[b,c]	2.27 ± 0.46[b,c]	0.91 ± 0.31[d]	3.18 ± 0.74[b,c]	0.42 ± 0.04[a]	2.23 ± 0.54[b,c]	2.65 ± 0.57[b,c]
	ROLL	4.65 ± 1.02[c]	1.54 ± 0.43[c]	0.68 ± 0.20[d]	2.22 ± 0.63[c]	0.55 ± 0.04[a]	1.88 ± 0.43[c]	2.43 ± 0.41[c]
	MUNG	5.51 ± 0.68[b,c]	2.03 ± 0.29[b,c]	0.82 ± 0.17[d]	2.92 ± 0.38[b,c]	0.47 ± 0.08[a]	2.20 ± 0.29[b,c]	2.67 ± 0.29[b,c]

Values are Mean ± SEM; [a,b,c,d] Non-similar superscripts indicate significant differences at $p < 0.05$

Table A7

Effect of Dietary Treatment on Circulating Glucose Levels in *Ad Libitum* Fed aP274-*Agouti* Transgenic Mice

A. Fasting Plasma Glucose Levels

	Fasting Plasma Glucose Levels		
	Baseline (mg/dL)	Final (mg/dL)	% Change
Basal	128.15 ± 6.56 [b]	142.70 ± 3.98 [a]	14.55 ± 5.59 [a]
IO-S	150.30 ± 3.58 [a]	149.50 ± 6.67 [a,b]	-0.80 ± 8.36 [a]
IO-F	141.90 ± 4.28 [a]	165.90 ± 6.79 [b]	24.00 ± 8.52 [a]
ROLL	148.05 ± 8.63 [a]	178.45 ± 8.76 [c]	30.40 ± 11.17 [a]
MUNG	154.83 ± 7.87 [a]	153.44 ± 6.50 [a,b]	-1.39 ± 11.05 [b]

Values are mean± SEM; [a,b,c] Non-similar superscripts indicate significant differences at $p < 0.05$.

B. Weekly Non-Fasting Blood Glucose Levels

	Blood Glucose (mg/dL)				
	Week 1	Week 2	Week 3	Week 4	Week 5
Basal	141.80 ± 5.00 [a,b]	136.00 ± 6.83 [b]	137.20 ± 5.72	152.10 ± 6.13	170.15 ± 2.74 [b]
IO-S	148.70 ± 7.18 [a,b,c]	143.75 ± 6.51 [b]	142.70 ± 6.31	147.20 ± 4.98	172.15 ± 5.41 [b]
IO-F	162.05 ± 4.88 [c]	161.95 ± 6.31 [a]	153.30 ± 4.81	158.70 ± 8.74	171.30 ± 4.97 [b]
ROLL	134.40 ± 4.89 [a]	136.20 ± 5.76 [b]	157.75 ± 8.43	139.40 ± 3.19	159.70 ± 4.22 [a,b]
MUNG	152.17 ± 6.04 [b,c]	145.22 ± 6.00 [a,b]	139.28 ± 7.79	149.44 ± 4.52	160.67 ± 6.21 [a,b]

Values are Mean ± SEM; [a,b,c] Non-similar superscripts indicate significant differences at $p < 0.05$.

Table A8

Effect of Dietary Treatment on Circulating Glucose Levels in Energy Restricted aP274-*Agouti* Transgenic Mice

A. Fasting Plasma Glucose Levels

		Fasting Plasma Glucose Levels		
		Baseline (mg/dL)	Final (mg/dL)	% change
	Basal ad lib	137.29 ± 7.17^{a}	122.57 ± 5.15^{a}	-9.82 ± 4.72^{a}
Energy Restricted	Basal-R	150.71 ± 3.15^{b}	116.33 ± 10.14^{a}	-23.52 ± 6.48^{a,b}
	IO-S	149.14 ± 2.63^{b}	113.00 ± 6.99^{b,c}	-24.32 ± 4.26^{a,b}
	IO-F	152.14 ± 3.58^{b}	103.14 ± 9.52^{b,c}	-32.34 ± 6.00^{b}
	ROLL	140.43 ± 2.70^{a}	91.00 ± 16.22^{c}	-35.27 ± 10.82^{b}
	MUNG	136.57 ± 4.19^{a}	91.57 ± 6.54^{c}	-32.48 ± 5.22^{b}

Values are Mean ± SEM; ^{a,b,c} Non-similar superscripts indicate significant differences at $p < 0.05$.

B. Weekly Fasting Blood Glucose Levels

		Fasting Blood Glucose Levels (mg/dL)				
		Week 1	Week 2	Week 3	Week 4	Week 5
	Basal ad lib	135.71 ± 1.61*	128.14 ± 3.61^{a}	130.00 ± 1.69^{a,b}	125.00 ± 5.35	129.00 ± 4.17^{a}
Energy Restricted	Basal-R	107.00 ± 1.05†	129.28 ± 5.18^{a,†}	122.71 ± 3.87^{a,†}	118.43 ± 5.50†	83.43 ± 7.45^{b,†}
	IO-S	116.00 ± 7.32†	129.29 ± 5.65^{a,†}	135.57 ± 2.61^{b,†}	118.29 ± 4.52†	69.57 ± 6.26^{b,†}
	IO-F	107.00 ± 4.76†	126.86 ± 2.67^{a,b,†}	132.71 ± 4.69^{a,b†}	112.14 ± 5.08†	72.43 ± 7.94^{b,c,†}
	ROLL	112.71 ± 5.08†	113.57 ± 4.77^{b,†}	121.30 ± 6.08^{a,†}	107.00 ± 9.92†	71.17 ± 3.73^{b,c,†}
	MUNG	104.71 ± 8.08†	114.43 ± 1.17^{b,†}	122.57 ± 5.01^{a,†}	108.86 ± 7.42†	63.43 ± 5.79^{c,†}

Values are Mean ± SEM; * Indicates a value significantly different from all others within column at $p<0.05$; † Indicates a value significantly different from baseline at $p<0.05$; ^{a,b,c} Non-similar superscripts indicate significant differences at $p < 0.05$

Table A9

Effect of Dietary Treatment on Fasting Plasma Metabolites in aP274-*Agouti* Transgenic Mice

A. *Ad Libitum* Phase

	Insulin (μU/ml)	Insulin:Glucose Ratio	Leptin (ng/ml)	Triglycerides (mg/dL)	Glycerol (μmol/L)
Basal	12.85 ± 1.82[a]	15.20 ± 3.74	15.53 ± 2.37[a]	124.69 ± 24.94[a]	631.74 ± 91.33[a]
IO-S	7.99 ± 0.71[b]	17.03 ± 1.78	16.27 ± 1.46[a]	112.08 ± 14.91[a,b]	550.69 ± 58.85[a]
IO-F	12.73 ± 2.83[a]	15.97 ± 2.71	16.05 ± 2.41[a]	168.32 ± 14.50[a]	676.92 ± 83.21[a]
ROLL	8.00 ± 1.10[b]	17.33 ± 2.48	10.96 ± 1.41[b]	79.60 ± 6.15[b]	537.29 ± 70.32[a]
MUNG	8.62 ± 0.85[b]	19.04 ± 3.29	15.60 ± 1.64[a]	86.15 ± 13.53[b]	664.47 ± 44.88[a]

Values are Mean ± SEM; [a,b,c]Non-similar superscripts indicate significant differences at $p < 0.05$.

B. Energy Restricted Phase

		Insulin (μU/ml)	Insulin:Glucose Ratio	Leptin (ng/ml)	Triglycerides (mg/dL)	Glycerol (μmol/L)
	Basal ad lib	11.46 ± 1.63[a]	11.67 ± 1.30	29.49 ± 2.62[a]	93.99 ± 7.61[a]	423.45 ± 36.52[a]
Energy Restricted	**Basal-R**	12.24 ± 1.71[a]	10.27 ± 1.62	21.08 ± 0.54[b]	87.82 ± 9.41[a,b]	448.47 ± 47.65[a]
	IO-S	9.57 ± 0.66[a,b]	12.08 ± 1.07	19.33 ± 2.97[b,c]	80.73 ± 8.87[a,b]	420.23 ± 27.30[a]
	IO-F	8.83 ± 0.35[b]	11.63 ± 1.01	16.35 ± 2.21[b,c]	81.61 ± 7.17[a,b]	473.30 ± 49.35[a]
	ROLL	9.85 ± 0.58[a,b]	9.38 ± 1.76	14.79 ± 3.32[c,d]	73.33 ± 3.56[b]	729.43 ± 92.94[b]
	MUNG	9.82 ± 0.35[a,b]	9.40 ± 0.71	11.63 ± 0.54[d]	72.84 ± 1.73[b]	527.49 ± 93.24[a,b]

Values are Mean ± SEM; [a,b,c]Non-similar superscripts indicate significant differences at $p < 0.05$.

Table A10

Effect of Dietary Treatment on Adipocyte Lipolysis in aP274-*Agouti* Transgenic Mice

A. *Ad Libitum* Phase

	Glycerol Release (μmol glycerol/μg protein)			Percent Stimulation
	Basal	Isoproterenol Stimulated	Absolute Increase	
Basal	8.42 ± 1.51	10.58 ± 2.37[a]	2.27 ± 0.87[a]	29.46 ± 11.41[a]
IO-S	6.26 ± 1.03	8.25 ± 1.46[a]	1.99 ± 0.68[a]	34.76 ± 15.27[a]
IO-F	6.13 ± 1.12	11.45 ± 1.78[a,b]	5.31 ± 1.42[b]	122.08 ± 47.49[b]
ROLL	7.43 ± 0.82	16.38 ± 3.13[b]	6.22 ± 0.90[b,c]	96.01 ± 22.83[b]
MUNG	6.72 ± 1.18	14.77 ± 1.78[b]	7.95 ± 1.49[c]	147.51 ± 38.65[b]

Values are Mean ± SEM; Matching superscripts indicate statistically equivalent values; [a,b,c] Non-similar superscripts indicate significant differences at $p < 0.05$

B. Energy Restricted Phase

		Glycerol Release (μmol glycerol/μg protein)			Percent Stimulation
		Basal	Isoproterenol Stimulated	Absolute Increase	
	Basal ad lib	7.32 ± 0.89[a]	6.91 ± 0.27[a,b]	-0.41 ± 0.95[a]	0.45 ± 13.42[a]
Energy Restricted	**Basal-R**	5.96 ± 1.19[a]	7.73 ± 1.25[a]	1.77 ± 0.74[b]	58.80 ± 31.51[b,c]
	IO-S	3.81 ± 0.71[b]	5.92 ± 1.14[a,b]	2.12 ± 0.52[b]	56.57 ± 14.00[b,c]
	IO-F	4.12 ± 0.92[b]	8.63 ± 2.31[a]	4.51 ± 1.47[c]	107.76 ± 26.60[c]
	ROLL	3.98 ± 1.26[b]	4.46 ± 0.37[b]	0.50 ± 1.00[a,b]	35.05 ± 21.29[b]
	MUNG	3.92 ± 0.77[b]	6.67 ± 1.36[a,b]	2.75 ± 1.10[b,c]	81.32 ± 29.93[b,c]

Values are Mean ± SEM; [a,b,c,d] Non-similar superscripts indicate significant differences at $p < 0.05$

Table A11

Effect of Dietary Treatment on Gene Expression in *Ad Libitum* Fed aP274-*Agouti* Transgenic Mice

A. Liver and Skeletal Muscle Gene Expression

	Liver		Gastrocnemius Muscle		Soleus Muscle	
	FAS:18s	PPAR-α:18s	UCP3:18s	PPAR-α:18s	UCP3:18s	PPAR-α:18s
Basal	4.42 ± 0.63^{a}	2.91 ± 0.59^{a}	0.56 ± 0.09^{a}	0.46 ± 0.12^{a}	0.28 ± 0.03^{a}	0.53 ± 0.11^{a}
IO-S	3.28 ± 0.55^{a}	$2.31 \pm 0.55^{a,b}$	0.65 ± 0.08^{a}	0.40 ± 0.08^{a}	0.23 ± 0.07^{a}	0.51 ± 0.15^{a}
IO-F	2.97 ± 0.41^{a}	$2.02 \pm 0.36^{a,b}$	0.55 ± 0.08^{a}	0.43 ± 0.12^{a}	0.20 ± 0.08^{a}	0.36 ± 0.05^{a}
ROLL	1.76 ± 0.33^{b}	1.63 ± 0.16^{b}	0.61 ± 0.10^{a}	0.26 ± 0.05^{a}	0.28 ± 0.07^{a}	0.71 ± 0.12^{a}
MUNG	1.61 ± 0.45^{b}	$2.86 \pm 0.63^{a,b}$	0.67 ± 0.08^{a}	0.46 ± 0.08^{a}	0.25 ± 0.05^{a}	0.66 ± 0.20^{a}

Values are Mean ± SEM; Matching superscripts indicate statistically equivalent values; ^{a,b,c} Non-similar superscripts indicate significant differences at $p < 0.05$.

B. Adipose Tissue Gene Expression

	Subscapular Adipose Tissue				Retroperitoneal Adipose Tissue			
	FAS:18s	PPAR-α:18s	UCP2:18s	PPAR-γ:18s	FAS:18s	PPAR-α:18s	UCP2:18s	PPAR-γ:18s
Basal	0.85 ± 0.27^{a}	1.08 ± 0.21^{a}	4.58 ± 0.87	1.40 ± 0.36^{b}	1.65 ± 0.13^{a}	2.32 ± 0.09^{a}	2.86 ± 0.32^{a}	1.55 ± 0.13^{a}
IO-S	0.34 ± 0.04^{b}	0.52 ± 0.16^{b}	3.72 ± 0.70	$1.00 \pm 0.24^{a,b}$	1.26 ± 0.12^{b}	1.79 ± 0.20^{b}	1.78 ± 0.24^{b}	0.73 ± 0.10^{b}
IO-F	0.29 ± 0.13^{b}	0.42 ± 0.05^{b}	3.88 ± 0.83	$0.92 \pm 0.28^{a,b}$	1.02 ± 0.12^{b}	$1.74 \pm 0.29^{b,c}$	1.69 ± 0.22^{b}	0.61 ± 0.10^{b}
ROLL	0.77 ± 0.17^{a}	1.17 ± 0.27^{a}	4.36 ± 0.93	0.76 ± 0.10^{a}	0.58 ± 0.08^{c}	1.31 ± 0.15^{c}	0.73 ± 0.15^{c}	0.26 ± 0.05^{c}
MUNG	$0.54 \pm 0.18^{a,b}$	1.79 ± 0.60^{a}	4.39 ± 0.86	1.38 ± 0.43^{b}	0.54 ± 0.06^{c}	1.31 ± 0.23^{c}	1.28 ± 0.09^{c}	$0.51 \pm 0.20^{b,c}$

Values are Mean ± SEM; ^{a,b,c} Non-similar superscripts indicate significant differences at $p < 0.05$

Table A12

Effect of Dietary Treatment on Gene Expression in Energy Restricted aP274-*Agouti* Transgenic Mice

A. Liver and Skeletal Muscle Gene Expression

		Liver		Gastrocnemius Muscle		Soleus Muscle	
		FAS:18s	PPAR-α:18s	UCP3:18s	PPAR-α:18s	UCP3:18s	PPAR-α:18s
	Basal ad lib	5.13 ± 0.81[a]	7.06 ± 1.07[a]	0.67 ± 0.05[a]	0.76 ± 0.13[a]	0.32 ± 0.05[a]	1.13 ± 0.31[a]
Energy Restricted	Basal-R	2.92 ± 0.82[b]	5.00 ± 1.10[b]	1.01 ± 0.17[b]	2.01 ± 0.34[b]	0.20 ± 0.02[b]	2.06 ± 0.27[b]
	IO-S	2.58 ± 0.62[b]	3.95 ± 0.36[c]	1.18 ± 0.15[b]	2.84 ± 0.50[c]	0.20 ± 0.03[b]	1.23 ± 0.26[a]
	IO-F	1.62 ± 0.44[b]	3.45 ± 0.39[c]	1.79 ± 0.32[c]	1.37 ± 0.45[a,b]	0.32 ± 0.07[a,b]	1.32 ± 0.26[a]
	ROLL	2.66 ± 0.38[b]	3.35 ± 0.60[c]	1.19 ± 0.05[b,c]	2.13 ± 0.29[b,c]	0.26 ± 0.03[a,b]	1.40 ± 0.41[a]
	MUNG	2.48 ± 0.73[b]	5.09 ± 0.70[b]	1.47 ± 0.24[c]	2.22 ± 0.49[b,c]	0.27 ± 0.04[a,b]	2.76 ± 0.49[b]

Values are Mean ± SEM; [a,b,c]Non-similar superscripts indicate significant differences at $p < 0.05$

B. Subscapular Adipose Tissue Gene Expression

		Subscapular Fat Pad			
		FAS:18s	PPAR-α:18s	UCP2:18s	PPAR-γ:18s
	Basal ad lib	0.55 ± 0.07[a]	0.61 ± 0.13[a]	0.83 ± 0.14[a]	0.88 ± 0.30[a]
Energy Restricted	Basal-R	1.43 ± 0.21[b]	0.60 ± 0.16[a]	0.63 ± 0.16[a]	1.25 ± 0.26[a,b]
	IO-S	0.80 ± 0.18[a]	0.74 ± 0.08[a]	0.56 ± 0.10[a]	0.55 ± 0.14[a]
	IO-F	2.84 ± 0.61[c]	0.52 ± 0.09[a]	0.65 ± 0.07[a]	1.11 ± 0.21[a,b]
	ROLL	2.16 ± 0.32[b,c]	0.85 ± 0.08[a]	0.58 ± 0.13[a]	1.43 ± 0.34[b]
	MUNG	1.00 ± 0.25[a,b]	0.75 ± 0.16[a]	0.53 ± 0.16[a]	0.72 ± 0.11[a]

Values are Mean ± SEM; Matching superscripts indicate statistically equivalent values; [a,b,c]Non-similar superscripts indicate significant differences at $p < 0.05$

VITA

Kristin Morris was born in October, 1967 in Jasper, Alabama. After graduating from high school, Kristin attended Samford University before transferring to the University of Alabama, where she later earned the degree of Bachelor of Science in Microbiology. Subsequently, Kristin worked for the State of Alabama Department of Public Health before returning to the University of Alabama to earn the degree of Master of Science in Nutrition. Kristin began her doctoral studies at the University of Tennessee in the fall of 1996.