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Role of Lipolysis and Lipogenesis in the Development of Diet-Induced Obesity

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ROLE OF LIPOLYSIS AND LIPOGENESIS IN THE DEVELOPMENT OF DIET-INDUCED OBESITY

by

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ABSTRACT

ROLE OF LIPOLYSIS OF LIPOGENESIS IN THE DEVELOPMENT OF DIET-INDUCED OBESITY

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Obesity is an increasingly common public health problem with approximately one-half of the American adult population overweight and one-quarter considered obese. This alarming trend has led researchers to determine potential causative factors of excess weight gain in humans. However, it is difficult to discern whether perturbations that result in obesity are the cause or simply the result of the obese state. Diet-induced obesity is one of the animal models that allow researchers to address temporal issues. Our laboratory utilizes a diet-induced obesity model in which Sprague-Dawley rats are placed on a purified moderately high fat diet and ultimately diverge into two distinct populations based on body weight gain. Approximately 50% of the rats gain more body weight and fat and are considered obesity-prone (OP), whereas the other half (obesity-resistant – OR) are similar in body composition to rats fed a low fat diet. Interestingly, rates of body weight gain and food consumption are greater for OP rats than OR rats during early phases of the dietary challenge, but not during later phases. Moreover, weight gain is associated with excess fat accretion in OP rats. These data led us to examine the potential causes of increased fat weight gain during the early phases. The major site of lipid storage is the adipose tissue. Two major processes occurring in adipocytes are lipolysis (lipid mobilization) and lipogenesis (lipid formation), which are controlled by different metabolic hormones. Potential differences in these processes or

hormone sensitivity may predispose OP rats to develop obesity or protect OR rats from the obese state. In experiment 1, *in vivo* lipolysis was measured in outbred OP and OR rats prior to exposure to an obesity-inducing diet. *In vitro* lipolysis was assessed in various adipocytes from inbred OP and OR rats in experiment 2. Early effects of moderately high fat feeding on insulin-stimulated glucose uptake and metabolism and body composition were examined in another set of experiments. Results demonstrated that *in vivo* lipolytic responses were not a causative factor in excess body weight and fat accretion in OP rats. Next, *in vitro* responses to various lipolytic agents were reduced in visceral adipocytes of inbred OP rats, which were already fatter than inbred OR rats. In the last set of experiments, MHF-feeding reduced insulin-stimulated glucose uptake and metabolism in adipocytes vs. LF feeding. Epididymal fat cells of OP rats synthesized more fatty acids from glucose than those of OR rats after short-term exposures to the same MHF diet. It may be speculated that altered lipolysis is not a causative factor for excess adiposity in OP rats. Moreover, increased insulin responsiveness (via lipid synthesis) may promote excess fat accretion in OP rats. As obesity develops, adipocytes of OP rats may become less responsive to lipolytic agents, which may exacerbate visceral fatness.

I dedicate this dissertation to my wife, Dana. This work would not have been possible without her sacrifice and support throughout my doctoral training.
I love you very much.

I also dedicate this dissertation to my parents, Beverly and Ron. Your love, support, and commitment throughout my academic pursuits have enabled me to achieve this goal.

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CHAPTER I

INTRODUCTION

BACKGROUND AND SIGNIFICANCE

Obesity in Humans

With the prevalence of overweight adults increasing dramatically over the last two decades in the United States, obesity has become a major public health problem. According to the third National Health and Nutrition Examination Survey (NHANES), 54% of the United States adult population is overweight (body mass index (BMI) ≥ 25 kg·m^{-2}) and 22% are obese (BMI ≥ 30 kg·m^{-2}) (93, 94). When comparing the increase in obesity between the 1976-80 and 1988-94 surveys, the number of obese men has increased by nearly 100% and the number of women has increased by 50% (53). In a recent study, Mokdad et al. (120) found that the prevalence of obesity among adults in the United States increased from 12.0% in 1991 to 17.9% in 1998. Moreover, a multi-state phone survey revealed that the number of overweight adults was increasing at a rate of 0.9% per year (61). Foreyt and Goodrick (55) have proposed that if these trends continued, the entire U.S. adult population would be overweight by 2230. The epidemic of overweight and obesity is also a global problem and not just limited to the United States (92). These alarming statsitics have researchers focusing on the primary causes of obesity.

The model journal used for this dissertation was the *American Journal of Physiology*

Obesity is a complex, multifactorial disease influenced by genetic, cultural, and environmental factors (20). Bouchard and colleagues (18) attempted to quantify the relative contribution of biological inheritance on the amount or distribution of body fat in a French Canadian cohort. Estimates of the variance contributed by transmissible (genetic or cultural factors) and nontransmissible (environmental components) were variable and dependent upon the outcome variable. Regardless of outcome variable (e.g., BMI, fat mass, ratio of subcutaneous fat to fat mass), environmental factors appear to explain a greater percentage of the total variation than genetic and cultural factors (18). In other words, an individual with an obesity-susceptible genotype may have an increased risk but it only becomes manifest when nurtured in a certain environment. A classic example of this is noted with the Pima Indian (133). Pima Indians are traditionally located in Northwestern Mexico, but another group of Pima Indians resides on reservations in southwestern areas of the United States. A majority of the U.S. Pima Indians are obese and Type II diabetics (non-insulin dependent diabetes mellitus), whereas their Mexican counterparts remain lean or normal weight. Introduction to and increased availability of the typical Western (i.e., increased dietary fats and simple sugars) diet to the U.S. Pima Indians have been suggested as the "environmental culprits" (133). These data reveal that members of the Pima Indians have the genotype to develop obesity, but phenotypic expression of obesity appears only in an obesity-inducing setting (e.g., exposure to higher-fat Western diet).

Although primary metabolic perturbations that cause obesity have not been completely elucidated, overeating, reduced physical activity, and exposure to calorie-dense diets are recognized as the three main determinants of obesity (72). Recently, the

Pound of Prevention study confirmed that these three factors were indeed related to body weight changes (142). Obesity research, however, is complicated by the fact that obesity is a component of the metabolic syndrome (syndrome X or insulin resistance syndrome), which includes insulin resistance, hypertension, dyslipidemia, and cardiovascular disease (130, 134). Consequently, obesity studies must also address these and other factors (e.g., pituitary and stress-related hormones). Another major obstacle with this research is the metabolic abnormalities associated with obesity are usually studied in humans or animals only after establishment of the obese state. This scenario makes it difficult to determine underlying causes. Drug and dietary interventions have been shown to be effective treatments for obesity (20). On the other hand, with the direct costs associated with obesity accounting for 5.7% of the National Health expenditure in the United States (161) and with long-term success for weight loss programs and regimens being limited (1), prevention may be the most effective treatment of obesity. Therefore, elucidation of the primary causes of obesity should be in the forefront of biomedical science research.

Animal Models of Obesity

Researchers have attempted to determine causative factors for human obesity. For example, early controlled overfeeding studies in normal weight subjects demonstrated that individuals predisposed to obesity or increased weight gain were unable to increase fat oxidation in response to energy dense diets (7). However, ethical issues pervade obesity research in humans. Researchers cannot make humans obese in order to study the basic mechanisms of obesity development for obvious reasons. Therefore, selectively bred animal models (e.g., *ob/ob* and *db/db* mice and Zucker rats) were developed to address this problem and identify the underlying mechanisms of

obesity (29, 169). These genetic models normally have a singular genetic defect, which result in massive obesity. Another model is hypothalamic obesity, which allows researchers to address the role of the central nervous system on regulating food intake and energy expenditure (19, 21). Although these models provide insight to the developmental causes of obesity, their applicability to humans is questionable because of the polygenic nature of human obesity. In contrast, animal models of diet-induced obesity appear to be more relevant with regard to human obesity. In these animal models obesity develops following administration of at least three different dietary regimens. High fat feeding is the most common dietary approach (26, 137). Other researchers have added either a sucrose solution (87, 88) or snack foods (140) to a regular chow diet to produce obesity. Similar to humans, rodents demonstrate divergent responses to high-fat or energy-dense (high fat and sucrose) diets. On comparable diets, some individuals become obese, whereas others maintain their ideal body weight. Schemmel et al. (137) demonstrated variance among seven rat strains in their propensity to develop diet-induced obesity on a high fat diet. Moreover, divergent weight gain patterns were noted within a single strain of outbred male Wistar rats fed "cafeteria" or semi-purified high fat diets (11, 26, 125). These early researchers have utilized diets with relatively high levels of dietary fat (>45% kcal as fat), which exceed those normally found in human diets.

Levin et al. (110, 111) noted that outbred Sprague-Dawley (SD) rats fed a semi-purified high-energy diet (HE = 31% kcal as fat) diverged into two distinct groups. Approximately half the SD rats (diet resistant – DR) on the HE diet gained weight at a similar rate as rats (controls) fed a standard chow-diet (5% kcal as fat), whereas the other half (diet-induced obese – DIO) accumulated more body weight and carcass adiposity

compared to DR and control rats. Semi-purified diets have also received criticism because development of obesity on these diets can only be attributed to the diet and not specific contents of the diet (e.g., saturated fat content or sucrose content). Conversely, purified diets allow researchers to examine the influence of different dietary components on body weight and fat gains.

To this end, Lauterio et al. (99) examined the development of diet-induced obesity in response to a purified moderately high fat (MHF) diet, which has a dietary fat level (32% kcal as fat) similar to the typical Western diet. The research with this diet and outbred male SD rats yielded similar results as Levin et al. (111) in that the body weight gain pattern was bimodal. Moreover, body weight and carcass adiposity were greater for obesity-prone (OP; similar to DIO from Levin's studies) rats compared to obesity-resistant (OR; similar to DR from Levin's studies) rats and control rats fed a low fat (or chow) diet (40, 99, 101). This diet-induced obesity model allows researchers to address the developmental aspects of obesity, as well as the influence of certain dietary components. Researchers are, however, restricted to plasma, urinary, and *in vivo* dialysis measurements because classification of rats as OP and OR can occur only after commencement of the dietary period and development of excess weight.

Predicting Development of Obesity - Outbred Rats

As useful as the diet-induced obesity model is to address the aforementioned measures, pre-obese comparisons of cellular mechanisms are limited. Obviously, rats must be killed to harvest tissues, but their classifications are unknown without the dietary challenge. The ability to predict which rats will become obese as a result of exposure to these diets would allow researchers to focus on metabolic events within rats prior to

changes in body composition. Berthoud et al. (11) reported that saccharin-induced insulin rise (termed cephalic phase, reflex insulin secretion) can be utilized to predict obesity susceptibility. These researchers noted that Wistar rats predisposed to obesity (termed high responders) had an elevated insulin response to saccharin compared with normal rats (termed low responders). Moreover, high and low responders did not differ in body weight or glucose-stimulated insulin response prior to exposure to a high-fat cafeteria diet (11). Similar findings were also observed in obese and lean Zucker rats. Obese Zucker (fa/fa) rats have a significantly stronger cephalic phase of insulin secretion than lean Zucker (FA/?) rats (80). This altered cephalic insulin response may predispose obese animals to increase glucose and lipid storage in different tissues in response to a meal.

Another prediction model developed by Levin (105) showed that obesity susceptibility can be predicted by measuring 24-hr urinary catecholamine output prior to exposure to an obesity-inducing diet. Rats destined to become obese on a HE diet had increased 24-hr urinary norepinephrine output compared with their lean counterparts. Catecholamine resistance may be present and/ or end-organ responsiveness may be reduced (e.g., catecholamine-stimulated lipolysis in adipocytes) in DIO rats. Potentially, a reduced lipolytic response in adipocytes may allow for increased lipid accretion and thus, obesity. However, reproducibility is a major problem with this prediction method because numerous researchers have been unable to replicate these findings.

Recently, our laboratory reported that plasma leptin concentrations or body weight gains after one week on a MHF diet were significant predictors of overall weight gain on a 14-week dietary challenge in outbred SD rats (Boozer & Lauterio, submitted).

Similar results using body weight gain data were found for Wistar rats (125). These data demonstrated that the majority of rats defined as OP and OR after 1 week usually remained within their grouping throughout the dietary challenge. Although these animals are exposed to moderately high fat diets, early prediction models allow researchers to address some of the early events associated with or preceding excess weight gain.

Predicting Development of Obesity - Inbred Rats

One major drawback with the outbred model is that rats must be exposed to a high fat diet before determining resistance or susceptibility to obesity. This limits investigations to those in diet-exposed rats. To circumvent this, Levin et al. (107) developed and characterized a sub-strain of SD rats that are selectively bred for DIO and DR traits. Briefly, adult male and female outbred SD rats were fed a HE diet for one month. At this time, the males gaining the most weight in response to the diet were bred with females gaining the most weight (i.e., selected for obesity response). Similarly, male and female rats exhibiting the lowest weight gain were bred to form the obesity-resistant group. This procedure was repeated for F_2 generation after a two week exposure to the diet. By the third generation (F_3), separation of DIO and DR rats was firmly established. Analysis of the F_5 generation revealed that DIO males and females had significantly greater body weights than DR males and females of the same age. Moreover, DIO rats consumed more energy (kcal) and gained more body weight than DR after one week on a chow diet. However, feed efficiency (body weight gain per energy consumed) on chow was not significantly different among the DIO and DR male and female rats. Increased chow intake appeared to be a function of increased metabolic mass (107).

In contrast to feeding these rats a chow diet, 2-wk (males) or 3-wk (females) on a HE diet revealed that DIO male and female rats gained more body weight, consumed more energy, and had greater feed efficiency than DR rats. DIO male rats were weight-matched with DR male rats and fed a chow diet for one week. DIO and DR rats consumed the same amount of food and had similar feed efficiency, but DIO rats had 109% heavier retroperitoneal adipose depots than DR rats (107). The combination of greater adiposity and increased feed efficiency (or increased energy storage) on HE diets noted in the DIO rats suggests that there are potential mechanistic perturbations occurring in adipose tissue in response to an energy dense diet.

Adipocyte Biology

Until recently, adipocytes were thought to be a passive participant in energy storage. The landmark discovery of leptin and its effects have led researchers to redefine their positions about adipose tissues (15). Leptin is a 16-kDa protein that is encoded by the obese gene and is mainly expressed and synthesized in adipose tissue (166). Plasma leptin concentrations are related to fat mass in rodents and humans (30, 58). Plasma leptin levels decrease with fasting and increase with food intake, suggesting that leptin acts as a signal of energy stores (15). The various effects of leptin appear to be mediated through its action on the hypothalamus. Leptin administration decreased food intake and stimulated energy expenditure in leptin-deficient *ob/ob* mice (70). In the arcuate nucleus, neuropeptide Y (NPY: potent stimulator of food intake and inhibitor of energy expenditure) expression and secretion were both inhibited by exogenous leptin (138, 145). Conversely, injecting leptin into the third ventricle increased corticotrophin releasing hormone (CRH: inhibitor of food intake) gene expression in the paraventricular

nucleus (138). Furthermore, leptin affects energy expenditure by increasing thermogenesis and physical activity via activation of the sympathetic nervous system (15). The recent leptin data seem to confirm the early lipostat theory of Kennedy (89), that adipose tissues regulate their mass by secreting a factor(s) that regulates food intake and energy expenditure.

Although adipose tissue as an endocrine organ has recently gained acceptance, body fat distribution was reported to influence certain metabolic diseases over four decades ago. Vague (155) reported that an upper body (truncal) fat distribution was associated with an increased incidence of diabetes mellitus and atherosclerosis. Specifically, intra-abdominal visceral adiposity is associated with insulin resistance and altered blood lipid profiles (91). Released free fatty acids from adipose tissue were increased in obese subjects when compared with lean ones and this augmented release was associated with the aforementioned metabolic complications (82). Free fatty acids interfere with hepatic insulin extraction and numerous actions of insulin including suppression of lipolysis and gluconeogenesis, recruitment of glucose transporters (e.g., GLUT4), and stimulation of glucose metabolism (141). Moreover, body fat distribution is different between genders. Males tend to have adiposity centered around the abdomen (android), whereas fat on women is located in the gluteo-femoral (gynoid) region and not in the truncal region (14, 155). Steroid hormones influence fat distribution patterns and its effects appear to be permissive (17). Androgens levels are associated with increased truncal fat and reduced subcutaneous fat in limbs of men. Conversely, subcutaneous fat is evenly distributed between trunk and limbs in women and is related to estrogen status. Regional differences in lipid accumulating and mobilization processes are also influenced

by sex steroids (17). For example, testosterone inhibits lipoprotein lipase (LPL) activity in abdominal subcutaneous fat, but not in femoral subcutaneous fat. On the other hand, estrogen stimulates LPL activity in gluteo-femoral subcutaneous fat, but not abdominal fat (14, 17). In summary, adipose tissue is an active endocrine organ that contributes to metabolic health. Elucidation of mechanisms regulating its size and distribution is important to reducing the complications associated obesity.

Additionally, because adipocytes are not homogeneous in their responses to stimuli, regional variations also need consideration when studying adipocyte biology. In humans, basal lipolytic activity is increased in subcutaneous fat cells vs. intra-abdominal (omental and mesenteric) fat cells (44a), but visceral adipocytes are more responsive to lipolytic, as well as, lipogenic stimuli (2, 44a). Catecholamine sensitivity is further increased in visceral adipocytes and reduced in subcutaneous fat cells with the obese state (3). This shift in lipolytic patterns within obese humans may contribute to the increased subcutaneous adiposity and metabolic perturbations associated with increased free fatty acid flux from visceral adipocytes (141). Similarly, visceral adipocytes are more responsive to lipolytic agents and insulin than subcutaneous adipocytes in rats (60, 151). Therefore, it is of interest to examine the two major metabolic processes occurring in adipocytes for possible differences in obesity-prone and obesity resistant rat populations. Adipocytes regulate their size by lipolysis (lipid mobilization) and lipogenesis (lipid accumulation via *de novo* synthesis or re-esterification) (73). These two processes are under strict control by several metabolic hormones. Catecholamines and growth hormone (GH) are the major regulators of lipolysis, whereas insulin controls lipogenesis.

Hormone-stimulated Lipolysis

Catecholamine-induced lipolysis is influenced by regional and sex-specific differences in adrenergic receptor populations (β_1, β_2, β_3 and α_2) in human and other animal models (96). Norepinephrine and epinephrine are catecholamine hormones that are secreted by the adrenal medulla (both) and postganglionic sympathetic neurons (norepinephrine) (65). The lipolytic properties of catecholamines and related compounds are mediated through β-adrenergic receptors, whereas activation of α_2-adrenergic receptors inhibits lipolysis (3). For lipolysis, catecholamines act via binding to β-adrenergic receptors and activating a stimulatory G-protein-related second messenger system that subsequently phosphorylates hormone-sensitive lipase (HSL) via a cAMP-dependent protein kinase A (PKA) mechanism (146). Upon phosphorylation, activated HSL is the rate-limiting step in the hydrolysis of triglycerides of intracellular lipid droplets to yield glycerol and fatty acids (164). PKA activity is also modulated by forskolin, which bypasses receptors to directly activate adenylyl cyclase (115). This compound is used to examine lipolytic mechanisms beyond receptor activation. In the basal state, non-activated HSL still has the capacity to hydrolyze triglycerides but at a much lower rate than activated HSL. Moreover, unphosphorylated perilipins are reported to protect the lipid droplet from HSL. However, isoproterenol-stimulated PKA activation results in translocation of activated HSL from the cytosol to the lipid droplet and phosphorylation of perilipins, which causes the perilipins to expose more of the lipid droplet for HSL-stimulated lipolysis (28). Differential sensitivity or responsiveness to catecholamines may predispose OP rats to or protect OR rats from accumulating lipids and increased adiposity.

At the adipocyte level, GH is known to have two different effects; transient insulin-like effects and delayed anti-insulin-like effects (33). GH induces insulin-like effects (anti-lipolysis and glucose uptake) in normal rat adipocytes that have not been exposed to GH for three hours (25, 44, 66). Exposing these cells to GH results in a net dephosphorylation of HSL and consequently, reduced lipolysis (13). The antilipolysis effect appears to reflect a GH-mediated activation of cGMP-inhibited cAMP phosphodiesterase, which results in increased degradation of cAMP (46). On the other hand, GH stimulates lipolysis in adipocytes indirectly and directly after a lag period (48). The indirect mechanisms by which GH stimulates lipolysis occur via increased mRNA expression and protein synthesis of β-adrenergic receptors and HSL (36, 162). Glucocorticoids act synergistically with GH to enhance lipid mobilization (50). Recently, Yip and Goodman (165) demonstrated that GH plus dexamethasone disrupts the translocation of the α-subunit of the inhibitory G-protein, thus blocking its inhibitory effects on adenylyl cyclase activation. The direct effects of GH on lipolysis have not been completely elucidated.

Steroid hormones also affect lipid mobilization, but their effects are considered permissive. Glucocorticoids stimulate lipolysis by indirect and direct mechanisms. Adrenalectomized rats have diminished lipolytic responses in adipocytes, but dexamethasone treatment reverses these alterations by increasing β-adrenergic receptor numbers (35). Dexamethasone also augments HSL mRNA expression *in vitro* (144). Moreover, dexamethasone alone was shown to stimulate lipolysis (50, 51), but its direct effect on HSL activation is not well understood. The indirect and directs effects of glucocorticoids appear to be mediated by glucocorticoid receptors found in rat adipocytes

(127). Conversely, excess cortisol levels in Cushing's Disease patients are associated with elevated truncal fat mass (135). Increased LPL activity and diminished lipolytic response are the mechanisms for increased truncal fat in these patients (14). Sex steroids also appear to influence lipid breakdown. Testosterone upregulates β-adrenergic receptor expression and works synergistically with GH to increase isoproterenol-stimulated lipolysis in adipocytes (163). Ovariectomized rats had impaired β-adrenergic receptor stimulation and adenylyl cyclase activity in parametrial fat cells, but not subcutaneous fat cells (95). Therefore, the effects of these steroids should be considered when evaluating differences in hormone-stimulated lipolysis.

Altered catecholamine or GH signaling, and thus reduced lipid mobilization, may result in excess lipid accumulation in adipocytes and predispose rats to obesity. For examples, GH may have increased insulin-like effects (i.e., glucose uptake) or reduced anti-insulin-like effects (i.e., lipolysis) at the adipocyte level in OP rats compared to OR rats. Furthermore, reduced expression of β-adrenergic receptors and consequently, lipolytic responsiveness may accompany reduced GH secretion noted in outbred OP rats before and after exposure to the MHF diet (98, 101). Moreover, OP rats also have increased urinary norepinephrine output when exposed to a novel environment, indicating increased sympathetic activation (111). It is possible that an overactive sympathetic nervous system in OP rats may affect lipolytic responses in adipocytes via altered receptor levels or post-receptor mechanisms. The lipolytic role of stress hormones (dexamethasone) can also be examined in adipocytes. The experiments in this proposal may help address these questions.

Insulin-stimulated Metabolism

Lipid storage in adipose tissue relies on many insulin-stimulated mechanisms. Insulin mediates glucose and free fatty acid uptake from bloodstream, stimulates synthesis of long-chain fatty acids from glucose (lipogenesis) and formation of triglycerides from fatty acid and glyceride-glycerol moieties (esterification), and inhibits breakdown of triglycerides (lipolysis) in adipocytes (65). All of these actions require different pathways and enzymes, but all rely on insulin binding to its receptor. Plasma insulin concentrations are low during the fasted state, but are elevated with feeding. There is an initial rise in insulin prior to or right at the beginning of the meal (cephalic phase insulin response). During this phase, insulin secretion is regulated by neurotransmitters released by the parasympathetic fibers of the vagus nerve that innervate pancreatic islet cells (11). The cells of origin for the cephalic phase insulin response are located in the dorsal motor nucleus of the medulla (12). After the initial insulin rise produced by this response, plasma insulin concentrations drop but as blood glucose levels rise during absorption, pancreatic β-cells secrete insulin to enhance substrate removal. Reduction in plasma glucose is stimulated by insulin via increased glucose uptake primarily into muscle (for muscle glycogen and energy needs) and adipose tissue (for triglyceride formation and energy needs) via receptor-mediated events (65). Insulin also stimulates enzymes that regulate glycogen and fatty acid synthesis in hepatocytes. Glucose transporters (GLUT) mediate glucose uptake across plasma membranes via formed channels. There are five isoforms of GLUT that are expressed in various cell types. GLUT1 is present in most cell types, whereas GLUT2 are found in liver and

pancreatic β cells and GLUT4 are located in pools of intracellular vesicles within fat and skeletal muscle cells (24).

For brevity, this review of insulin action will focus on the effects of insulin on adipose tissues. On fat cells, insulin binds to the extracellular domain of its receptor (tyrosine kinase receptor family), which results in phosphorylation of tyrosine residues on the intracellular portion of the receptor (63). These phosphotyrosines attract and activate other second messengers, which initiate cascades of phosphorylations and dephosphorylations that activate or deactivate numerous enzymes and transcription factors. Insulin receptor substrate-1 (IRS-1) is recruited by phosphotyrosines and is thought to mediate many of the activities of insulin binding (103). IRS-1 activates and recruits GLUT4 from intracellular pools, which fuse with the plasma membranes to increase glucose uptake (24). Insulin also stimulates the synthesis of fatty acids by increasing glucose uptake (substrate availability) and the activity of enzymes necessary for fatty acid synthesis (e.g., fatty acid synthase and acetyl CoA carboxylase) (6, 57). In contrast, insulin inhibits lipolysis to enhance lipid storage. This is achieved by inhibiting the formation of and accelerating the breakdown (via increased phosphodiesterase) of cAMP, which is necessary to activate PKA and subsequently hormone sensitive lipase (65).

Adipocyte uptake of circulating free fatty acids is controlled by LPL, which is secreted from adipocytes and adheres to the vascular endothelium near the cells (31). Very-low density lipoproteins (VLDL) and chylomicrons containing triglycerides are transported in the bloodstream and release free fatty acids upon hydrolysis by LPL. The released free fatty acids are transported into adipocytes and re-esterified to triglycerides

(43). LPL expression and secretion are also regulated by insulin in adipocytes (57). Possibly, increased sensitivity to insulin or alterations in expression, synthesis, or activation of the above enzymes and proteins may predispose animals or humans to obesity.

Summary

Obesity is a major contributor to many of the metabolic perturbations found in humans and animals. It is of the utmost importance to determine and understand the mechanisms regulating body weight gain, specifically fat weight. Body weight is regulated by energy consumed and energy utilized. Calories consumed beyond energy requirements are stored in fat cells. Adipose tissue is not a passive storage site, but rather an important endocrine organ whose secreted hormones and metabolites regulate numerous metabolic processes. Moreover, adipose tissue is composed of adipocytes that are expanding, contracting, and proliferating as energy stores dictate. Two major processes occurring in adipocytes are lipolysis and lipogenesis. These processes are under the strict control of different metabolic hormones. Alterations in one or both of these processes may be predispose OP rats (or humans) to or protect OR rats from developing obesity.

PURPOSE

Overall Objective

The overall objective of these experiments was to determine whether lipolysis, insulin-stimulated glucose uptake and metabolism or both were altered *in vivo* or in isolated adipocytes of OP and OR outbred or inbred SD rats prior to or during exposure

to an obesity-inducing diet. Lipid accumulation in adipocytes is balanced by the intricate interplay between lipolysis and lipogenesis, which are influenced by different metabolic hormones. Recently, we reported that although outbred OP rats were more efficient at storing energy than outbred OR rats throughout the dietary challenge, relative food consumption and relative growth were greater in OP rats compared to OR rats only during the early phase of the dietary challenge (100). Therefore, dysregulation of lipolysis, lipogenesis, or both may predispose OP rats to develop obesity or protect OR rats from it. The animal model of diet-induced obesity allowed the opportunity to examine these processes at various time points during the development of obesity. Exposure to a MHF diet resulted in approximately one half of a SD rat population to become obese (OP), whereas the other half (OR) gains weight at a similar rate as rats fed a chow diet (low fat) (40, 98, 99, 100, 101). Earlier research with this model showed that plasma GH was reduced in outbred OP rats compared to outbred OR rats prior to and after the onset of obesity (98, 101). Hyperinsulinemia, hyperleptinemia, and dyslipidemia were noted in outbred OP rats compared with OR rats after the onset of obesity (40, 100). All of the above hormones and metabolites influence adipocyte biology. Utilizing both outbred and inbred SD rats, we determined whether lipid-regulating mechanisms in adipocytes were different between OP and OR rats. The following aims were conducted to achieve these goals:

Specific Aim I

Specific Aim I determined whether basal and hormone-stimulated lipolysis was different between outbred or inbred OP and OR rats. In experiment 1, *in vivo* lipolytic responses were measured in outbred rats prior to a dietary challenge to ascertain whether

these responses predicted obesity-susceptibility or resistance. This was accomplished with a whole body lipolytic challenge and a local lipolytic challenge via microdialysis of an individual fat depot prior to the MHF dietary challenge. After divergence, glycerol release variables were compared retrospectively between outbred OP and OR rats. Since weight-matched inbred OP and OR rats were reported to have differential body composition (107), these inbred rats were used to compare *in vitro* lipolytic responsiveness in adipocytes of OP and OR rats in experiment 2. Fat cell type and size also influence lipid mobilization. Therefore, this experiment enabled us to address these issues.

Specific Aim II

Specific Aim II focused on the role of insulin sensitivity on the early development of diet-induced obesity. Insulin regulates glucose transport and metabolism (including lipogenesis) in adipose tissue among other tissues. Increased insulin sensitivity was reported in adipose tissues of rats with hypothalamic obesity and genetically obese rats and mice early in obesity development. The role of insulin-stimulated glucose uptake and metabolism on excess fat accretion was investigated in adipocytes of outbred OP and OR rats during the early stages of obesity development. Moreover, dietary fat levels influence insulin sensitivity and responses in adipocytes and other cell types. The above comparisons were also performed in adipocytes of moderately high fat and low fat-fed rats. Similar to hormone-stimulated lipolysis, insulin-stimulated glucose metabolism is influenced by cell size. Therefore, adipocyte profiles were measured and compared among groups. Expression of fatty acid synthase mRNA, a key enzyme for fatty acid synthesis was determined in the proposed experiments.

CHAPTER II

MATERIALS AND METHODS

Experiment 1 investigated the question as to whether lipolytic responses prior to a dietary challenge predicted obesity-susceptibility in outbred rats as measured *in vivo* (specific aim I). Experiment 2 examined *in vitro* lipolysis in inbred SD rats to determine if differences in visceral and subcutaneous adipocyte lipolytic responses contributed to obesity susceptibility or resistance (specific aim I).

Experiment 3 determined whether insulin-stimulated glucose uptake was different between epididymal adipocytes of OP and OR rats during an early phase of a MHF dietary challenge (specific aim II). Additionally, the responses of adipocytes from rats fed either a moderately high fat diet or low fat diet (controls = C) were also compared. Experiment 4 evaluated insulin-stimulated glucose metabolism in epididymal adipocytes of OP and OR rats during early phases of a MHF dietary challenge (specific aim II). Comparisons were also made with rats fed a low fat diet. Experiment 5 compared fatty acid synthase messenger ribonucleic acid (mRNA) expression in pooled epididymal fat tissue of OP, OR, and C rats (specific aim II).

ETHICAL TREATMENT OF ANIMALS

All rats were housed in facilities approved by the American Association for the Accreditation of Laboratory Animal Care. These studies were approved by the Animal Care and Use Committee of Eastern Virginia Medical School and carried out according to NIH Guidelines.

METHODS: EXPERIMENT 1

Animals

Thirty (30), cannulated male Sprague-Dawley (250 to 300 g, Charles River, Waltham, MA) rats were obtained and housed in individual stainless steel hanging cages in a temperature-controlled room (22 ± 2°C) with a 12 light-dark cycle. Rats were fed a standard rat chow diet (Harlan Teklad, Madison, WI) *ad libitum* during a one week acclimation period. Rats were handled daily to assess and maintain patency of jugular cannulae. A baseline blood sample (one ml) was collected after the acclimation period and prior to lipolytic challenges for plasma hormone analyses.

Whole Body Lipolytic Challenge

Rats were subjected to a whole body lipolytic challenge after removing food in the morning or approximately 3 to 4 hours prior to the following procedures. Experimental procedures were performed in a sampling area within the same room that they were housed. Each rat had a remote catheter fixed to the cannula to reduce handling. Following 10-min acclimation period in sampling area, basal blood samples (two 500 μl samples) were drawn five minutes apart. After the second basal blood draw, isoproterenol (20-μg/kg; (4)) was injected via cannula. Isoproterenol was chosen as the lipolytic agent because it is a non-selective β-adrenergic receptor agonist, which has more potent lipolytic properties than epinephrine and norepinephrine (48). Repeated blood sampling occurred at 5, 10, 15, and 25 min after isoproterenol injection. After each blood draw, an equal volume of normal saline was injected into the animal to minimize volume depletion effects. Blood samples were centrifuged for 20 minutes at 1500 rpm at 4°C. Plasma was transferred to a labeled microfuge tube and frozen at -20°C until

glycerol analysis. Glycerol was used as an index of lipolysis instead of free fatty acids because glycerol must exit adipocytes due to reduced glycerol kinase levels (156).

Plasma Glycerol Assay for Whole Body Lipolytic Challenge

Plasma glycerol was measured with the Sigma Kit #337-40A (Sigma Chemical, St. Louis, MO) according to supplied protocol. Basal and peak glycerol release were compared within and between groups.

Local Lipolytic Challenge via Microdialysis

Rats were allowed to recover for at least one week after whole body challenge. During this week, rats were familiarized with the Awake Animal System (CMA 177, Carnegie Medicin, Worcester, MA). After recovery, local lipolytic responses were determined in the epididymal adipose pad using microdialysis according to published procedures (4). This visceral fat pad was chosen for size and location. Surgery at this site was less invasive resulting in less trauma to the rat than surgery on other visceral fat pads (mesenteric and retroperitoneal). Microdialysis works by inserting a probe into the tissue of interest and then infusing that tissue with the drug or hormone of interest. These probes are bi-directional in that they allow for infusion through one tube and collection of dialysis perfusate via another tube, which is then analyzed for the metabolite or hormone of interest.

Prior to insertion, individual microdialysis probes (CMA/20) were perfused with 2-ml of Ringer solution at a rate of 15-µl/min with the microinfusion pump (CMA/100) to remove any glycerol in the probe membrane. On the day of probe insertion, rats were anesthetized with an interperitoneal injection of ketamine (67 mg/kg) and xylazine (13 mg/kg) and placed on a heated operating surface. Surgical areas (lower abdomen and

back of neck) were shaved and washed with betadine solution. An incision was made in the abdomen above the scrotal area and the left epididymal fat pad was exteriorized onto a moist sterile gauze pad. A trocar needle and sheath were inserted into the midpoint of the fat pad. After placement of sheath, the trocar needle was removed and the microdialysis probe was inserted into the sheath. The sheath was then removed after the probe was secured by suturing the probe to an adjacent tissue (e.g., abdominal muscle wall). The probe tubes were tunneled subcutaneously with a tunnel trochar, exteriorized through the back of the neck, and secured with a restraining collar. Internal incisions were sutured and external incisions were closed with surgical clips. Rats were allowed to recover on a heated source. Lipolytic tests were performed after an overnight recovery period.

On the day of local lipolytic challenge, food was removed early in the morning or approximately 3 to 4 hours before the following procedures. Rats were placed in an Awake Animal System, which does not restrict animal movement. In this system, probe tubing is connected to a swivel and thus it allows rats to be tested in the conscious state and relatively undisturbed. Other components of the system included two microdialysis pumps (1 for vehicle and 1 for drug) and three liquid switches (CMA 110), which were connected by tubing. Prior to connecting the pump tubing to the probe tubing of the rats, pump tubing was flushed (10 μl/min) with 1 ml of Ringers to ensure that it was functional and to remove any air bubbles and glycerol. This set-up enabled simultaneous testing of three rats. After connecting rats to the pump tubing, rats were acclimated to the Awake System for 60-min. Ringer's solution was infused at a rate of 5-μl/min to check the integrity of the connections between the pump and probe tubing during this period.

After 60-min acclimation period, basal dialysis perfusate samples were collected in ten minute intervals in labeled collection tubes. To start isoproterenol (10 μM) infusion, a liquid switch was turned and drug was infused at a rate of 5 μl/min for 30 min. The dialysis perfusate collection tubes were changed every ten minutes and stored on ice. At the end of the infusion period, rats were disconnected from the pump tubing and quickly anesthetized with halothane to remove the microdialysis probe. Wounds were closed with surgical clips.

Microdialysis Glycerol Assay

Due to the small volumes collected during the experiment, a highly sensitive glycerol assay was necessary. Therefore, samples were analyzed using the CMA 600 microanalyzer at Carnegie Medicin (Worcester, MA). Basal and peak glycerol release were compared within and between groups.

Dietary Challenge

After completing the whole body and local lipolytic challenges, surviving rats (n = 24) were placed on a moderately high fat (MHF = 31.8% kcal as fat; Research Diets, New Brunswick, NJ, (99)) diet for 14 weeks. At the end of 14 weeks, rats were classified as obesity-prone (OP) or obesity-resistant (OR) based on body weight gain (99, 111). Body weight and food intake were measured weekly. These data were used to calculate feed efficiency, relative growth, and relative food consumption. Feed efficiency is calculated as body weight gain (g) divided by energy consumed (MJ) for a given week and represents storage of energy as body weight. Relative food consumption is determined by dividing weekly food consumption (g) by the mean body weight for that week and indicates consumption of food as a function of body mass. Relative growth is

calculated by dividing weekly body weight gain by the mean body weight for that week and depicts weight gain as function of body mass. Rats were killed by decapitation and trunk blood was collected for plasma. Epididymal and retroperitoneal fat pads were harvested and weighed for each rat. Fat pad weight was used to calculate adiposity index, a measure of fatness (42).

Plasma Hormone Measurements

Plasma leptin and insulin concentrations were determined by radioimmunoassay (RIA) according to supplied protocols (Linco Research, Inc., St. Louis, MO).

Statistics

Glycerol release data from either the whole body or local lipolytic challenge were compared between groups with Student's t-test. Total body weight gain, visceral fat pad weight, cumulative food intake data were all compared with Student's t-tests. Pre- and post-dietary plasma leptin and insulin concentrations were analyzed with 2 x 2 repeated measures analysis of variance (ANOVA). Repeated measures ANOVA were also used to compare weekly data (body weight and food intake-related data). Comparisons of weekly means for each variable were made with Student's t-test corrected for Type I error rates (Tukey-Kramer test). A correlation (Pearson) matrix was generated to evaluate relationships among certain variables. All data are expressed mean ± standard error of the mean (SE) and level of significance was set at $p < 0.05$ for all comparisons.

METHODS: EXPERIMENT 2

Animals and Dietary Challenge

Inbred OP and OR male SD rats (n = 6 per group) were used for the experiments in this section. Rats were purchased at approximately 4 weeks of age from Columbia

University, which received breeder pairs from the original stock of Levin et al. (107). Rats were individually housed in hanging stainless steel cages in a temperature-controlled room (22 ± 2°C) with a 12 h light-dark cycle. Rats were acclimated to new surroundings before conducting any experiments and fed a standard rat chow (Chow = 4.5% kcal as fat; Harlan Teklad, Madison, WI) *ad libitum* for four weeks. Body weight, naso-anal length and food intake were measured over the last 2 weeks to evaluate feed efficiency on a chow diet. Feed efficiency was calculated as body weight gain (g) divided by energy consumed (kcal) for 2 weeks. At 8 weeks of age, OP and OR rats were killed by decapitation and trunk blood was collected in EDTA-coated glass tubes. Visceral fat depots (epididymal and retroperitoneal) were excised and weighed and a sample of subcutaneous fat from the inguinal region was also removed for analysis. Retroperitoneal, epididymal, and subcutaneous adipose tissues were placed in warm PBS buffer (37°C) after dissection.

Adipocyte Isolation

Adipose tissues from the same depot and group (e.g., OP epididymal) were pooled and collagenase digested according to the method of Rodbell (136). Approximately 5-g of each pooled fat depot were minced and placed in 50-ml centrifuge tubes containing 5-mg of collagenase (Type I; Worthington Biochemical Corp., Lakewood, NJ) and 5-ml of Krebs-Ringers-Hepes-Bicarbonate-Albumin (KRBH-A) buffer plus 50-µl of 50 mM glucose. Tubes were shaken (60 cycles/min) at 37°C for 1-hr and adipocytes were separated from cellular debris and undigested tissue by filtering over 250-µm nylon mesh. Adipocytes were washed several times with collagenase-free KRBH-A buffer to remove collagenase and centrifuged to separate adipocytes from

preadipocytes, stromal cells, and vascular membranes. Adipocytes were diluted to a final concentration of 10% cells (e.g., 2-ml of packed adipocytes in 18-ml of KRBH-A buffer), which is approximately 100,000 cells/ml. This cell suspension was utilized for the procedures outlined below.

Cell Diameter and Size

Cell diameter (μm) of approximately 1,200 cells (~200 cells per fat pad per rat) was measured with the Image 1 Analysis System (Universal Image Corporation, West Chester, PA). The 10% cell suspension was used for the cell diameter measurements. Briefly, one slide with two vacuum grease wells was made and 3-4 drops (~100 μl /well) were placed in each well. Six slides per fat pad per group were used to measure approximately 200 cells per slide. The Image 1 Analysis System allowed for images to be stored and analyzed at a later time. Mean cell diameter was used to estimate mean cell volume (64) and cell surface area (167). Cell size (μg lipid/cell) was calculated by multiplying cell volume (pl) by lipid density (lipid density is approximately 0.915 g/ml) (39).

Cell Lipid Content

Cell lipid content was determined according to the method of Dole (41). The 10% cell suspension (1-ml per sample) was utilized to determine cell lipid content. Six replicates of cell suspension were added to 5 ml of lipid extraction solution (2-propanol: heptane: sulfuric acid). Tubes were vortexed and 3 ml of distilled water and heptane were added. After vortexing and centrifuging, the upper heptane phase containing lipids was quantitated and recorded. Duplicate, 1.5-ml samples of this phase were evaporated (heptane) on tared aluminum weigh boats. Weigh boats were re-weighed after

evaporation and the difference was equal to the lipid weight (mg). Cell lipid content and cell size were used to calculate cell number (39), which was used with cell surface area (167) to normalize glycerol release data.

In vitro Lipolysis Assay

Studies of hormone-stimulated lipolysis were performed on isolated cells with various concentrations of hormone, drug, or both. In plastic tubes (17 x 100 mm), 160-µl of 10% cell suspension were incubated with 60-µl of lipolysis buffer (*LIPO* buffer: adenosine deaminase, PBS and KRBH-A buffer) and 25-µl of various concentrations of either isoproterenol (final concentrations = 10^{-8} to 10^{-6} M, Sigma, St. Louis, MO), bovine growth hormone (GH; final concentrations = 10^{-8} to 10^{-6} M, Monsanto, St. Louis, MO), or forskolin (final concentration = 10^{-5} M, Calbiochem, La Jolla, CA). Preliminary research in our laboratory revealed that isoproterenol and GH-stimulated lipolytic responses were not present at hormone concentrations below 10^{-8} M (Davies and Lauterio, unpublished observations). Moreover, the findings of Tavernier et al. (151) revealed that 10^{-6} M isoproterenol is a maximal dose for all types of adipocytes. Therefore, the dose range of 10^{-8} to 10^{-6} M was chosen for both isoproterenol and GH. They (151) also showed that forskolin at 10^{-5} M is a maximum dose. Dexamethasone (5-µl: final concentration = 2.5×10^{-7} M, Phoenix Pharmaceuticals, St. Joseph, MO) was also added to tubes alone or with GH (50). KRBH-A buffer (5-µl) was added to non-dexamethasone tubes for proper dilution. Basal (control) tubes contained 160-µl of 10% cell suspension, 60-µl of LIPO and 30-µl of KRBH-A buffer. Six replicate tubes were utilized for each condition with visceral adipocytes and 4 replicates with subcutaneous cells due to lack of cells. Tubes were incubated at 37°C in a shaking water bath for 4 hr.

The 4 hr incubation period was chosen because the effects of GH on lipolysis are reported to occur after a lag period of approximately 2 hr (50). Standardization of the time periods also allowed for comparisons among all test conditions. After incubation, 200-µl of media below cells were deproteinized with 200-µl of cold 0.65N perchloric acid (PCA) in a separate tube, vortexed and frozen at –20°C until glycerol analysis (60).

Glycerol Assay for In vitro Lipolysis Assay

Thawed samples were centrifuged at maximum speed (13,000 rpm) for 20 minutes, after which, PCA was neutralized with 50 µl of imidazole buffer (0.4 M imidazole, 2 N KOH, 0.4 M KCl) in the same microfuge tube (60). Glycerol was measured in 100 µl of the neutralized sample according to the Sigma Glycerol Kit (Sigma #337-40A, St. Louis, MO) protocol with slight modifications and calculated by an equation determined by a standard curve. Glycerol was used as an index of lipolysis instead of free fatty acids because glycerol must exit adipocytes due to reduced glycerol kinase levels (156). Glycerol release was expressed per cell surface area per 4 hr to account for cell size differences (167) and as fold increase above basal to assess responsiveness (condition – basal/ basal).

Plasma Profiles

Plasma glucose was measured immediately after plasma separation with the Sigma Glucose kit (St. Louis, MO). The remaining plasma was stored at –20°C until specific hormone analyses. Plasma insulin concentrations were measured with a rat-specific RIA kit according to the provided protocol (Linco Research, Inc., St. Louis, MO). Plasma leptin concentrations were measured with a murine leptin ELISA

according to the provided protocol (Diagnostics System Laboratory Inc., Webster, TX). This ELISA has been reported to have 100% cross-reactivity with the rat leptin species.

Statistics

Glycerol release normalized to cell surface area was compared between different adipocyte populations for OP and OR with appropriate statistical tests (ANOVA, Student's t-tests). Cell sizes and diameters from each fat depot were compared between OP and OR with Student's t-tests, as needed. Body weight, feed efficiency, fat pad weight, plasma leptin and insulin concentrations were all compared between groups with Student's t-tests, as needed. All data are expressed mean ± SE and level of significance was set at $p < 0.05$ for all comparisons.

METHODS: EXPERIMENTS 3, 4, & 5

Animals and Dietary Challenge

Sixty, outbred male Sprague-Dawley rats (175 - 200 g) were used for Experiments 3, 4, and 5. Rats were individually housed in hanging stainless steel cages in a temperature-controlled room (22 ± 2°C) with a 12 h light-dark cycle and allowed to acclimate to their new surroundings for one week. After initial weight and length measurements, six rats were randomly selected and killed to determine baseline data from rats fed a chow diet (Harlan Teklad, Madison, WI) *ad libitum*. Of the remaining animals, twelve rats were randomly selected to remain on a purified low fat (LF = 10.6% kcal as fat; Research Diets, New Brunswick, NJ) diet and the other rats (n = 42) were challenged with the MHF (MHF = 31.8% kcal as fat; Research Diets) diet for 1 or 3 (see Experiment 4) weeks. All rats were fed *ad libitum*. Body weight and food intake (corrected for spillage) were measured weekly and used to calculate feed efficiency, relative growth,

and relative energy consumption. Feed efficiency was calculated as body weight gain (g) divided by energy consumed (kcal) for a given week. Relative energy consumption was determined by dividing weekly energy consumption (kcal) by the mean body weight for that week. The term, relative energy consumption, is used in the present experiments instead of relative food consumption because rats consumed diets (MHF and LF) with different energy densities. Relative growth was estimated by dividing weekly body weight gain by the mean body weight for that week.

After 1-wk of dietary challenge, rats with greatest (n = 6) and least (n = 6) amount of body weight gain were killed to examine the early effects of diet and weight gain on insulin-stimulated glucose uptake in isolated epididymal adipocytes (Experiments 3, 4, & 5). Six rats consuming the LF diet for 1 week were also killed and served as controls (C). The remaining rats continued their diets for 2 more weeks, after which the 6 highest and 6 lowest weight gainers on the MHF diet along with the other 6 C rats were examined for insulin responses in isolated adipocytes (Experiment 4). High weight gainers were referred to as OP and low weight gainers, OR. Remaining rats were not utilized in these experiments. Rats were killed in the postprandial rather than fasting state because lipogenic enzyme activity is increased during the former period (149). They were killed by decapitation and blood was collected in EDTA-coated glass tubes. Plasma was separated by centrifugation and used to determine plasma hormone and metabolite concentrations. Epididymal fat and retroperitoneal fat were excised and weighed. Retroperitoneal fat was snap-frozen in liquid nitrogen. Epididymal fat tissues were placed in 37°C PBS after dissection.

Adipocyte Isolation

A piece of epididymal fat tissue was weighed for each animal and then pooled according to group and fat depot. Pooled fat was minced and then collagenase digested to isolate adipocytes according to the method of Rodbell (136). Approximately 5-g of each pooled fat were placed in 50-ml centrifuge tubes containing 5-mg of collagenase and 5-ml of KRBH-A buffer plus 50-µl of 50 mM glucose. The KRBH-A buffer also included an adenosine analog, (-)-N^6-2-phenylisopropyl adenosine (PIA), to minimize lipolysis (60). These tubes were shaken (60 cycles/min at 37°C) for 1-hr and adipocytes were separated from cellular debris and undigested tissue by filtering over 250-µm nylon mesh. Adipocytes were washed several times with collagenase-free KRBH-A buffer to remove collagenase and centrifuged to separate adipocytes from preadipocytes, stromal cells, and vascular membranes. Adipocytes were diluted to a final concentration of 10% cells (e.g., 2-ml of packed adipocytes in 18-ml of KRBH-A buffer), which is approximately 100,000 cells/ml. This cell suspension was utilized for the procedures outlined below.

Cell Diameter and Size

Cell diameter of approximately 1,000 cells (~200 cells per fat pad per replicate) was measured with the Image 1 Analysis System. The 10% cell suspension was used for the cell diameter measurements. Briefly, one slide with two vacuum grease wells was made and 3-4 drops (~100 µl /well) were placed in each well. Five slides per group were used to measure approximately 200 cells per slide. The Image 1 Analysis System allowed us to store images for later review and analysis. This facilitated concurrent cell profiling with *in vitro* experimentation. Mean cell diameter was used to estimate mean

cell volume (64). Cell size was calculated by multiplying cell volume (pl) by lipid density (lipid density is approximately 0.915 g/ml) (39).

Cell Lipid Content

Cell lipid content was determined according to the method of Dole (41). The 10% cell suspension (1-ml per sample) was utilized to determine cell lipid content. Five replicates of cell suspension were added to 5 ml of lipid extraction solution (2-propanol: heptane: sulfuric acid). Tubes were vortexed and 3 ml of distilled water and heptane were added. After vortexing and centrifuging, the upper heptane phase containing lipids was quantitated and recorded. Duplicate, 1.5-ml samples of this phase were evaporated (heptane) on tared aluminum weigh boat. Weigh boats were re-weighed after evaporation and the difference was equal to the lipid weight (mg). Cell lipid content and cell size were used to calculate cell number (39), which was used to normalize glucose uptake and metabolism data.

Glucose Uptake Assay (Experiment 3)

Glucose uptake assay was used as an index of insulin sensitivity and performed according to the method of Livingston and Lockwood (114), as modified by Fried et al. (60). This assay measured the total uptake of radiolabeled 2-deoxyglucose (^{14}C-2-DG, Sigma, St. Louis, MO) instead of glucose. This isoform (2DG) is preferable as it is transported and phosphorylated by the same processes as glucose, but it cannot be further metabolized (159), thus providing a reliable endpoint. Five replicates were run for each condition with cells from week 1 only. 250-µl aliquots of the 10% cell suspensions were added to plastic test tubes (17x100 mm) containing 0.1, 1.0 or 3.5 (maximal dose) nM of insulin (in 3 µl). Tubes without insulin (Humulin R, Lilly, Indianapolis, IN) served as

basal glucose uptake. Phloretin (3 nM in 3-µl, Sigma, St. Louis, MO)-treated cells were used to assess non-specific glucose uptake. After incubation in shaking water bath at 37°C for 15-min, 50-µl of a cocktail containing 0.1 mM cold 2-DG and ^{14}C-2-DG (0.16 µCi per 50 µl) were added to each tube. Incubation was stopped after 2 minutes by centrifuging tube contents through 100-µl of silicon oil. Centrifuge tubes were cut through the silicon layer and fat cell pellets were dropped into scintillation vials. Ten ml of scintillation fluid were added to each vial and radioactivity was counted in a liquid scintillation counter.

Total counts per minute (CPM) for 25 µl of the radioactive cocktail were used to calculate specific activity by dividing CPM by 2500 to yield CPM per picomole (pmol). Sample counts minus blanks were divided by specific activity and cell number to obtain relative rates of glucose uptake (pmol/10^6 cells/2 min). Basal glucose uptake, condition minus basal glucose uptake to account for differences in basal values and percent increase above basal to assess insulin responsiveness were compared among groups.

Lipogenesis Assay (Experiment 4)

The lipogenic effects of insulin were determined by measuring glucose incorporation into lipids. Aliquots of pooled epididymal cells were incubated with radiolabeled glucose in the absence or presence of insulin (0.1, 1.0 and 3.5 nM in 10-µl for the cells from week 1) according to the protocol of Lavau et al. (102). Due to insulin sensitivity issues, comparisons at week 3 were made only with insulin at 3.5 nM (maximum concentration). In polypropylene scintillation vials, 500-µl of 10% cell suspension were added to 500-µl of a cocktail containing 5mM glucose and [U-^{14}C]-glucose (0.5 µCi per tube, Sigma, St. Louis, MO). Basal glucose metabolism was

measured by incubating cells without insulin. Blanks were run in parallel with each experiment by incubating buffer alone (no cells) with radiolabeled cocktail to account for non-specific radiolabelling. Five replicates were measured for each condition. Prior to incubation, cells were gassed with 95% CO_2:5% O_2 for 30-seconds and capped with a stopper that was equipped with a center well (Kimble-Kontes, Vineland, NJ) containing a strip of Whatman #3 filter paper (Whatman, Maidstone, England). After 2-hr incubation, the reaction was terminated by adding 0.5 ml of 6N sulfuric acid (Labchem Inc, Pittsburgh, PA) to the cells at the bottom of the vial and 0.3 ml of Hyamine hydroxide (Packard, Meriden, CT) to the center well to trap radiolabeled carbon dioxide (CO_2). Vials were re-capped and incubation was allowed to continue for an additional 90 min to capture radiolabeled CO_2. Radioactive counts were measured in two 25-µl aliquots of the radioactive cocktail to determine specific activity. Specific activity was used to convert counts per minute (CPM) to glucose (µmoles).

The following sections address the individual procedures utilized to measure the various products of glucose metabolism.

Total Lipids (Triglycerides)

Incorporation of ^{14}C into total lipids was determined by isolating lipids from the media and cells at the bottom of the vials by method of Dole (41). First, 5-ml of lipid extraction solution were added to the media and cells at the bottom of the vials. After transferring to 15-ml Falcon tubes and vortexing, 3 ml of distilled water and heptane were added and tubes were vortexed and centrifuged. After centrifugation, lipids in the upper heptane phase (i.e., lipid extract) were quantitated by evaporating 1-ml of this phase in tared scintillation vials. After evaporation, the scintillation vials were re-

weighed and the difference was equal to the lipid content (mg/ml). Scintillation fluid was added to the vials and radioactivity was counted. After subtracting radioactivity associated with the blank, radioactivity (CPM) was converted to μmoles of glucose incorporated into lipids and this value was normalized for mg of cellular lipids and cell number, thus both cell size and cell number adjustments were performed.

Fatty Acid Moities of Triglycerides

To determine incorporation of ^{14}C into fatty acids (FA), two ml of the aforementioned lipid extract were saponified with 40% KOH in ethanol and then acidified with 3N HCl (102). FA moieties were separated by adding heptane and then centrifuged. Two ml samples of the upper heptane phase containing FA were added to scintillation vials and allowed to dry overnight. After drying, scintillation fluid was added and radioactivity was measured in a scintillation counter. After subtracting blank, the radioactive counts associated with FA moiety were determined and this was calculated as a percentage of total lipids. This percentage was then used to estimate μmoles of glucose incorporation by multiplying it by the μmoles of glucose associated with total lipids.

Glyceride-glycerol Moiety of Triglycerides

Since triglycerides are composed of FA and glycerol, incorporation of ^{14}C into glyceride-glycerol moiety was assumed to be the difference between total lipids and FA.

Carbon Dioxide

Incorporation of ^{14}C into CO_2 was determined by clipping the center wells into scintillation vials containing scintillation fluid and allowing vials to set for 24-hr in the

dark. After 24-hr, radioactive counts were measured, converted to μmoles of glucose and normalized to cell number.

Plasma Profiles

Plasma glucose was measured immediately with a Sigma kit (Glucose HK, St. Louis, MO). The remaining plasma was stored at –20°C until specific hormone analyses. Plasma insulin and leptin concentrations were measured with rat specific RIA kits (Linco, Inc, St. Louis, MO). Plasma free fatty acids (FFA) concentrations were measured with the NEFA C kit (Wako Chemical, Richmond, VA). Plasma and lipoprotein triglyceride and cholesterol concentrations were measured with Sigma kits (Sigma Chemical, St. Louis MO). Lipoprotein fractions were obtained as previously described (40).

Statistics (Experiments 3 & 4)

Body weights, fat pad weights, cell size, plasma leptin, insulin, FFA, and glucose concentrations were all compared among groups with either one-way ANOVA (Tukey-Kramer for post-hoc test) or Student's t-tests, as needed. Feed efficiency, relative growth, and relative energy consumption were calculated as described earlier and compared among groups with one-way ANOVA. Glucose uptake expressed as either relative to cell number, as condition minus basal or as percent increase above basal were compared among groups or between OP and OR with either one-way ANOVA or Student's t-tests, as needed. Insulin-stimulated ^{14}C-glucose metabolism (CO_2, lipids including fatty acids and glycerol) expressed as either relative to cell number, as condition minus basal or as percent increase above basal were compared among groups or between OP and OR with either one-way ANOVA or Student's t-tests, as needed. Due to the large differences between adipocyte glucose metabolism responses between MHF

and LF fed rats, initial data analyses included all three groups to account for dietary differences and then a secondary analysis was performed on OP and OR data. All data were expressed mean ± standard error of the mean and level of significance was set at $p < 0.05$ for all comparisons.

FAS mRNA Expression via RT-PCR (Experiment 5)

Total RNA was isolated from pooled epididymal fat tissue of OP, OR, or C rats from week 1 according to the method of Chomczynski and Sacchi (27). RNA yields and quality were assessed by measuring absorbance at 260 and 280 nm and by electrophoresis on 1.0% agarose gels. cDNAs were synthesized by reverse transcription (RT) of total RNA according to the protocol provided with the First Strand cDNA Synthesis kit (Promega, Madison, WI). RNA (1 μg in a volume of 7.5 μl) was heated at 70°C for 5 min and then placed ice on for 2 min. RT reaction mixture containing 1X RT buffer, 1 mM dNTP, 5 mM $MgCl_2$, 20 U RNasin, 20 U AMV reverse transcriptase, 0.5 μg oligo $(dT)_{15}$, and 2 μl of nuclease-free water in a total volume of 12.5 μl was added to RNA to yield a final reaction volume of 20μl (47). After brief centrifugation, reaction tubes were incubated at 42°C for 45 min, then at 95°C for 5 min. Reaction tubes were stored at -80°C overnight or used for polymerase chain reaction (PCR) immediately.

The cDNA equivalent of 250 ng (for fatty acid synthase) or 50 ng (for β-actin) of starting RNA was amplified by PCR with published oligonucleotide primers (Life Technologies/ Gibco BRL, Rockville, MD) specific for fatty acid synthase (FAS; (5)) and β-actin (45). The specific primer sequences and product sizes are listed in Table 1. PCR mixes contained 1X Reaction buffer (Promega), 200 μM dNTP, 0.2 μM of forward and reverse primers for FAS or 0.1 μM of forward and reverse primers for β-actin, one TAQ

Hot Start bead (Promega) and RT mix in a final volume of 50 µl. The thermocycler was programmed for 95°C for 2 min followed by 36 cycles at 95°C for 45 sec, 45 sec at 55°C (annealing temperature), and 45 sec at 72°C (47). Following PCR, amplification products were visualized on 1.5% agarose gels and a digital photograph was recorded with the Eagle Eye II Still Video System and Software (Stratagene, La Jolla, CA). Band intensities for each amplicon were measured using the SigmaGel (Jandell Corporation, San Rafael, CA) program. FAS/β-actin ratios were calculated to control for variations in RNA loading.

Statistics (Experiment 5)

Data were expressed as percent of control (C = 100%) and compared between groups. No formal statistics were performed because total RNA was isolated from pooled tissues, which essentially yields only one data point per group.

Table 1. *Primer sequences and PCR product sizes for fatty acid synthase (FAS) and β-actin*

	Sense Primer	Antisense Primer	Product size
FAS	5'-ctgaatctgagtatcctgctg-3'	5'-tgttgatgatagactccaggc-3'	838 bp
β-actin	5'-ctctttaatgtcacgcacgat-3'	5'-agtgctgtgggtgtaggtact-3'	534 bp

CHAPTER III

IN VIVO LIPOLYTIC RESPONSIVENESS IS NOT A PREDICTOR OF OBESITY SUSCEPTIBILITY IN OUTBRED SPRAGUE-DAWLEY RATS

INTRODUCTION

Landerholm and Stern (97) reported that epinephrine-stimulated lipolysis *in vitro* was a predictor of obesity susceptibility in female Sprague-Dawley (SD) rats. In their study, excised subcutaneous adipose tissue was incubated with epinephrine prior to an 11-wk very high-fat (84% kcal) dietary challenge. Glycerol release was measured for each animal and data were separated into quartiles. Rats in the lowest quartile (i.e., lowest lipolytic response) were compared with rats in the highest quartile. Although initial body weights did not differ, the lowest lipolytic responders had significantly elevated body weights and carcass adiposity compared to the highest lipolytic responders after the dietary challenge (97). Moreover, preliminary data from Brown et al. (23) noted similar findings for male Holtzman rats. In their study, epididymal fat tissue examined *in vitro* for catecholamine-stimulated lipolysis prior to a high fat dietary challenge. These researchers found that body weight gained on a high fat diet and mesenteric depot weight were negatively correlated with catecholamine-stimulated lipolysis (23). These data suggested that reduced lipolytic responsiveness to catecholamines predisposed rats to obesity.

In the Landerholm and Stern study (97), lipolytic responsiveness was assessed in excised subcutaneous adipose tissue, which is less sensitive to lipolytic agents than

visceral adipose tissues in rats (60, 151). Since the intent of their experiment was to develop a rapid screening tool for a large group of rats, invasive surgery to remove visceral fat was not warranted. It was of interest to determine whether these *in vitro* data could be replicated *in vivo* because of the difficulty extrapolating *in vitro* responses to actual *in vivo* conditions (4). Therefore, we attempted to confirm these results by examining lipolysis *in vivo* by two separate methods; whole body and local approaches. The whole body approach addressed overall responsiveness to a lipolytic agent via plasma sampling, whereas the local approach allowed for evaluation of an individual adipose (epididymal) depot via microdialysis. Visceral fat was chosen for study because of its association with the metabolic syndrome (91, 134, 141). Finally, to determine whether lipolytic sensitivity predicted obesity-susceptibility, rats were challenged with a moderately high fat (MHF) diet for 14 weeks. After the dietary period, rats were classified as OP or OR and pre-diet lipolytic responses (i.e., glycerol release) were compared retrospectively between groups.

RESULTS

Body Composition and Plasma Hormone Profiles

After a 14 wk dietary challenge, rats were classified as obesity-prone (OP; n = 8) or obesity-resistant (OR; n = 8) based on body weight gain. The middle eight rats were not used for analyses. OP and OR rats did not differ in body weight prior to the MHF diet. Body weights differed significantly between OP and OR rats after two weeks on the MHF diet and this remained throughout the study (Figure 1). As a result of the diet, OP rats gained more body weight and accumulated more visceral fat, expressed as total fat and as an index of adiposity, than OR rats after the 14-wk period (Table 2).

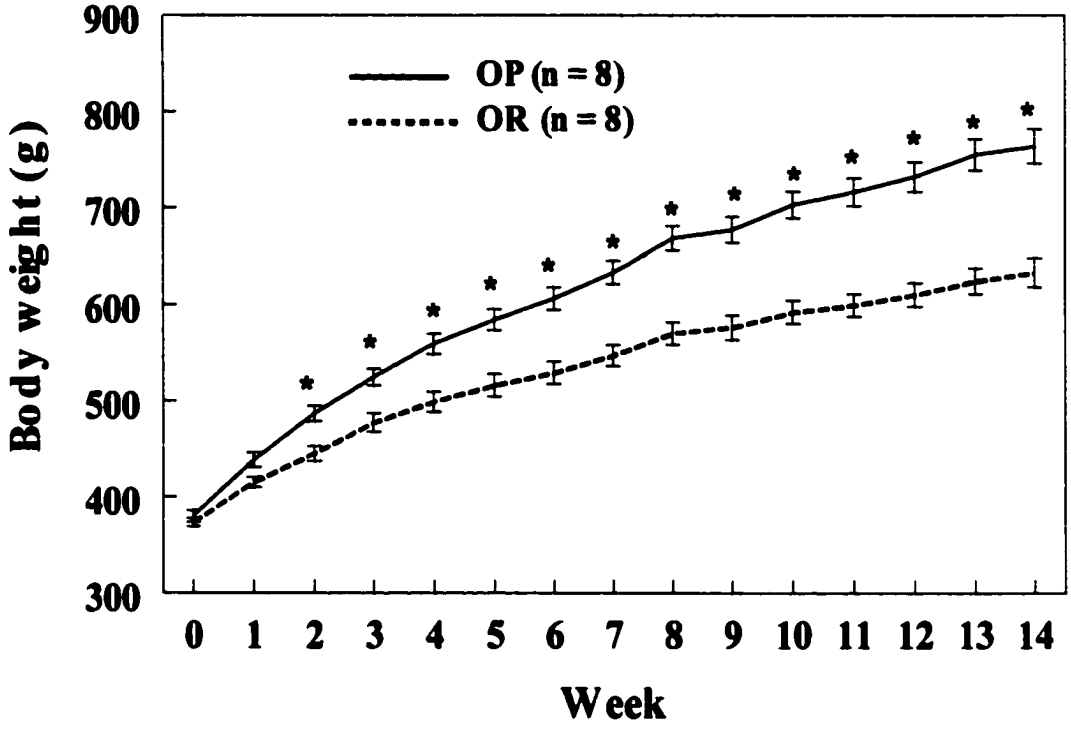

Fig. 1. Weekly body weights of obesity-prone (OP; solid line) and obesity-resistant (OR; dashed line) rats fed a moderately high fat diet for 14 weeks. Data are represented as mean ± SE. *Difference between OP and OR rats at a given time point ($p < 0.05$ or less).

Table 2. *Adiposity variables and food intake data of obesity-prone (OP) and obesity-resistant (OR) rats fed a moderately high fat diet for 14 weeks*

	OP (n = 8)	OR (n = 8)
Total visceral fat (g)	67.0 ± 2.8[a]	42.5 ± 3.3[b]
Adiposity index (%)	8.8 ± 0.32[a]	6.7 ± 0.42[b]
Cumulative energy intake (kcal)	11186 ± 287[a]	9698 ± 208[b]
Cumulative feed efficiency (g/MJ)	8.1 ± 0.1[a]	6.3 ± 0.03[b]

Data are presented as mean ± SE.
Adiposity index = visceral fat (g)/ [carcass wt – visceral fat] (g) * 100% (42)
Feed efficiency = total body weight gained (g)/ total energy intake (MJ)
Different letters within row denote significant differences ($p < 0.01$ or less)

Baseline plasma leptin and insulin concentrations were not different between groups. However, there was a significant group (OP and OR) by time (pre and post) interaction ($p = 0.004$) for leptin. Both groups significantly increased plasma leptin concentrations from pre- to post-diet, but the increase for the OP rats was significantly greater than that for OR rats (Table 3). Similar findings were noted for plasma insulin concentrations, except pre- and post-dietary plasma insulin concentrations for OR rats were not different (Table 3). Moreover, plasma leptin concentration was positively correlated with fat mass ($r = 0.71$, $p < 0.05$), thus group differences for plasma leptin may have resulted of fat mass differences. To account for this, plasma leptin concentrations were normalized to visceral fat mass (i.e., relative leptin). OP rats still had significantly ($p < 0.05$) increased relative leptin when compared to OR rats (0.79 ± 0.11 vs. 0.52 ± 0.03 ng/ml/g, respectively), indicating increased leptin secretion per unit fat mass.

Rates of Food Consumption and Growth

Although rats consumed the same diet, OP rats had greater feed efficiency (i.e., the ratio of body weight gained for the amount of energy consumed) throughout the dietary challenge, except at weeks 3 and 12 (Figure 2). OP also consumed greater cumulative amounts of energy for the 14 weeks (Table 2). Interestingly, relative food consumption (i.e., ratio of food consumed (g) by the average body weight (g) for a given week) was increased in OP rats only during the first two weeks of the MHF diet (Figure 3). Additionally, OP rats grew at a greater rate (i.e., ratio of body weight gained (g) by the average body weight (g) for a given week) than OR rats only for the first two weeks of the MHF diet (Figure 4).

Table 3. *Pre- and post-diet plasma hormone profiles of obesity-prone (OP) and obesity-resistant (OR) rats fed a moderately high fat diet for 14 weeks*

	OP (n = 8)	OR (n = 8)
Pre-diet plasma insulin (ng/ml)	0.7 ± 0.1^a	0.9 ± 0.2^a
Post-diet plasma insulin (ng/ml)	5.1 ± 1.0^a	2.0 ± 0.3^b
Pre-diet plasma leptin (ng/ml)	1.2 ± 0.1^a	1.2 ± 0.1^a
Post-diet plasma leptin (ng/ml)	52.3 ± 6.2^a	22.1 ± 2.1^b

Data are presented as mean ± SE. (See text for pre and post-diet comparisons)
Different letters within row denote significant group differences ($p < 0.05$ or less)

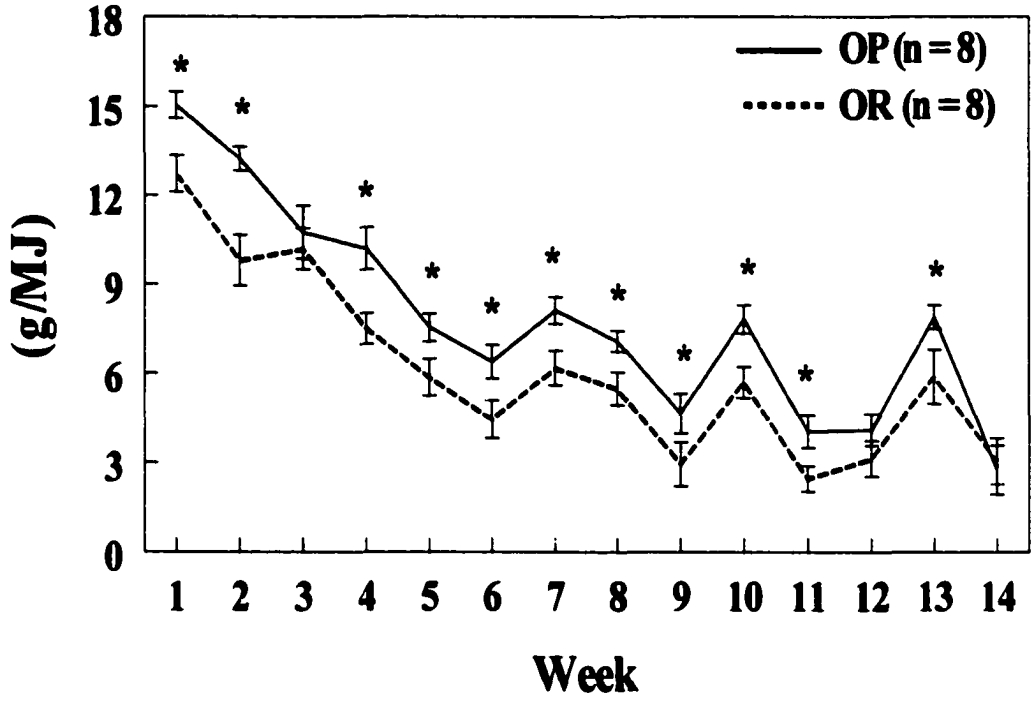

Fig. 2. Weekly feed efficiency of obesity-prone (OP; solid line) and obesity-resistant (OR; dashed line) rats fed a moderately high fat diet for 14 weeks. Data are represented as mean ± SE. * Difference between OP and OR rats at a given time point ($p < 0.05$ or less).

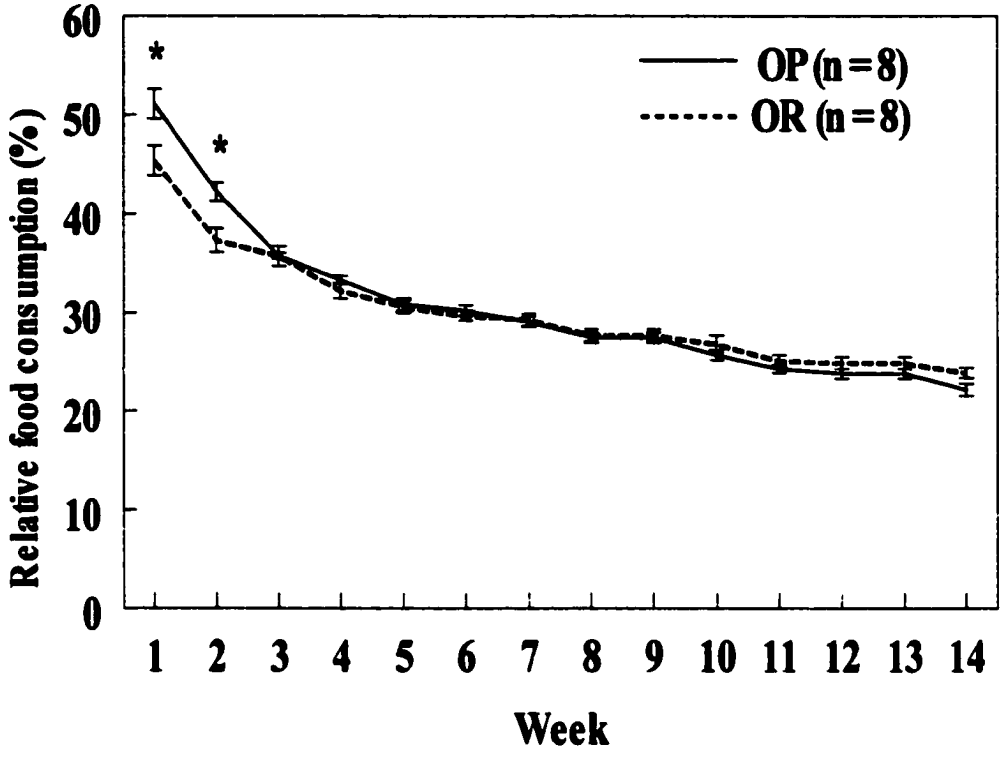

Fig. 3. Relative food consumption (RFC) of obesity-prone (OP; solid line) and obesity-resistant (OR; dashed lines) rats fed a moderately high fat diet for 14 weeks. Data are represented as mean ± SE. * Difference between OP and OR rats at a given time point ($p < 0.05$ or less).

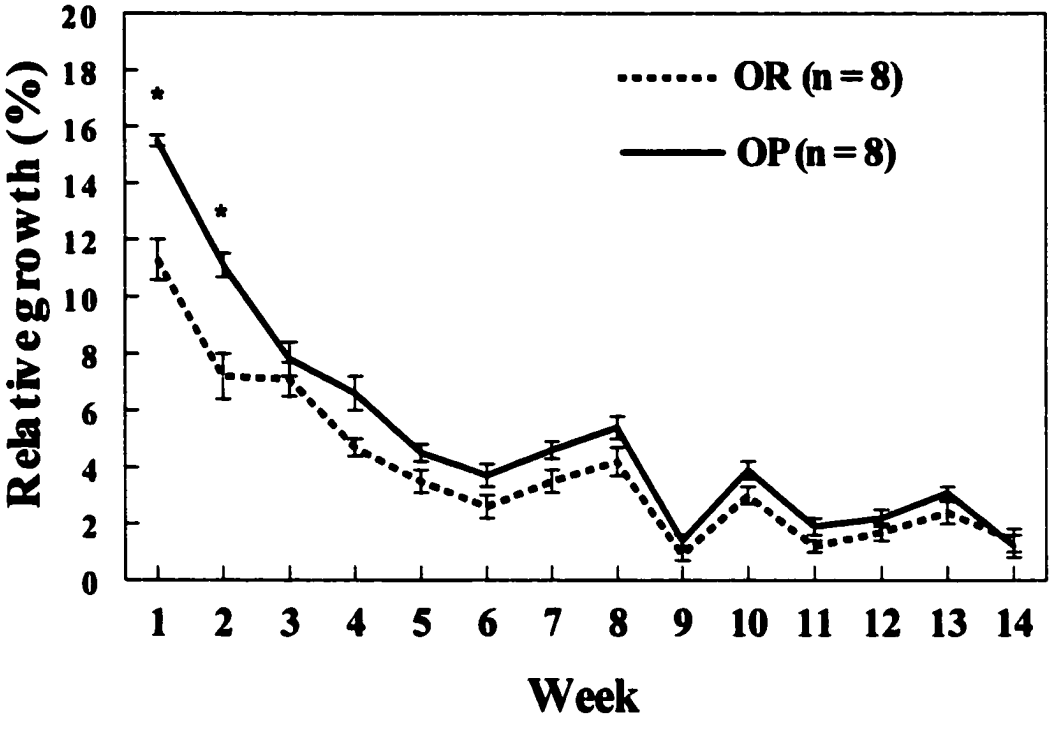

Fig. 4. Relative growth of obesity-prone (OP; solid line) and obesity-resistant (OR; dashed line) rats fed a moderately high fat diet for 14 weeks. Data are represented as mean ± SE. * Difference between OP and OR rats at a given time point ($p < 0.05$ or less).

Glycerol Release in vivo

As expected, basal and peak glycerol release were greater for the whole body method compared to that measured from one epididymal fat pad via the microdialysis method. For the whole body method, basal and peak glycerol release did not differ between groups (data not shown). Isoproterenol injections increased plasma glycerol concentrations by approximately 65% over basal levels, but the differences (peak – basal) in isoproterenol response were similar between OP and OR rats (Figure 5). Comparable findings were also obtained from measuring local lipolytic responses in epididymal fat pad via microdialysis. Basal and peak glycerol concentrations did not differ between OP and OR rats (data not shown). The isoproterenol response (peak – basal) was not different in epididymal fat of OP and OR rats (Figure 6).

DISCUSSION

As in previous experiments utilizing this model, Sprague-Dawley (SD) rats diverged into two distinct populations (obesity-prone, OP and obesity-resistant, OR) based on body weight gain while consuming a moderately high fat (MHF) diet for 10 to 14 weeks (40, 98, 101, 105, 106, 108, 110, 111). Weight gain was associated with increased fat accretion in these studies. This experiment and those that follow largely were designed to determine how this divergence occurs. Decreased lipid mobilization within fat stores may predispose rats to accumulate excess adiposity. The first study utilized the universal lipolytic agent, isoproterenol, to determine if lipolytic responses predicted obesity susceptibility in outbred SD rats. Lipolysis was assessed *in vivo* by two separate methods, plasma sampling (whole body challenge) and microdialysis in epididymal adipose depot (local challenge). Glycerol release was used as an index of

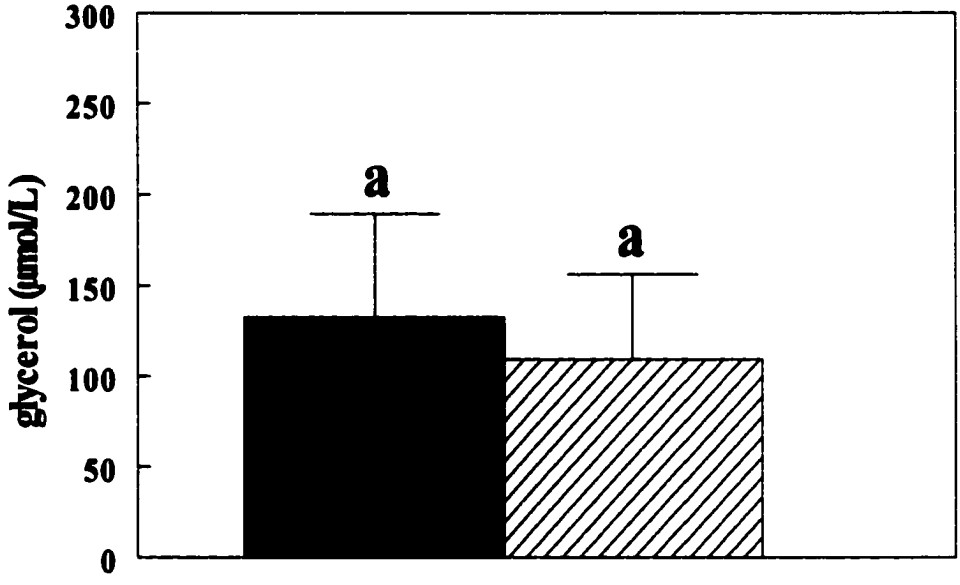

Fig. 5. Isoproterenol-induced responses (peak minus basal plasma glycerol release) during a whole body lipolytic challenge in obesity-prone (n = 8; solid bars) and obesity-resistant (n = 8; striped bars) rats prior to a 14 week dietary challenge.
Data are represented as mean ± SE. Different letters denote significant differences ($p < 0.05$ or less).

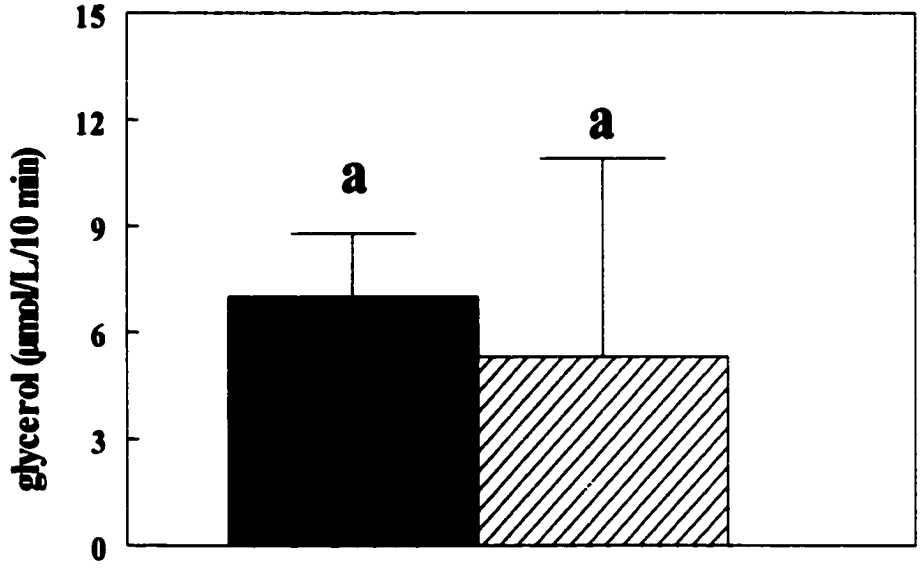

Fig. 6. Isoproterenol-induced responses (peak minus basal plasma glycerol release) during a local lipolytic challenge via microdialysis in the left epididymal fat pad of 8 obesity-prone (solid bars) and 8 obesity-resistant (hatched bars) rats prior to a 14 week dietary challenge. Data are represented as mean ± SE. Different letters denote significant differences ($p < 0.05$ or less).

lipolysis instead of free fatty acids because glycerol must exit adipocytes due to reduced glycerol kinase levels (156). Since the lipolytic and anti-lipolytic regulators exist *in vivo*, it was necessary to examine lipolysis in the intact animal to assess the net effect of isoproterenol as it existed in the intact animal. However to sort out isoproterenol-specific effects, as well as other lipolytic regulators, an *in vitro* analyses were also necessary (see Chapter IV).

After lipolytic challenges, rats were placed on a moderately high fat (MHF) diet for 14 weeks to obtain group classifications (i.e., OP or OR). The main finding was that *in vivo* basal and isoproterenol-stimulated glycerol release did not differ between OP and OR rats prior to dietary challenge, regardless of method. In agreement with these data, outbred SD rats predicted to become obese or remain lean based 24-urinary norepinephrine output prior to a high energy diet (111) had similar hormone-stimulated lipolysis in isolated adipocytes (Dr. Susan Fried, Rutgers University, personal communication). Moreover, Levin et al. (108) reported that *in vivo* basal and norepinephrine-induced thermogenesis (via oxygen consumption measurements) were not different between OP and OR rats before exposure to a high-energy diet. These data indicated that end organ responses to β-adrenergic agonists were not altered in outbred OP and OR rats prior to the obese state. It is, however, not known whether lipolytic responsiveness is altered with the obese state in these rats. Interestingly, catecholamine-induced lipolysis was blunted in obese humans compared with lean ones (3). If similar events occurred in these rats, reduced hormone-stimulated lipolysis would allow OP rats to maintain their increased adiposity, but it was not a primary cause of the obese state.

In the present study, isoproterenol (20 μg/kg) produced a 60 to 70% increase in whole body glycerol release. This isoproterenol dose was chosen because it elicited at least a 100% increase in glycerol release in similar size rats (4). Peak values in the whole body study were similar but slightly higher than that found by Arner (385 [OR] and 435 [OP] vs. 375 μmol/L (4), respectively). Additionally, our basal values were also greater than that previously observed (276 [OR] and 303 [OP] vs. 175 μmol/L, respectively). One limitation of our study was that only one dose was evaluated in the rats. The possibility still exists for different effects with other doses. However, we were attempting to measure lipolytic responses via two different methods before the rats reached a certain age and weight. Additional doses and days would have interfered with the start date of the dietary challenge. Moreover, differences between studies may be related the state of consciousness. Our rats were conscious and placed in a small sampling area, whereas they were sedated in the Arner study (4). Anesthesia is known to cause acute stress. Anesthetic drugs are also lipophilic and alter somatostatin, which may add other confounding factors since endpoints include fat and somatostatin-regulated hormones. Although conscious rats may experience increased stress during plasma sampling, extension catheters were added to minimize handling and reduce stress. Increased stress from the plasma sampling and inadequate habituation time may have accounted for the increased basal lipolysis, which may have reduced the effect of isoproterenol. Stress hormones, however, were not measured in this study.

In the second part of Experiment 1, local lipolytic responses were measured in epididymal fat pads via microdialysis and compared between OP and OR rats. Microdialysis allows one to assess individual tissues for different metabolic responses.

Epididymal fat was chosen to represent visceral fat, which is strongly associated with many metabolic perturbations (91). Hyperinsulinemia and hyperleptinemia are examples of these perturbations and both were present in our OP rats. Confirming the whole body data, local lipolytic responses were similar at basal and peak between OP and OR rats. These data suggested that β-adrenergic receptor populations or responsiveness were not altered in epididymal adipose tissues of outbred OP and OR rats. Previous studies have shown that body composition profiles were not different between outbred OP and OR rats prior to an obesity-inducing diet (105, 106) and this may be related to the similar lipolytic responses in OP and OR groups. However, these findings do not preclude possible differences in epinephrine-stimulated lipolysis between groups as found previously (97). Epinephrine stimulates both anti-lipolytic mechanisms via α_2-adrenergic receptors and lipolytic mechanisms via β-adrenergic receptors (96). Although α_2-adrenergic receptor mRNA expression is low in rat epididymal fat (151), differential regulation of fat α_2-adrenergic receptor expression may contribute to divergent responses noted with MHF feeding. Moreover, other fat depots (e.g., mesenteric, retroperitoneal, or subcutaneous) should be evaluated to confirm our findings.

For comparison, our basal values were approximately 2.5 times greater than published values (4) utilizing similar methodology and microdialysis equipment. Peak glycerol values were similar between OP and OR rats and that found by Arner (4) when compared at the same isoproterenol concentration (10 μM). Due to basal differences, isoproterenol-stimulated response was approximately 10-fold greater in the Arner study vs. our data (250% vs. 23%, respectively). Using only one dose was a limitation to our study but as mentioned earlier, we were trying to perform two different experiments

within a short amount of time. The major differences between Arner and our study are fat depot and sedation state. Dorsal subcutaneous fat was examined in the Arner study, whereas epididymal fat was probed in the present study. In another study examining gonadal fat responses via microdialysis, Darimont et al. (32) showed that glycerol release from parametrial fat was increased approximately 25%, which was similar to the 23% increase found within 30-min in the present study. Similar to whole body results, stress and anesthesia may have played a role in the disparate results between studies. In the present study rats were placed in an Awake System, which allowed for unrestrained movement. Moreover, rats had 1 hr to acclimate to the system prior to sampling. This time period might not have been sufficient to allow rats to re-establish basal stress and lipolytic levels although turnover and plasma half-lives for these neurohormones are short. To minimize these stress-related effects, all rats were familiarized with the system in the week preceding this set of experiments.

In summary, the present study was the first one to attempt predicting obesity-susceptibility utilizing *in vivo* approaches. Our data contrasted with earlier research that demonstrated *in vitro* glycerol release was significantly reduced in rats destined to become obese (23, 97). Methodological issues could account for the differences among the studies. *In vivo* approaches were employed in the present study, whereas *in vitro* analyses were performed by previous researchers (23, 97). Landerholm and Stern (97) also measured epinephrine-stimulated glycerol release from subcutaneous adipose tissue of female SD rats. Although Brown et al. (23) measured glycerol release in epididymal fat tissue of male Holtzman rats, their results did not agree with the *in vivo* microdialysis results of the present study. Strain-related differences may have accounted for the

divergent results between studies. In conclusion, *in vivo* lipolytic responses, as assessed by two distinct methods, were not predictive of obesity susceptibility in male SD rats.

Although OP and OR rats did not differ in body weights and *in vivo* lipolytic responses prior to the MHF diet, numerous differences were found throughout the dietary challenge. Feed efficiency was increased in OP rats throughout the dietary challenge and this increased efficiency was associated with excess fat accretion (100). Interestingly, relative rates of food consumption and growth were greater in OP compared to OR rats during the early phase (i.e., first two weeks) of the dietary challenge, but were similar during the late phases (100). These results suggested that there were two phases (dynamic and static) occurring throughout the dietary challenge, which confirmed earlier findings in rats with hypothalamic obesity (17). The dynamic phase is represented by marked hyperphagia, hyperinsulinemia, and increased body weight gain (78). Hyperinsulinemia has been implicated as the primary event in obesity development with hypothalamic lesions (79). In the present study, plasma insulin concentrations did not differ between groups prior to the MHF dietary challenge and were not measured during the early phase of this challenge. On the other hand, OP rats consumed more energy and gained more body weight than OR rats upon introduction of a highly palatable diet. Consistent with these findings was the increased hypothalamic neuropeptide Y (NPY, a potent stimulator of food intake) mRNA expression found in outbred OP rats vs. OR rats prior to and after two weeks on a high energy diet (106). After the dynamic phase, both OP and OR rats grew and ate at similar relative rates (static phase) for the remainder of the dietary period. In agreement with these data, we reported that NPY mRNA expression was similar in OP and OR rats after 14 wk on a MHF diet (100). Moreover,

NPY expression and synthesis are regulated by leptin and insulin (139). Plasma leptin and insulin concentrations were greater in OP rats than in OR rats, but NPY expression was similar between groups after the dietary challenge. It appeared that augmented levels of these hormones were necessary to regulate NPY expression in OP rats, suggesting both leptin and insulin resistance in these rats (100).

Other early diet-induced differences have also been reported for OP and OR rats. OR rats responded to a high fat diet by lowering 24-hr respiratory quotient with respect to OP rats at week 4, indicating increased fat oxidation for OR rats (26). Lipoprotein lipase (LPL) mRNA expression and activity were up-regulated in epididymal fat and down-regulated in the gastrocnemius muscle of OP rats after 1 wk on a high fat diet (125). This scenario possibly allowed for increased uptake of dietary fatty acids in adipose tissue and ultimately increased fat pad size. Therefore, processes other than lipolysis appeared to contribute to the increased adiposity and weight gain found in outbred OP rats during exposure to a MHF diet. These findings also indicated that the early phase of the dietary challenge should continue to be the research focus.

CHAPTER IV

ASSESSMENT OF THE LIPOLYTIC RESPONSES IN ISOLATED ADIPOCYTES OF INBRED OP AND OR RATS

INTRODUCTION

As noted in Experiment 1, relative food consumption and growth were greater in OP rats compared to OR rats during the early phase of a dietary challenge but were similar during later phases (100). Furthermore, feed efficiency was augmented throughout the dietary challenge (100). Early weight gain in OP rats was associated with increased adiposity (40). Lipid mobilization (lipolysis) and responsiveness to lipolytic agents are possible factors contributing to the excess fat accretion in OP rats. Previous *in vitro* findings indicated that reduced lipolytic sensitivity in excised adipose tissues was associated with obesity susceptibility in male and female rats (23, 97). However, results from Experiment 1 revealed that *in vivo* lipolysis as measured by two distinct methods was not a predictor of obesity susceptibility in outbred rats. In agreement, predicted OP and OR outbred rats (based on pre-dietary 24-hr urinary norepinephrine output (111)) did not differ in the lipolytic effect to β-adrenergic agonists *in vitro* (Dr. Susan Fried, Rutgers University, personal communication). Although the above results may contrast, one consistent finding was that rats had similar body weights or body compositions at the time of the lipolytic assessment, regardless of future grouping (i.e., OP or OR).

Recently, Levin and colleagues developed inbred strains of Sprague-Dawley rats that were bred for obesity-prone or obesity-resistant traits (107). Moreover, Levin et al. (107) reported that weight-matched, inbred OP rats were fatter than OR rats, indicating

that OP rats were already obese. OP rats were reported to have increased feed efficiency and insulin resistance in response to a high-energy diet and might be related to the increased adiposity found in OP rats (107). The exact reasons for these physiological differences have not been completely elucidated. Catecholamine-induced lipolysis is blunted in obese humans compared to lean ones *in vivo* and in isolated subcutaneous fat cells (3). On the other hand, visceral adipocytes from obese individuals are reported to have increased catecholamine sensitivity (3). Differences in lipolytic responses have not, however, been addressed in isolated adipocytes with this inbred rat model. Additionally, a major advantage with these rats is that group assignments (i.e., OP and OR) are known and physiologic comparisons can be made without dietary intervention.

Furthermore, earlier studies compared responses from a single fat pad or lipolytic agent (23, 97, experiment 1). Tavernier et al. (151) noted that rat adipocytes from different fat pads had variable responses to lipolytic agonists. They also found that visceral adipocytes (e.g., epididymal and retroperitoneal) had increased numbers of β_1/β_2 adrenoceptors, β_3 adrenoceptor mRNA expression, and hormone sensitive lipase activity and expression when compared to subcutaneous adipocytes (151). Isolated adipocytes approximate the *in vivo* condition because cells have equal exposure to hormone or drug, whereas cells within excised tissues have different exposure levels to these agents due to diffusion issues. Therefore, lipolytic responses to isoproterenol, forskolin, and growth hormone (GH) were investigated in various types of isolated adipocytes from weight-matched, inbred OP and OR rats. Dexamethasone was also examined alone or with GH because it acts synergistically with GH to increase lipolysis (50).

RESULTS

Body Composition, Plasma Hormone Profiles and Food Consumption

Although 8-wk old, inbred OP and OR rats had similar body weights, OP rats have 33% more visceral adiposity than OR rats while consuming a chow diet (Table 4). This was comparable to the 52% difference in carcass adiposity reported for weight-matched, inbred OP and OR rats (2.5 mo-old) on chow (107). Body lengths did not differ between groups (data not shown). Feed efficiency, energy intake, and body weight gain were similar between OP and OR rats in the present study (Table 4), which was consistent to that reported for weight-matched OP and OR rats after 1 week on a chow diet (107). Post-absorptive, plasma insulin, glucose, and leptin concentrations were not different between groups in our study (Table 5). In contrast, Levin et al. (107) found that post-absorptive plasma insulin and glucose levels were increased in young inbred OP rats, but were not different from OR rats in the fasted state.

Cell Size and Diameter

Cell sizes of isolated adipocytes from OP and OR inbred rats are located in Figure 7. Briefly, mean cell size and diameter (56.6 ± 0.6 vs. 34.4 ± 1.9 μm, respectively) from RP fat cells were significantly increased in OP vs. OR rats. Groups did not differ in mean cell size and diameter (data not shown) of EPI fat cells. Mean cell sizes of subcutaneous (SC) fat from the inguinal region were not statistically different between OP and OR rats ($p = 0.052$), whereas cell diameter was significantly larger in OP compared to OR rats (58.0 ± 0.7 vs. 44.3 ± 3.7 μm, respectively).

Table 4. *Body composition and food intake data of 8 wk old inbred Sprague-Dawley obesity-prone (OP) and obesity-resistant (OR) rats*

	OP (n = 6)	OR (n = 6)
Body weight (g)	264.5 ± 6.6[a]	261.3 ± 8.1[a]
Epididymal fat (g)	2.82 ± 0.15[a]	2.16 ± 0.09[b]
Retroperitoneal fat (g)	2.55 ± 0.22[a]	1.89 ± 0.11[b]
Adiposity index (%)	2.03 ± 0.12[a]	1.55 ± 0.06[b]
Energy Intake (kcal)	1085.7 ± 24.1[a]	1076.6 ± 30.0[a]
Feed efficiency (g/kcal)	0.094 ± 0.002[a]	0.098 ± 0.003[a]

Data are presented as mean ± SE.
Adiposity index = visceral fat (g)/ [carcass wt – visceral fat] (g) * 100% (42)
Feed efficiency = body weight gained (g)/ energy intake (kcal)
Different letters within row denote significant differences ($p < 0.05$ or less)

Table 5. *Plasma profiles of inbred obesity-prone (OP) and obesity-resistant (OR) rats at 8 weeks of age*

	OP (n = 6)	OR (n = 6)
Plasma glucose (mg/dl)	156.8 ± 5.9[a]	145.7 ± 5.0[a]
Plasma insulin (ng/ml)	1.1 ± 0.2[a]	1.0 ± 0.1[a]
Plasma leptin (ng/ml)	2.5 ± 0.3[a]	2.8 ± 0.2[a]

Data are presented as mean ± SE.
Different letters within row denote significant differences ($p < 0.05$ or less)

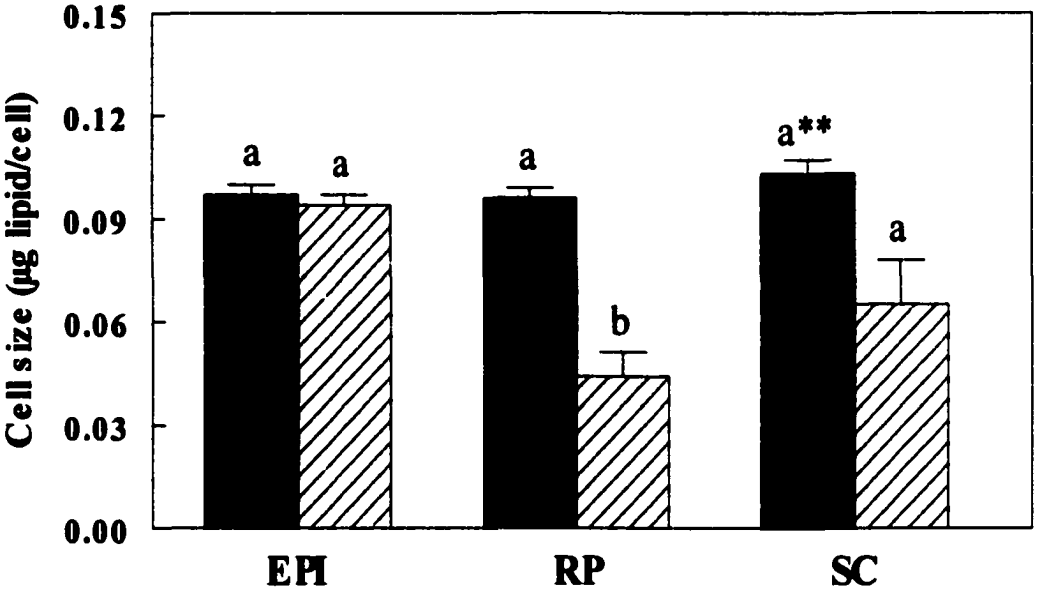

Fig. 7. Mean cell size (µg lipid/ cell) of epididymal (EPI), retroperitoneal (RP), and subcutaneous (SC) adipocytes of 8 wk old inbred obesity-prone (OP; solid bars) and obesity-resistant (OR; striped bars) rats. Data are presented as mean ± SE. Different letters within specific cell type indicate significant differences ($p < 0.05$). **$p = 0.052$ for SC.

Glycerol Release in vitro

Since there were cell size differences and cell size influences lipolytic responses (10, 67, 104, 121, 131, 158, 168), glycerol release data were normalized to cell surface area to account for these differences (167). Cell surface area adjustments were performed on all cell types for comparisons among cell types. There were group and site-specific differences in hormone-stimulated lipolysis in response to various lipolytic agents in pooled RP, EPI and SC adipocytes from OP and OR rats. The following sections are arranged according to cell type and agonists.

Retroperitoneal Adipocytes and Isoproterenol & Forskolin

After adjusting for cell size differences, basal glycerol release was still significantly increased in RP adipocytes of OP rats vs. those of OR rats (Figure 8). Compounds that stimulate lipolysis via activation of the protein kinase A pathway were evaluated in RP adipocytes from OP and OR rats. Isoproterenol stimulated glycerol release in RP fat cells of OP and OR rats, but all doses produced maximal or near maximal glycerol output. Absolute glycerol release was significantly reduced in RP fat cells of OP compared with OR rats (Figures 8). Group differences in glycerol release were also found with forskolin in RP adipocytes (Figures 8). To evaluate responsiveness to lipolytic agonists and to control for differences in basal responses, data were expressed as fold increase over basal. Pooled RP cells of OP rats were significantly less responsive to isoproterenol and forskolin than those of OR rats (Figures 9).

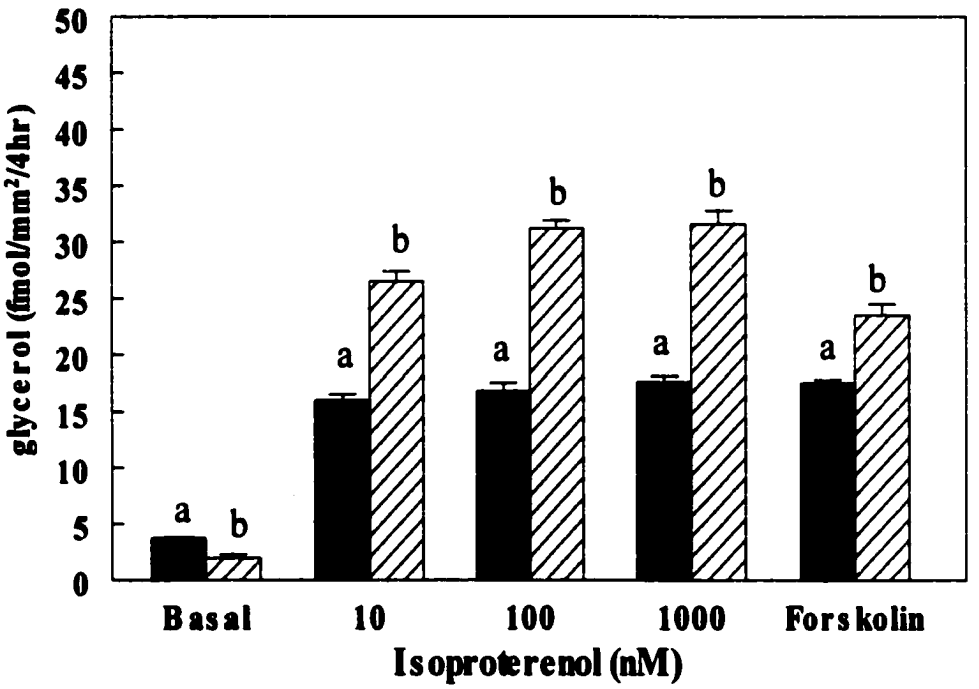

Fig. 8. Absolute rates of glycerol release in pooled retroperitoneal adipocytes of 6 inbred obesity-prone (solid bars) rats and 6 inbred obesity resistant (striped bars) rats in response to isoproterenol and forskolin (10 μM). All stimulated values are significantly greater than basal unless indicated in the text. Data are expressed as mean ± SE.
Different letters at specific concentration indicate significant differences ($p < 0.05$).

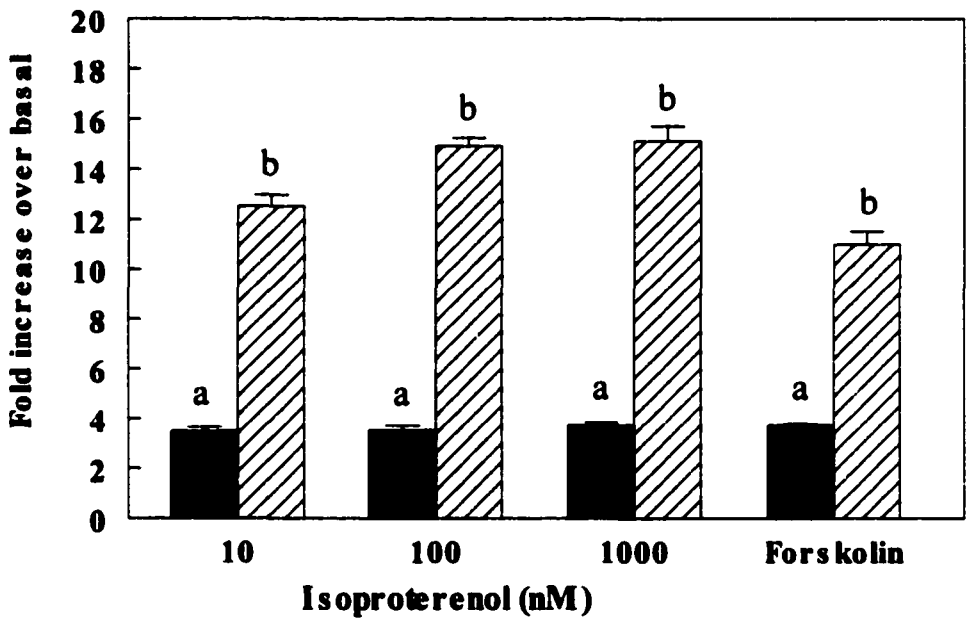

Fig. 9. Fold increase over basal glycerol release in response to isoproterenol and forskolin (10 μM) in pooled retroperitoneal adipocytes of 6 inbred obesity-prone (solid bars) rats and 6 inbred obesity resistant (striped bars) rats. Data are expressed as mean ± SE. Different letters at specific concentration indicate significant differences ($p < 0.05$).

Epididymal Adipocytes and Isoproterenol & Forskolin

Basal glycerol release was significantly reduced in EPI adipocytes of OP rats vs. those of OR rats (Figure 10). Isoproterenol stimulated glycerol release in EPI fat cells of OP and OR rats, but all doses produced maximal or near maximal glycerol output. Glycerol release, however, was significantly reduced in EPI fat cells of OP compared with OR rats (Figures 10). Group differences in glycerol release were also found with forskolin in EPI adipocytes (Figures 10). To evaluate responsiveness to lipolytic agonists and to control for differences in basal responses, data were expressed as fold increase over basal. Pooled EPI cells of OP rats were significantly less responsive to isoproterenol and forskolin than those of OR rats (Figures 11).

Subcutaneous Adipocytes and Isoproterenol & Forskolin

Basal glycerol release was similar between SC adipocytes from OP rats and OR rats (Figure 12). Compounds that stimulate lipolysis via activation of the protein kinase A pathway were evaluated in SC adipocytes from OP and OR rats. Isoproterenol stimulated glycerol release in SC fat cells of OP and OR rats, but all doses produced maximal or near maximal glycerol output. Absolute glycerol release was not significantly different between SC fat cells of OP and OR rats, except at 100 nM isoproterenol (Figures 12). Group differences in glycerol release were also found with forskolin in SC adipocytes (Figures 12). To evaluate responsiveness to lipolytic agonists and to control for differences in basal responses, data were expressed as fold increase over basal. Pooled SC cells of OP and OR rats responded similarly to isoproterenol and forskolin, except at the lowest concentration of isoproterenol (Figures 13).

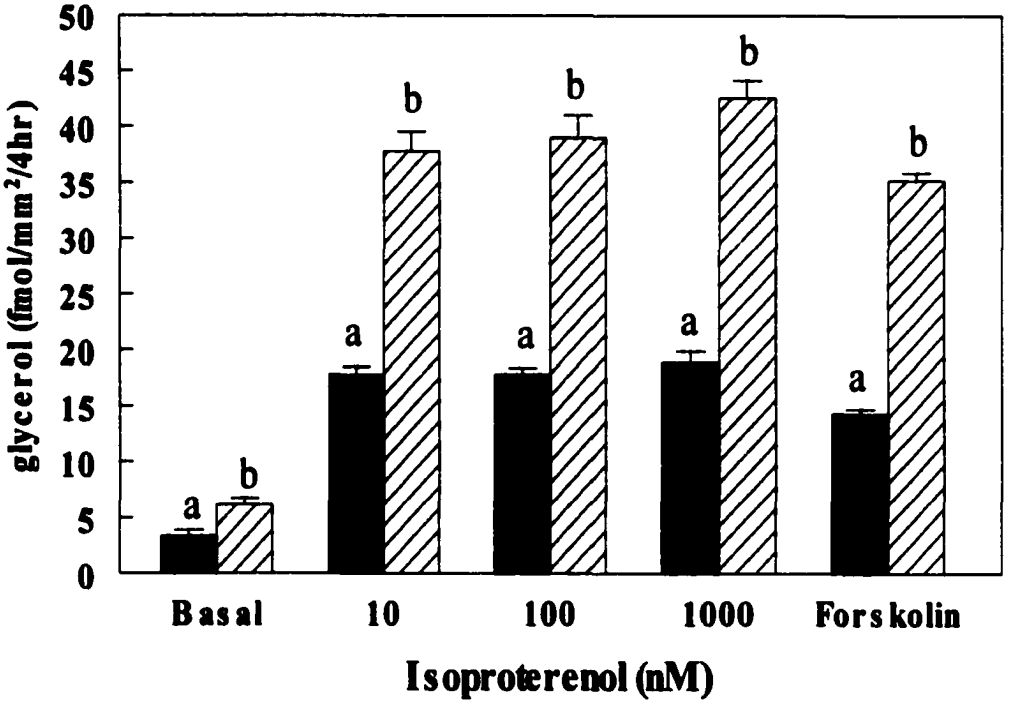

Fig. 10. Absolute rates of glycerol release in pooled epididymal adipocytes of 6 inbred obesity-prone (solid bars) rats and 6 inbred obesity resistant (striped bars) rats in response to isoproterenol and forskolin (10 μM). All stimulated values are significantly greater than basal unless indicated in the text. Data are expressed as mean ± SE. Different letters at specific concentration indicate significant differences ($p < 0.05$).

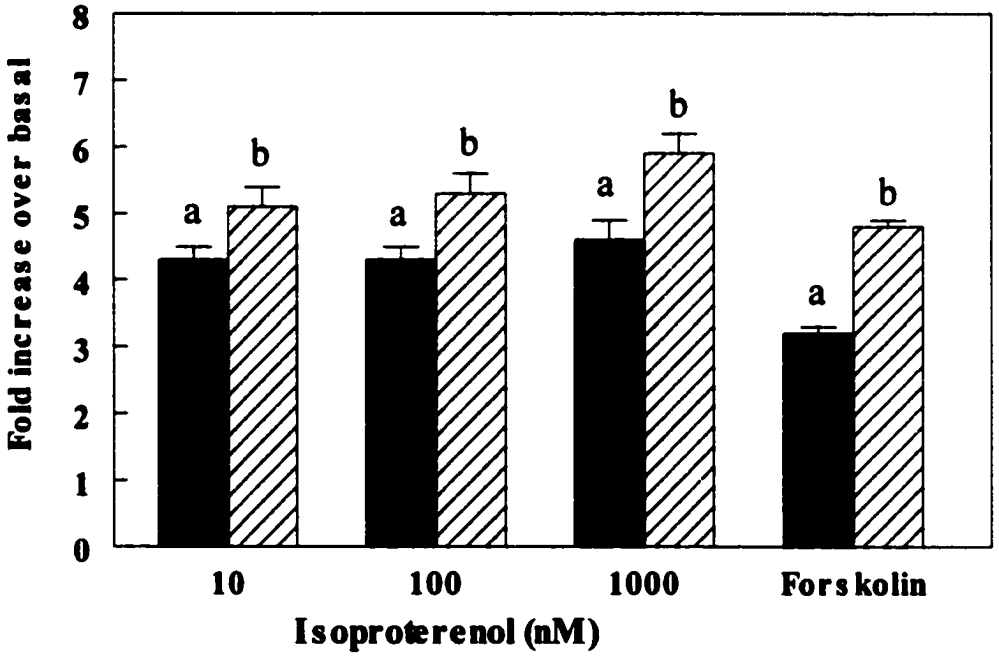

Fig. 11. Fold increase over basal glycerol release in response to isoproterenol and forskolin (10 μM) in pooled epididymal adipocytes of 6 inbred obesity-prone (solid bars) rats and 6 inbred obesity resistant (striped bars) rats. Data are expressed as mean ± SE. Different letters at specific concentration indicate significant differences ($p < 0.05$).

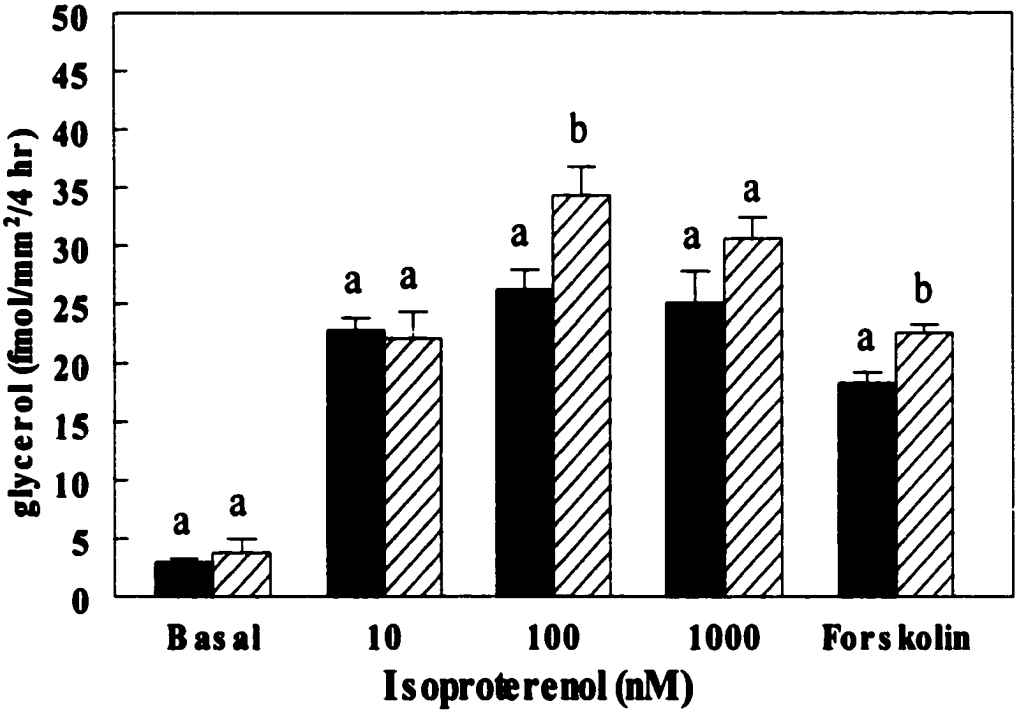

Fig. 12. Absolute rates of glycerol release in pooled subcutaneous adipocytes of 6 inbred obesity-prone (solid bars) rats and 6 inbred obesity resistant (striped bars) rats in response to isoproterenol and forskolin (10 μM). All stimulated values are significantly greater than basal unless indicated in the text. Data are expressed as mean ± SE.
Different letters at specific concentration indicate significant differences ($p < 0.05$).

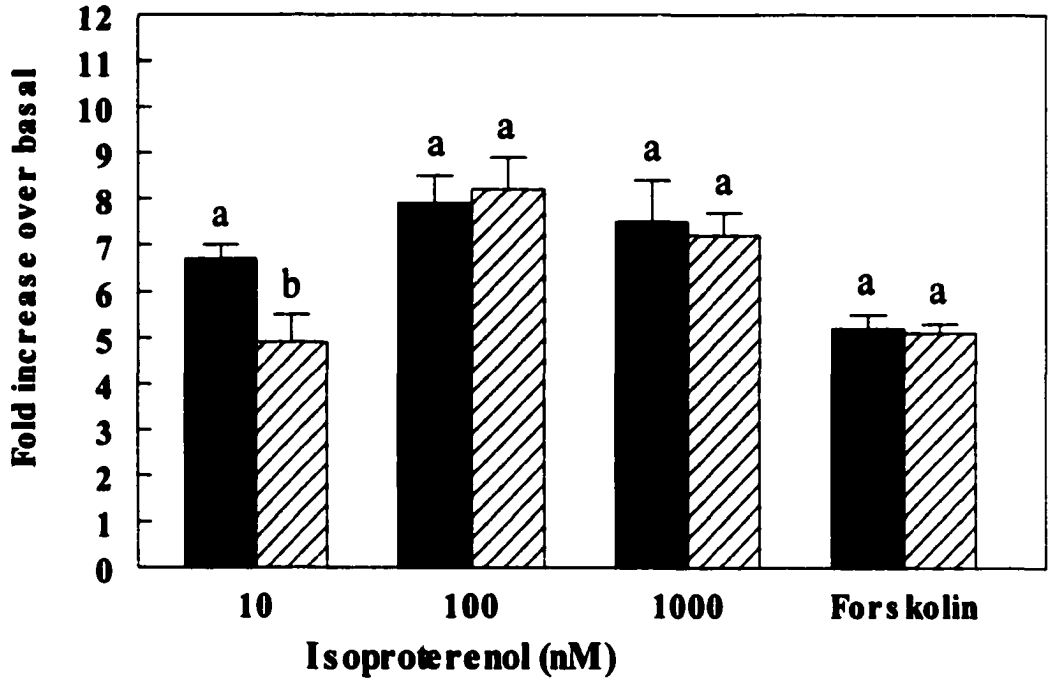

Fig. 13. Fold increase over basal glycerol release in response to isoproterenol and forskolin (10 μM) in pooled subcutaneous adipocytes of 6 inbred obesity-prone (solid bars) rats and 6 inbred obesity resistant (striped bars) rats. Data are expressed as mean ± SE. Different letters at specific concentration indicate significant differences ($p < 0.05$).

Retroperitoneal & Epididymal Adipocytes and Growth Hormone & Dexamethasone

Hormones that stimulate lipolysis via pathways distinct from isoproterenol and forskolin were evaluated in RP and EPI adipocytes from OP and OR rats. Responses were not measured in SC fat due to insufficient cell amounts. GH alone did not stimulate glycerol release above basal values in either group or fat cell (data not shown). DEX-induced glycerol release was not different in RP cells of OP and OR rats (Figure 14). Since dexamethasone (DEX) and GH have synergistic effects on lipolysis (50), the combination of these two lipolytic agents was evaluated *in vitro*. DEX and GH did not increase glycerol release beyond DEX alone in RP cells of OP rats (Figure 14). There was a significant increase in glycerol release when DEX and GH was compared with DEX alone in RP cells of OR rats. In EPI cells, there was a group difference in DEX-stimulated glycerol release (Figure 15). Similar to RP cells, DEX and GH-induced glycerol release was not different from that of DEX alone in EPI cells of OP rats (Figure 15). There was a significant increase in glycerol release when DEX and GH was compared with DEX alone in EPI cells of OR rats, but not at the lowest GH concentration (Figure 15).

When expressed as fold increase over basal, pooled RP cells of OP rats were significantly less responsive to DEX than those of OP rats (2.45 ± 0.13 vs. 6.16 ± 0.92 fold increase, respectively). Similar DEX results were noted in EPI cells of OP and OR rats (2.1 ± 0.4 vs. 3.8 ± 0.1 fold increase, respectively). Since fat cells of OP rats did not respond to the DEX and GH combination, it is not surprising that responsiveness measures were less in OP than OR rats (data not shown).

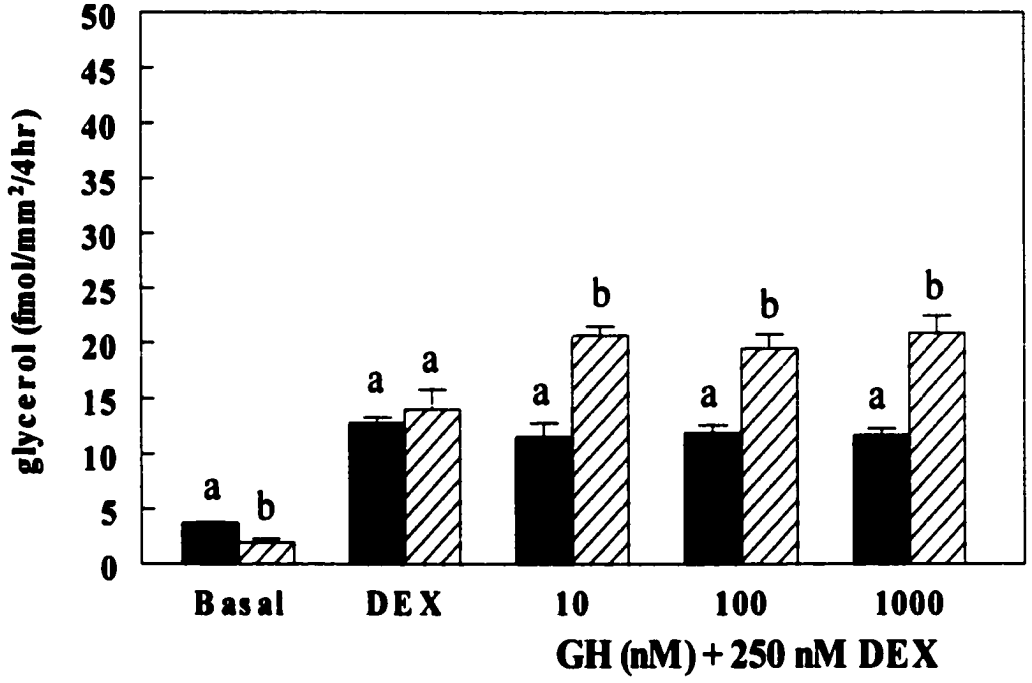

Fig. 14. Absolute rates of glycerol release in pooled retroperitoneal adipocytes of 6 inbred obesity-prone (solid bars) rats and 6 inbred obesity resistant (striped bars) rats in response to Dex alone (250 nM) and growth hormone plus dexamethasone (DEX). All stimulated values are significantly greater than basal unless indicated in the text. Data are expressed as mean ± SE. Different letters at specific concentration indicate significant differences ($p < 0.05$).

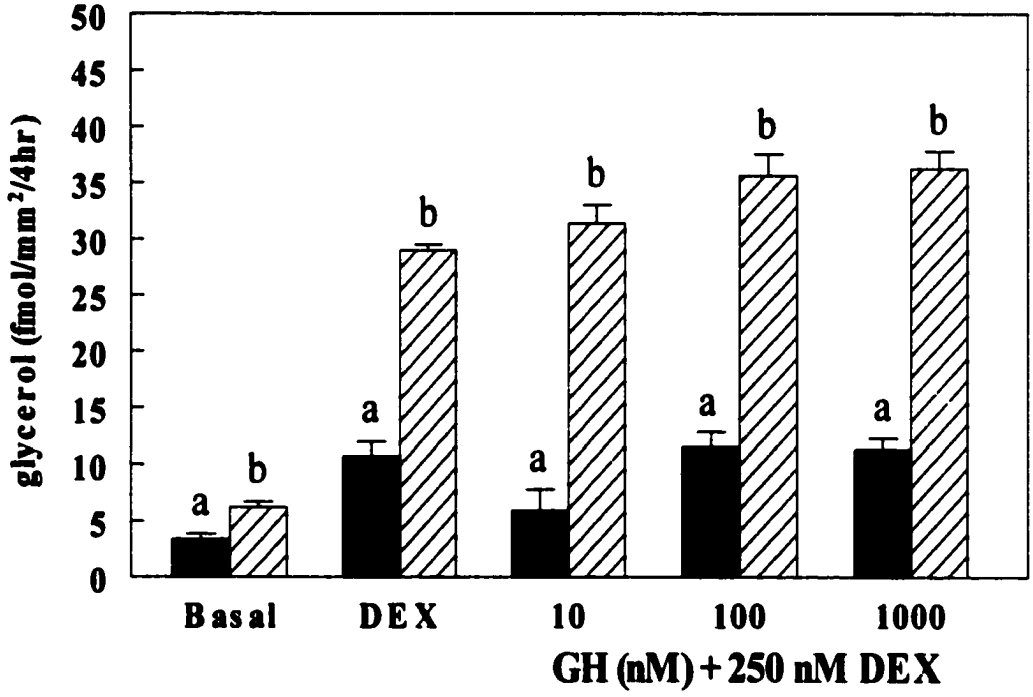

Fig. 15. Absolute rates of glycerol release in pooled epididymal adipocytes of 6 inbred obesity-prone (solid bars) rats and 6 inbred obesity resistant (striped bars) rats in response to Dex alone (250 nM) and growth hormone plus dexamethasone (DEX). All stimulated values are significantly greater than basal unless indicated in the text. Data are expressed as mean ± SE. Different letters at specific concentration indicate significant differences ($p < 0.05$).

DISCUSSION

In the present study, we attempted to ascertain whether lipolytic responses were altered in weight-matched OP and OR inbred rats. Visceral adiposity was increased in OP rats even though body weight was similar to OR rats. This is in agreement with Levin et al. (107). Elevated visceral fatness may be related to the major finding in the present study that responses to lipolytic agents, except GH alone, were reduced in isolated visceral adipocytes of OP rats. Similarly, obese humans have differential responses to catecholamines compared with lean ones *in vivo* and *in vitro* (3). Subcutaneous fatness was not measured in the present study. However, it may not be different because lipolytic responsiveness to isoproterenol and forskolin were comparable between groups. No differences in response to isoproterenol were found in abdominal and femoral subcutaneous adipocytes of obese and lean men (119). Reduced hormone-induced lipid mobilization may have contributed to the 33% increase in visceral fatness within inbred OP rats. Alternatively, increased hormone-stimulated lipolysis may have protected OR rats from increased visceral adiposity. Unfortunately, definitive conclusions cannot be drawn because differences in hormone-stimulated lipolysis and adiposity are both present in the inbred OP and OR rats. Future studies are necessary to confirm these differences and explore the underlying mechanisms for them.

Previous *in vitro* findings indicated that reduced lipolytic sensitivity in excised adipose tissues was associated with obesity susceptibility in outbred male and female rats (23, 97). Conversely, hormone-stimulated lipolysis was similar in isolated adipocytes of outbred OP and OR rats predicted to become obese or remain lean based 24-urinary norepinephrine output prior to a high energy diet (Dr. Susan Fried, Rutgers University,

personal communication). In experiment 1, *in vivo* lipolysis was also not different between OP and OR rats prior to dietary challenge. Regardless of outcome, outbred OP and OR rats do not differ in body composition prior to a high energy diet (111), which is not the case with inbred rats in the present study. Therefore, comparisons among these studies are difficult because inbred OP and OR rats have differential body composition.

In addition to the general reduction in lipolysis found in inbred OP rats, there were hormone-dependent and site-specific differences in the lipolytic responses from adipocytes of OP and OR rats. The following sections are divided into basal and hormone-stimulated responses, which was subdivided into lipolytic agents with similar mechanisms for activating lipolysis.

Basal Lipolysis

Basal lipolysis is proportional to fat cell size in rats (10, 104, 121, 131, 158, 168). Basal lipolysis displayed marked variations for both fat depot and group in the present study. RP cells of OP rats had increased basal lipolysis when compared to OR rats even after accounting for cell size differences, suggesting mechanisms other than cell size accounting for this discrepancy. Recently, Berger and Barnard (10) showed that high fat feeding increased both omental cell size and basal lipolysis in 2-mo old female Fischer rats. They also found that basal hormone-sensitive lipase (HSL) activity was increased in larger fat cells (10). Another mechanism for increased basal lipolysis in larger fat cells is related to reduced intracellular phosphatidlycholine concentrations, which protects lipid droplets from HSL (154). These factors may account for the increased basal glycerol release from RP cells of OP rats in the present study.

Conversely, EPI cell size was not different between OP and OR rats, but basal glycerol release was diminished for OP rats, which agreed with reported findings (67). Subcutaneous cell size tended to be increased in OP rats compared with OR rats, but basal lipolysis was equal between groups. In another study, Obst et al. (124) compared cell sizes from various fat depots between young Osborne-Mendel and S 5B/P1 rats, which are dietary obesity susceptible and resistant, respectively. Their results for RP, EPI, and SC cell size differences between groups prior to dietary challenges paralleled those found in the present study. These observations suggested cell size may influence basal lipolysis differently across fat depots, but it was not the only factor influencing basal adipocyte metabolism. Since perilipins are shown to protect lipid droplets from unstimulated HSL (i.e., basal activity) (28, 115), differences in perilipin concentrations among cell types and groups could contribute to these results.

In retroperitoneal fat, the combination of increased basal lipolytic activity and fat pad mass may contribute to the altered metabolic profiles found in inbred and outbred OP rats. At basal conditions, elevated free fatty acids (FFA) via lipolysis in RP fat are drained by the hepatic portal vein into the liver (2). Excess FFAs serve as substrate for hepatic triglyceride synthesis, thus possibly promoting dyslipidemia noted in OP rats (40). FFA may also disrupt hepatic extraction of insulin, thereby contributing to hyperinsulinemia (100, 107, 109) and hyperglycemia (107) noted in inbred and outbred OP rats. Increased free fatty acid turnover may also decrease glucose utilization by the Randle cycle and increase endogenous glucose production via elevated gluconeogenic substrates (e.g., excess glycerol release via lipolysis) (132). In weight-matched OP and OR inbred rats, Levin et al. (107) found that post-absorptive plasma insulin and glucose

levels were increased in young inbred OP rats. We, however, did not find group differences in these variables. OP rats in the Levin study were slightly older and had 47% more RP fat mass than the ones utilized in the present study. Since increased fat mass is associated with insulin resistance, differences in fat mass may account for differential findings between studies. Moreover, two weeks on a high energy diet (similar to the MHF diet) resulted in impaired glucose tolerance, as measured by an oral glucose tolerance test, for OP rats vs. OR rats (107). Hyperinsulinemia also influences vasoconstriction and blood pressure (141). Outbred OP rats have increased systolic blood pressure compared with OR rats after 10 wk on a MHF diet (40). These data demonstrate the complex interaction between adipocyte metabolism and variables associated with the metabolic syndrome.

Isoproterenol and Forskolin-stimulated Lipolysis

Obesity and increased adiposity are associated with blunted *in vivo* catecholamine-induced lipolysis in humans (3). In the present study, isoproterenol-stimulated glycerol release expressed relative to cell surface area was reduced in visceral adipocytes of OP rats. Comparable results were found with forskolin, a direct activator of adenylyl cyclase, suggesting that group differences were related to post-receptor mechanisms. Moreover, isoproterenol and forskolin-stimulated lipolytic responsiveness (expressed as fold increase over basal) were reduced in RP and EPI cells of OP rats, but the reduction was not as great in EPI cells. This may be related to the lack of EPI cell size differences between groups. In agreement, Portillo et al (131) showed that high fat feeding increased cell diameter of pooled visceral fat by only 7.7% and reduced lipolytic responsiveness to β_1 and β_3 adrenoceptor agonists modestly but significantly. Portillo et

al. (131) and our data are in contrast to the findings of DiGirolamo et al. (38) and Berger et al. (10) that showed either omental or EPI adipocytes of comparable size have comparable basal and hormone-stimulated lipolytic capacity regardless of nutritional state (e.g., obese vs. lean or high-fat vs. low-fat fed).

In the present study, inbred OP rats were already obese when compared with OR rats of the same age and body weight. Therefore, it is interesting to compare our lipolytic response data with results from other genetically obese animal models, which display increased adiposity for a given size (169). Genetically obese Zucker rats and leptin-deficient ob/ob mice have reduced responsiveness to catecholamines when compared to their lean littermates (77, 85, 104, 158, 168). Mild hypothyroidism in obese Zucker rats has been implicated as a mechanism for reduced responsiveness to isoproterenol in EPI cells (104). Hypothyroidism is associated with reduced intracellular cAMP content and adenylyl cyclase activity in fat cells (104). Hollenga et al. (77) also observed that maximum stimulation of adenylyl cyclase was similar in EPI cells of obese and lean rats, but the relationship between cAMP production and lipolytic response was shifted to the right for obese Zucker rats. This indicated that more cAMP was necessary to achieve a given response in adipocytes of obese rats. Similar results were observed between adipocyte responsiveness of older and younger rats (76), a model of large and small adipocytes. Although β-adrenergic receptor amounts were increased in older rat adipocytes, the ability to produce cAMP in response to β-adrenergic agonists was severely diminished, thus lowering glycerol production (76). Since cAMP is the direct activator of protein kinase A, which phosphorylates and activates HSL, it is possible that reduced cAMP production may explain differences found in the present study. This

would agree with the speculated post-receptor defect in the present study because isoproterenol and forskolin responses are comparable.

The hormone-stimulated results in SC adipocytes from OP and OR rats were not quite as clear as the ones from visceral adipocytes. OP SC cells had reduced glycerol release when exposed to forskolin and 100 nM isoproterenol. Conversely, responsiveness of SC cells did not differ between groups when expressed as fold increase over basal, except that OP was more responsive than OR at 10 nM isoproterenol. The exact reason for this last finding was not clear. Future studies should clarify this result. In agreement, Portillo et al. (131) found that responsiveness to various lipolytic agents was similar in SC adipocytes of rats fed high and low fat diets for one week, even though cell size was increased in high fat-fed rats. Altogether, it appeared that isolated adipocytes from visceral fat but not subcutaneous fat of OP and OR inbred rats differed in their responses to lipolytic agents, which activated adenylyl cyclase via different mechanisms.

Growth Hormone and Dexamethasone-stimulated Lipolysis

Growth hormone (GH) is a metabolic hormone reported to reduce body fat by increasing lipolysis and decreasing lipogenesis *in vivo* (33). GH-deficient individuals have increased visceral adiposity compared to normal ones, but this difference was reversed with GH treatment (9). Dexamethasone (DEX) is a synthetic glucocorticoid, which has permissive effects on glucose and lipid metabolism (14). Adrenalectomized rats have diminished lipolytic responses in pooled visceral adipocytes, but dexamethasone treatment reversed these alterations by increasing components of the β-adrenergic receptor system (35). Moreover, Fain and colleagues (49, 50, 51) showed that

DEX potentiated the lipolytic response to GH in parametrial adipocytes and adipose tissue via increased RNA and protein synthesis. In the present investigation, adipocytes were incubated in the presence of GH alone, DEX alone or together. Similar to reported findings with isolated adipocytes or adipose tissues of rats and humans (44, 49, 117), GH alone failed to induce lipolysis above basal levels after 4 hr regardless of cell type (EPI and RP) or group (OP and OR). A possible explanation for these findings was that adipocytes needed to be incubated with GH prior to metabolic experiments. Pre-incubation with GH increased responsiveness to catecholamines in human fat cells (117). Similarly, priming rat fat cells and tissues with GH enhanced the lipolytic action of GH (44). Additionally, GH acted by increasing GH and β-adrenergic receptors and hormone sensitive lipase gene expression in adipose tissue (33, 36, 157, 162).

When DEX alone was incubated with EPI and RP cells, there were significant increases above basal lipolysis noted for both groups. There were group differences in this response for EPI cells only. In previous studies, DEX alone increased lipolysis in excised adipose tissue (51), 3T3-F442A adipocytes (36), and EPI adipocytes (144) during short (4 hr) or long (24 hr)-term incubations. DEX also stimulated hormone-sensitive lipase expression in fat cells after 24 hr (144). These effects may be mediated by glucocorticoid receptors located in rat adipocytes (127). The differential responses to DEX in EPI cells of OR and OP rats may be due to augmented levels of glucocorticoid receptors, but this needs further examination. The direct effects of DEX on activation of the lipolytic cascade are not clear, but Fain et al. (51) attributed the increased fatty acid release to the inhibitory effects of DEX on glucose metabolism, thus reducing re-esterification. However, glucose was not present in the media in our study. Therefore,

the exact intracellular mechanisms for the elevated lipolytic response to DEX were unclear.

Adrenal glucocorticoids play a role in the development of hypothalamic obesity, gold thioglucose-induced obesity, and dietary obesity (19). Adrenal glucocorticoids were shown to be necessary for the development of diet-induced obesity in rodents and obesity in humans. Adrenalectomy prevented rats from becoming obese in response to a high fat diet and DEX replacement reversed this effect (116). Moreover, excess cortisol production was found in patients with Cushing's Disease, who tended to have increased truncal fatness (135). Diminished lipolytic and increased LPL activity were the mechanisms for increased truncal fat in these patients (14). Plasma corticosterone concentrations were not measured in the present study. In another model of diet-induced obesity, corticosterone levels were similar between OP and OR rats after 1, 2, and 5 weeks on a high fat diet (62). This indicates that corticosteroids do not play a primary role in the early stages of diet-induced obesity, but are necessary for its development, indicating the permissive nature of this hormone.

After accounting for DEX-stimulated glycerol release, the combination of GH and DEX increased glycerol release from EPI and RP cells of OR rats only, except at the lowest GH concentration in EPI cells. The combination had no effect on lipolysis in the adipocytes of OP rats. GH and DEX together were reported to interfere with mechanisms associated with the inhibitory G-protein (G_i), which inhibits adenylyl cyclase activity and promotes anti-lipolysis. GH and DEX disrupted translocation of the α_2-subunit of the G_i in adipocytes, thus diminishing the anti-lipolytic effects of G_i (165). Alterations in this response may be related to the present findings with GH and DEX. The differential

responses to GH and DEX may also be related to the GH status of the rats. Hypophysectomy reduced lipolytic responses in isolated adipocytes, but GH replacement normalized the lipolytic actions of catecholamines by increasing β-adrenoceptor number (162). Furthermore, GH ablation decreased GH receptor expression in rat adipose tissue, which was restored with GH treatment (157). Recently, Lauterio et al. (98, 101) reported that the GH status of outbred OP rats was compromised before, as well as, after exposure to a MHF diet. Outbred OP rats also have reduced GH storage and sensitivity to GH releasing hormone in cultured somatotrophs after 14 weeks on MHF diet (101). GH influences both somatic and metabolic processes (33, 65). Body length did not differ between groups, suggesting that possible GH differences did not disrupt linear growth. Although GH status was not measured in inbred SD rats, it was possible that reduced GH status of inbred OP rats influenced certain metabolic processes that affected lipolytic responses in visceral adipocytes and accounted for increased adiposity.

Unfortunately, comparisons cannot be made between this experiment and earlier research because these inbred OP and OR rats were already physically different at 8 weeks of age. The differences in adiposity and lipolytic responsiveness occurred without placing rats on a high fat diet, suggesting that they may be the result of the inbreeding process. Moreover, lipolytic responses to isoproterenol, forskolin, and growth hormone plus dexamethasone were reduced in visceral adipocytes of OP rats. Adipocytes were not responsive to GH alone, regardless of group or cell type. The finding of similar responses in subcutaneous adipocytes revealed that there were depot-specific differences within these rats. It should be pointed out these findings were for *in vitro* conditions. Although OP rats have reduced lipolytic responsiveness in isolated adipocytes, OP rats

also have more adiposity, thus more fat cells to possibly compensate for reduced cellular responses *in vivo*. Extrapolation to the *in vivo* environment should be done with caution. In conclusion, the lipolytic effect of drugs acting on β-adrenergic receptors and at post-receptor levels was impaired or not present in isolated visceral adipocytes of inbred obese rats.

CHAPTER V

ASSESSMENT OF INSULIN-STIMULATED GLUCOSE UPTAKE IN ISOLATED ADIPOCYTES FROM AN EARLY PHASE OF WEIGHT GAIN IN OP AND OR RATS

INTRODUCTION

Researchers have reported that adipose tissue of rats with ventromedial hypothalamic lesions had normal to increased insulin sensitivity prior to the onset of obesity or during the early stages of obesity, whereas insulin sensitivity of skeletal muscle was normal during these time periods *in vivo* (128, 129). Similar results have been reported for young Zucker rats (69). Eberhart et al. (42) reported that epididymal adipocytes of obesity-prone mice (AKR/J) were more sensitive to insulin-mediated glucose uptake than cells of obesity-resistant mice (SWR/J) after one week of a high fat diet. Moreover, Levin and Dunn-Meynell (106) reported that outbred DIO rats were hyperinsulinemic but euglycemic compared to DR rats after only two weeks on a high-energy diet. Experimental chronic hyperinsulinemia with euglycemia was shown to promote increased glucose uptake via up-regulation of GLUT4 and glucokinase protein and mRNA expression (6). In summary, early stages of obesity development are associated with reduced sensitivity to insulin-stimulated actions in skeletal muscle, whereas sensitivity in adipose tissue may be normal to increased. This time course in the development of differential insulin sensitivity and resistance and potentially favors the shunting of glucose and fatty acids into the insulin-responsive adipose tissue.

As found in Experiment 1, feed efficiency was increased in OP rats compared to OR rats throughout a 14 week dietary challenge (100). Conversely, relative growth and relative food consumption were increased in OP rats compared to OR rats only during the first two weeks of the dietary challenge (100). These data suggest that this early divergent growth period (i.e., dynamic phase) was important in terms of obesity development. As previously reported, rats that demonstrated the greatest body weight gains after only one week on a MHF diet were found to be obese by the end of the study (Boozer and Lauterio, submitted). Increased insulin sensitivity in adipocytes of OP rats may play a role in increased body weight and fat accretion during this early divergent period.

Moreover, insulin sensitivity is influenced by cell size. Numerous researchers have reported that smaller cells were more responsive to insulin compared with larger cells (37, 74, 81, 102, 113). Insulin binding was not responsible for the differences in insulin sensitivity (54, 102, 113). In our laboratory, we examined insulin-stimulated glucose uptake in epididymal adipocytes of 5-6 wk old rats vs. those of 20 wk old rats (OP or OR rats on a moderately high fat (MHF) diet for ~10 wk). We found that glucose uptake in larger adipocytes of older rats fed a moderately high fat diet was approximately 1-fold over basal with maximal insulin stimulation (3.5 nM) compared to a 4 to 7-fold increase in glucose uptake in smaller adipocytes of younger rats (Davies & Lauterio, unpublished observations). Our results demonstrated that regardless of nutritional state (i.e., obese or lean), adipocytes of older rats lose their responsiveness to insulin, which is consistent with results of Dr. Susan Fried (Rutgers University, personal communication). Therefore, insulin-stimulated glucose uptake was measured in younger OP and OR rats

after only one week on a MHF diet in the present study. Moreover, dietary effects on insulin-stimulated glucose uptake were compared between MHF-fed and low fat-fed rats.

RESULTS

Body Composition, Plasma Hormone Profiles and Food Consumption

Rats were classified as OP or OR based on body weight gain after 1 week on a MHF diet. Body composition data are located in Table 6. Initial body weights and lengths did not differ among OP, OR, and C rats. OP rats gained significantly more body weight than OR and C rats after 1 week on the MHF diet (Table 6). Relative growth was also greater for OP vs. OR and C rats. OP rats had significantly more visceral fat than OR rats, but the difference was not significant between OP and C rats (Table 6). When expressed as an adiposity index, the difference was still significant between OP and OR rats (Table 6). Similar to visceral fat, plasma leptin concentrations were greater for OP vs. OR rats ($p = 0.054$), while there was a trend for plasma leptin to be increased in OP compared to C rats. There were no differences among groups for plasma glucose, insulin, and free fatty acid (FFA) concentrations (Table 7). Plasma and lipoprotein triglycerides tended to be increased in OP rats vs. OR rats but large standard errors for OP resulted in non-significant differences (Table 7). Cholesterol did not differ between OP and OR, C not determined at week 1 (data not shown).

OP rats consumed more energy than OR rats, which in turn consumed more energy than C rats after 1 week of either MHF or LF diet (Table 8). When energy consumption was expressed relative to body weight, OP rats were still greater than OR rats, while OR rats were greater than C rats. Feed efficiency was similar between OR and C rats but both were significantly reduced when compared to OP rats.

Table 6. *Body composition data of obesity-prone (OP), obesity-resistant (OR), and control rats after 1 week on a moderately high fat (OP & OR) or low-fat diet*

Variable	OP (n = 6)	OR (n = 6)	Control (n = 6)
Body weight 0 (g)	245.2 ± 3.2^{a}	240.3 ± 4.2^{a}	236.7 ± 2.6^{a}
Body weight 1 week (g)	282.7 ± 3.6^{a}	260.8 ± 4.5^{b}	255.4 ± 2.8^{b}
Body weight gain (g)	37.5 ± 0.6^{a}	20.4 ± 0.4^{b}	18.7 ± 1.5^{b}
Relative growth (%)	14.2 ± 0.1^{a}	8.2 ± 0.1^{b}	7.6 ± 0.6^{b}
Visceral fat# (g)	4.44 ± 0.16^{a}	3.39 ± 0.27^{b}	$3.77 \pm 0.28^{ab*}$
Adiposity Index (%)	1.59 ± 0.05^{a}	1.32 ± 0.11^{b}	1.50 ± 0.11^{ab}
EPI Cell size (μg lipid/cell)	0.129 ± 0.005^{a}	0.080 ± 0.005^{c}	0.106 ± 0.002^{b}

#Visceral fat = epididymal + retroperitoneal fat depots
Adiposity index = visceral fat (g)/ [carcass wt – visceral fat] (g) * 100% (42)
Data are presented as mean ± SE.
Different letters within row denote significant differences ($p < 0.05$ or less)
*OP > C; $p = 0.064$

Table 7. *Non-fasted plasma profiles of obesity-prone (OP), obesity-resistant (OR), and control (C) rats after 1 week on a moderately high fat (OP & OR) or low-fat diet (C)*

Variable	OP (n = 6)	OR (n = 6)	C (n = 6)
Plasma leptin (ng/ml)	2.91 ± 0.52[a]	1.67 ± 0.23[a*]	2.30 ± 0.30[a]
Plasma insulin (ng/ml)	0.99 ± 0.11[a]	0.93 ± 0.16[a]	1.06 ± 0.25[a]
Plasma glucose (mg/dl)	127.3 ± 2.2[a]	117.3 ± 3.3[a]	131.2 ± 12.4[a]
Plasma FFA (mEq/L)	0.64 ± 0.07[a]	0.54 ± 0.05[a]	0.50 ± 0.05[a]
Plasma triglycerides (mg/dl)	95.8 ± 19.6[a]	65.5 ± 4.0[a]	ND
VLDL triglycerides (mg/dl)	69.6 ± 22.1[a]	33.6 ± 3.7[a]	ND

FFA = free fatty acids, VLDL = very low density lipoproteins
Data are presented as mean ± SE
Different letters within row denote significant differences ($p < 0.05$ or less)
*OP > OR; $p = 0.054$
ND = not determined

Table 8. *Energy intake data of obesity-prone (OP), obesity-resistant (OR), & control (C) rats after 1 week on a moderately high fat (OP & OR) or low-fat diet (C)*

Variable	OP (n = 6)	OR (n = 6)	C (n = 6)
Energy intake (kcal)	589.3 ± 14.7[a]	510.8 ± 12.0[b]	434.5 ± 11.0[c]
Feed efficiency (g/kcal)	0.064 ± 0.001[a]	0.040 ± 0.001[b]	0.043 ± 0.003[b]
REC* (kcal/gBW)	2.23 ± 0.04[a]	2.04 ± 0.04[b]	1.77 ± 0.05[c]

* Relative energy consumption = energy consumed (kcal)/ body weight (g)
Data are presented as mean ± SE
Different letters within row denote significant differences ($p < 0.05$ or less)

Cell Size of Epididymal Adipocytes

Mean cell sizes of pooled EPI adipocytes from OP, OR, and C rats are located in Table 6. Mean cell size was greater for OP rats than C rats, which in turn was greater than OR rats. Mean cell sizes for all three groups were larger than that of chow-fed baseline rats (0.058 ± 0.003 μg lipid/cell).

Insulin-stimulated 2-deoxyglucose Uptake

Insulin-stimulated (^{14}C)-2-deoxyglucose (2DG) uptake was measured in pooled EPI cells of OP, OR, and C rats after 1 week of a dietary challenge. 2DG and glucose are transported into adipocytes by similar mechanisms and thus 2DG serves as a surrogate for glucose uptake (159). When compared with values from baseline chow-fed rats, all three groups had significant reduction in responsiveness to insulin. There was approximately a 9.5 ± 1.2 (mean ± SE) fold-increase in 2DG uptake in response to maximal dose of insulin (3.5 nM) in EPI cells of baseline rats not exposed to high fat diets (data not shown). This was in stark contrast to that found with EPI cells of OP (0.86 ± 0.04 fold increase), OR (0.66 ± 0.11 fold increase), and C (3.5 ± 0.4 fold increase) at the maximal insulin concentration. Clearly, short-term exposure to moderately high or low fat diets (MHF = 32% fat and LF = 11% fat) reduced insulin sensitivity, as measured by 2-deoxyglucose uptake, in EPI cells compared to a chow diet (approximately 5% kcal as fat).

When comparing insulin-stimulated responses in EPI cells of rats fed different diets for one week, there were diet-induced effects on 2DG uptake. Basal 2DG uptake was significantly increased in EPI cells of OP and OR rats compared with those of C rats, but there were no differences between OP and OR (Figure 16). Due to increased basal

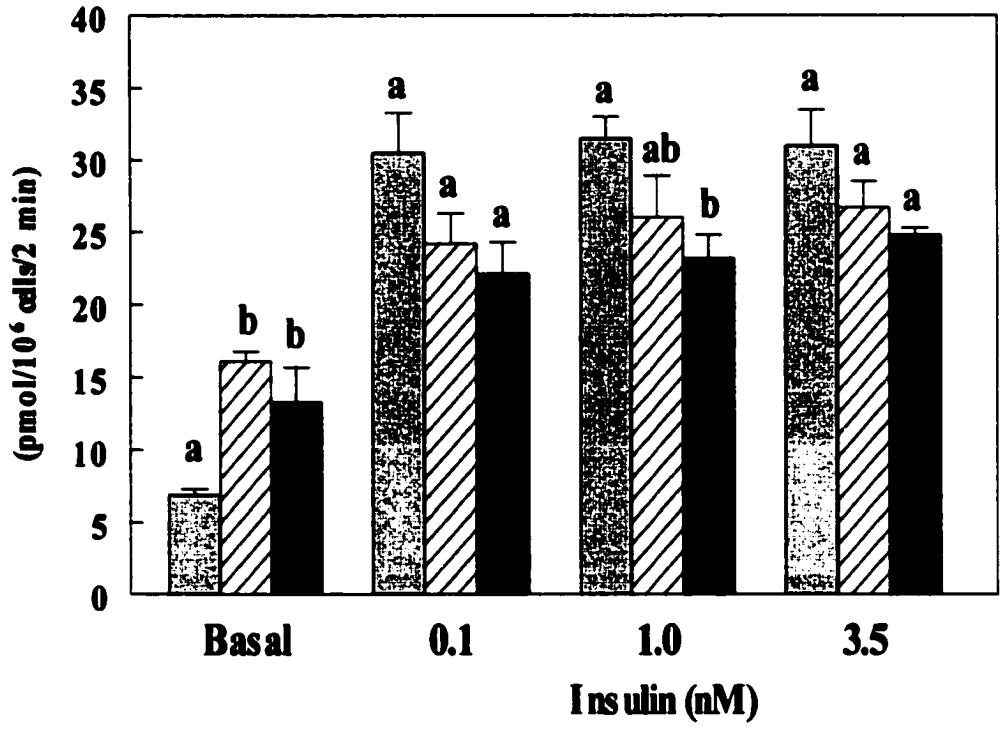

Fig. 16. Basal and insulin-stimulated rates of (^{14}C)-2-deoxyglucose uptake in epididymal adipocytes of obesity-prone (black bars), obesity-resistant (striped bars), & control (gray bars) rats after 1 week on a moderately high fat (OP & OR) or low-fat diet (C). Different letters within specific concentration denote significant differences ($p < 0.05$ or less).

levels found in EPI cells of OP and OR rats, absolute rates of 2DG uptake did not differ among groups, except for insulin at 1.0 nM (Figure 17). In contrast, relative rates (insulin-stimulated minus basal) of 2DG uptake were significantly greater in EPI cells of C rats than that found in OP and OR rats when accounting for differences in basal glucose uptake (Figure 18). Similarly, when expressed as percent increase over basal, C rats were still increased when compared to OP and OR rats (Figure 18).

DISCUSSION

It is well established that high fat feeding reduces insulin-stimulated glucose uptake in adipose tissue or adipocytes (75, 102, 112, 147, 148). However, insulin sensitivity (i.e., glucose uptake) was elevated in adipocytes young obesity-susceptible rodents (42, 69). Epididymal (EPI) fat cells of AKR/J (diet-sensitive) mice have increased insulin-stimulated glucose uptake compared with SWR/J (diet-resistant) mice, regardless of diet (high or low fat diets) (42). Obese Zucker rats also have elevated basal and insulin-stimulated glucose transport in inguinal adipocytes compared to lean Zucker rats (69). Therefore, we examined whether insulin-stimulated glucose uptake was different between rats fed a moderately high fat (OP and OR) or low fat (C) diet and also between rats classified as OP and OR after 1 week of a dietary challenge. Basal 2-deoxyglucose (2DG) uptake was increased in EPI cells of OP and OR rats when compared with cells of C rats in the present study. Previous researchers showed that basal 2DG uptake tended to be greater in adipocytes of rats fed a high fat diet than in fat cells of rats fed a high carbohydrate diet (160). Additionally, basal glucose uptake has also been reported to be greater in larger fat cells than smaller ones (114). This contrasted other findings that showed depressed basal 2DG uptake in EPI cells of rats fed

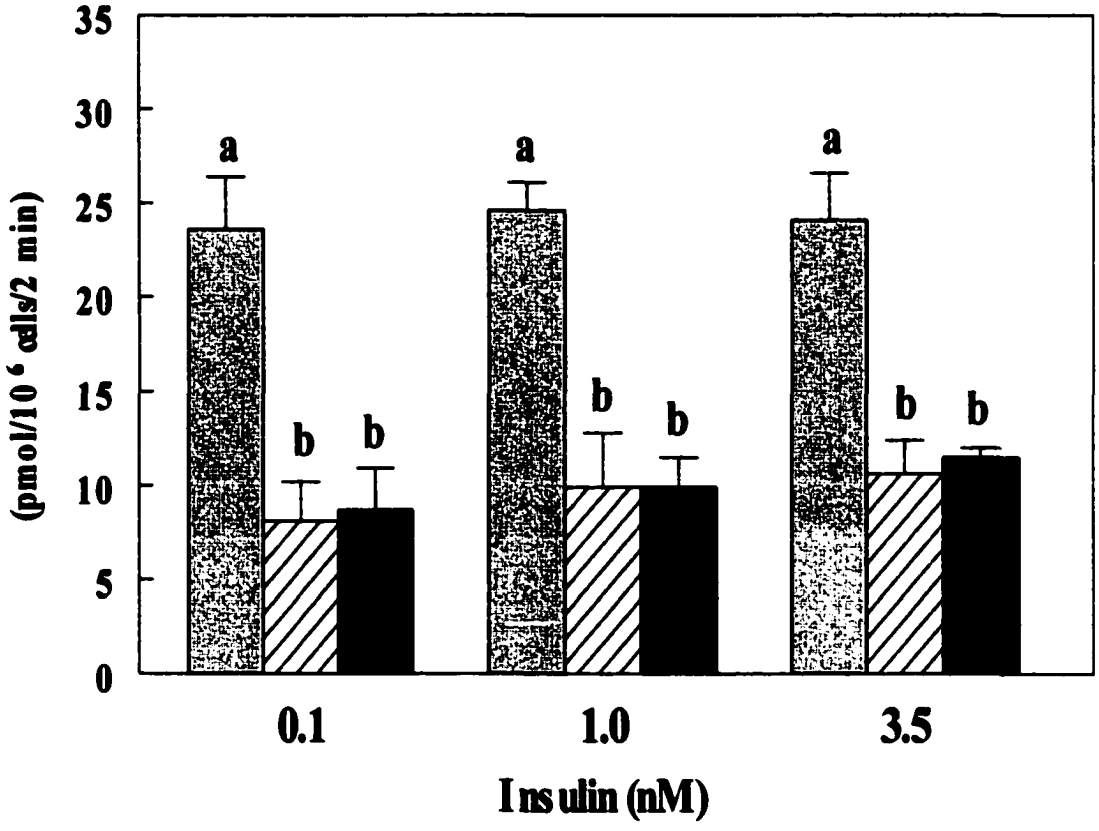

Fig. 17. Relative rates of insulin-stimulated (minus basal) (^{14}C)-2-deoxyglucose uptake in epididymal adipocytes of obesity-prone (black bars), obesity-resistant (striped bars), & control (gray bars) rats after 1 week on a moderately high fat (OP & OR) or low-fat diet (C). Different letters within specific concentration denote significant differences ($p < 0.05$ or less).

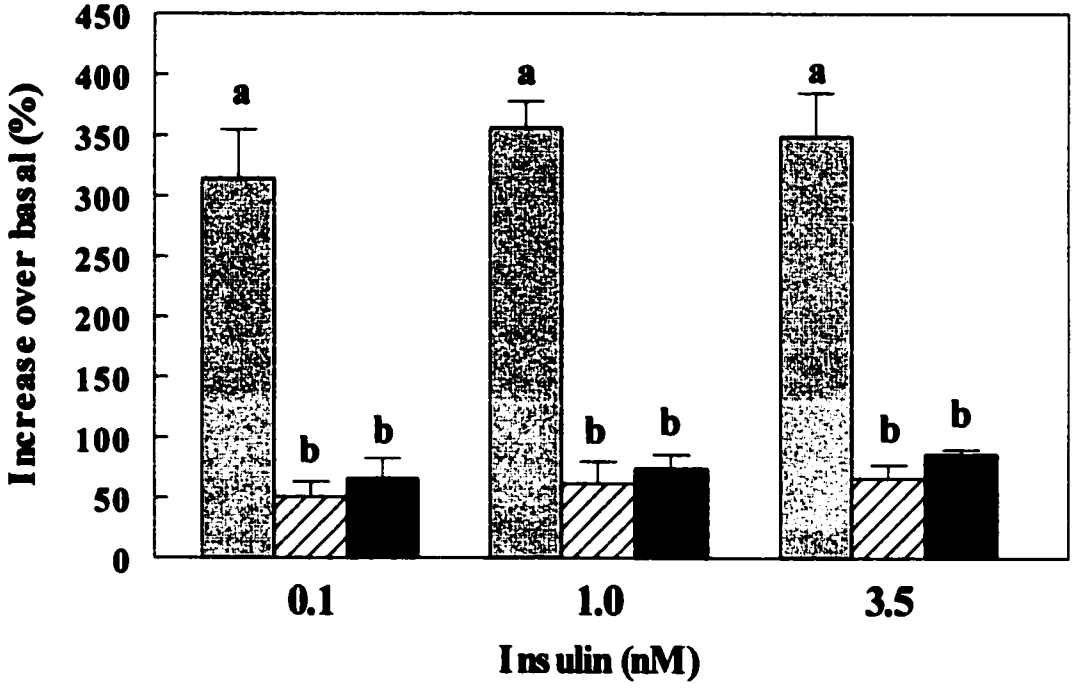

Fig. 18. Insulin-stimulated (^{14}C)-2-deoxyglucose uptake expressed as percent increase over basal in epididymal adipocytes of obesity-prone (black bars), obesity-resistant (striped bars), & control (gray bars) rats after 1 week on a moderately high fat (OP & OR) or low-fat diet (C). Different letters within specific concentration denote significant differences ($p < 0.05$ or less).

a high fat diet (102). The value for C rats was similar to the baseline value of chow fed rats in the present study (6.9 ± 0.4 vs. 6.5 ± 0.2 pmol/10^6 cells/2 min, respectively). Thus differences would not be due to methodological error. Differences were unrelated to cell size because the group order for EPI cell size was OP, C, and OR (highest to lowest) in our rats after 1 week. It should be pointed out that C rats were a combination of OP and OR rats and may have contributed to differences in cell size measurements. Moderately high fat feeding was found to increase basal glucose uptake in adipocytes in the present study, but the exact mechanism was unclear.

In agreement with other research (75, 102, 112), feeding rats a MHF diet for only one week reduced insulin sensitivity (expressed as percent increase over basal or insulin-stimulated condition minus basal) in isolated adipocytes. EPI cells of rats fed a high fat diet experienced less than a 1-fold increase in insulin-stimulated glucose uptake vs. the greater than 3-fold increase noted in C rats at all insulin concentrations. These effects appeared to be independent of cell size because OR cells were smaller than C cells and smaller cells were shown to be more insulin responsive (37, 74). Interestingly, maximal insulin stimulation was also dramatically reduced in EPI cells of C rats vs. those of chow fed rats (3.5-fold vs. 9.5 fold increase). The low fat diet had approximately 11% fat compared with 5% fat found in the chow diet, indicating even a modest increase in dietary fat content compromised insulin sensitivity. In another study, AKR/J (i.e., OP) mice were still more responsive to the stimulatory effects of insulin than SWR/J (i.e., OR) after consuming a diet similar in composition to our diet (42). The AKR/J mice, however, were also more responsive to insulin prior to the diet, suggesting these obesity-susceptible mice had a genetic predisposition to enhanced insulin responsiveness.

Reduced insulin sensitivity in adipocytes with high fat feeding is related to altered mechanisms beyond the insulin receptor. Lavau et al (102) found that insulin binding was not affected with high fat feeding. Moreover, Livingston et al. (113) showed that insulin receptor number was not depressed in adipocytes of insulin resistant individuals. It has been shown that high fat feeding affects glucose transporters 4 (GLUT4). GLUT4 are stored in large intracellular pools within adipose and muscle tissues and mediate insulin-stimulated glucose transport (8). Hissin and colleagues (74, 75) reported that obese rats and rats fed a high fat diet have relatively depleted intracellular pools of GLUT4 in isolated adipocytes. Similar effects of GLUT4 expression and recruitment were found in skeletal muscle of rats fed a high diet (68, 71, 86). Since no differences in glucose uptake were found between OP and OR EPI cells, it seemed that MHF feeding impaired insulin-stimulated glucose uptake mechanisms within adipocytes equally for both groups. These data indicated that glucose delivery and uptake were not related to the differences in epididymal fat weights between OP and OR rats. However, glucose metabolism may be altered in EPI fat cells of OP and OR rats.

CHAPTER VI

ASSESSMENT OF INSULIN-STIMULATED GLUCOSE METABOLISM IN ISOLATED ADIPOCYTES FROM EARLY PHASES OF WEIGHT GAIN IN OP AND OR RATS

INTRODUCTION

Insulin stimulates glucose metabolism within adipocytes, in addition to promoting glucose uptake. Levin et al. (106) reported that OP rats were hyperinsulinemic but euglycemic compared to OR rats after only two weeks on a high-energy diet. Experimental chronic hyperinsulinemia with euglycemia promoted excess lipid accretion via up-regulation of certain lipogenic enzymes (acetyl CoA carboxylase and fatty acid synthase) in adipose tissue (6). Differential lipogenic responses to insulin were reported in other animal models of obesity. Hypothalamic obesity was associated with increased insulin-stimulated lipogenesis *in vivo* 1 week post-lesions (128, 129). Young obese Zucker rats have increased lipogenic enzyme activity in adipose tissues with marked insulin resistance in skeletal muscles (22, 69). Therefore, adipocytes of obesity-prone (i.e., OP) Sprague-Dawley rats may have an increased capacity for insulin-stimulated lipogenesis at the onset of a hypercaloric diet, thus promoting excess lipid accumulation.

Furthermore, Fried et al. (60) found regional variations in insulin-induced glucose metabolism in rat adipocytes. They reported that epididymal (EPI) adipocytes were more responsive than retroperitoneal adipocytes to insulin, whereas effect of insulin on isolated subcutaneous adipocytes was low. These regional variations in glucose metabolism were related to differential lipogenic enzyme content (60). High fat feeding also influences

glucose metabolism in adipocytes among other cells in humans and animals (112). Lipogenic enzyme activity was reduced in adipocytes of high fat-fed rats within 1 week (102, 149). Since EPI adipocytes were the most responsive to insulin and high fat feeding rapidly reduced insulin-stimulated actions in adipocytes, we examined EPI adipocytes to determine potential differences in insulin-stimulated glucose metabolism among OP, OR, and C rats. Furthermore, fatty acid synthase (FAS) activity is upregulated in hepatocytes and adipocytes from Zucker rats, rats with hypothalamic obesity, and diet-induced obese rats (22, 118, 143, 152, 153). FAS gene expression is regulated at the transcriptional level by hormonal and nutritional controls, which suggests that enzyme activity is related to mRNA expression (16). Therefore, to help explain potential differences in lipogenic responses among groups, FAS mRNA expression was measured in total RNA from EPI adipose tissue of OP, OR and C rats.

RESULTS

Body Composition, Plasma Hormone Profiles and Food Consumption (Week 1)

Rats were classified as OP or OR based on body weight gain after 1 week on a MHF diet. Body composition data are located in Table 6 (see Chapter V). Initial body weights and lengths did not differ among OP, OR, and C rats. OP rats gained significantly more body weight than OR and C rats after 1 week on the MHF diet (Table 6). Relative growth was also greater for OP vs. OR and C rats. OP rats had significantly more visceral fat than OR rats, but the difference was not significant between OP and C rats (Table 6). When expressed as an adiposity index, the difference was still significant between OP and OR rats (Table 6). Similar to visceral fat, plasma leptin concentrations were greater for OP vs. OR rats, while there was a trend for plasma leptin to be increased

in OP compared to C rats. There were no differences among groups for plasma glucose, insulin, and free fatty acid (FFA) concentrations (Table 7). Plasma and lipoprotein triglycerides tended to be increased in OP rats vs. OR rats but large standard errors for OP resulted in non-significant differences (Table 7). Cholesterol did not differ between OP and OR, C not determined at week 1 (data not shown).

OP rats consumed more energy than OR rats, which in turn consumed more energy than C rats after one week of either a MHF or LF dietary challenge (Table 8). When energy consumption was expressed relative to body weight, OP rats were still greater than OR rats, while OR rats were greater than C rats. Feed efficiency was similar between OR and C rats but both were significantly reduced when compared to OP rats.

Body Composition, Plasma Hormone Profiles and Food Consumption (Week 3)

Rats were classified as OP or OR after based on body weight gain after 3 week on a MHF diet. Physical characteristics for OP, OR, and C rats are located in Table 9. Although initial body weights and lengths did not differ among OP, OR and C rats, OP rats had gained significantly more body weight than OR and C rats after 3 weeks, body lengths did not differ among groups (data not shown). Relative growth was also greater for OP rats vs. OR and C. OP rats had significantly more visceral fat weight than OR and C rats. When visceral fat was expressed as an adiposity index, OP rats were still greater than OR and C rats. Plasma leptin concentrations were greater for OP vs. OR and C rats. There were no differences among groups for plasma glucose, insulin, and FFA concentrations (Table 10). Plasma and very-low density lipoprotein triglycerides were significantly increased in OP rats vs. OR and C rats (Table 10).

Table 9. *Body composition data of obesity-prone (OP), obesity-resistant (OR), and control (C) rats after 3 weeks on a moderately high fat (OP & OR) or low-fat diet (C)*

Variable	OP (n = 6)	OR (n = 6)	C (n = 5)
Body weight 0 (g)	250.5 ± 3.3[a]	238.0 ± 4.3[a]	243.0 ± 2.2[a]
Body weight 3 wk (g)	323.2 ± 3.7[a]	293.8 ± 5.5[b]	302.4 ± 2.3[b]
Body weight gain (g)	72.7 ± 1.6[a]	55.9 ± 1.3[b]	59.4 ± 1.5[b]
Relative growth^ (%)	25.3 ± 0.58[a]	21.0 ± 0.22[b]	21.8 ± 1.01[b]
Visceral fat# (g)	6.39 ± 0.36[a]	4.37 ± 0.36[b]	4.81 ± 0.41[b]
EPI cell size (μg lipid/cell)	0.122±0.005[a]	0.096±0.002[c]	0.108±0.003[b]
Adiposity Index (%)	2.02 ± 0.11[a]	1.50 ± 0.10[b]	1.62 ± 0.13[b]

^Relative growth is calculated over three week dietary period.
#Visceral fat = epididymal and retroperitoneal fat depots.
Adiposity index = visceral fat (g)/ [carcass wt – visceral fat] (g) * 100% (42)
Data are presented as mean ± SE
Different letters within row denote significant differences ($p < 0.05$ or less)

Table 10. *Non-fasted plasma profiles of obesity-prone (OP), obesity-resistant (OR), and control (C) rats after 3 weeks on a moderately high fat (OP & OR) or low-fat diet (C)*

Variable	OP (n = 6)	OR (n = 6)	C (n = 5)
Plasma leptin (ng/ml)	3.82 ± 0.43^a	2.61 ± 0.22^b	2.44 ± 0.30^b
Plasma insulin (ng/ml)	1.16 ± 0.19^a	1.19 ± 0.11^a	0.80 ± 0.06^a
Plasma glucose (mg/dl)	133.8 ± 3.5^a	134.7 ± 3.3^a	130.0 ± 5.0^a
Plasma FFA (mEq/L)	0.58 ± 0.05^a	0.57 ± 0.03^a	0.55 ± 0.04^a
Plasma triglycerides (mg/dl)	234.0 ± 17.6^a	170.0 ± 17.7^b	171.0 ± 8.1^b
VLDL triglycerides (mg/dl)	172.0 ± 3.0^a	112.5 ± 6.7^b	123.0 ± 10.7^b

FFA = free fatty acids, VLDL = very low density lipoproteins
Data are presented as mean ± SE
Different letters within row denote significant differences ($p < 0.05$ or less)

After 3 weeks on either a MHF or LF diet, OP rats consumed more energy than OR and C rats (Table 11). Feed efficiency was similar between OR and C rats but both were significantly reduced when compared to OP rats. When energy consumption was expressed relative to body weight, OP rats were still greater than OR, whereas there was a trend for relative energy consumption to be greater in OP vs. C rats ($p = 0.07$).

Cell Size of Epididymal Adipocytes

Mean cell sizes of pooled EPI adipose tissue from either OP, OR, or C rats from weeks 1 and 3 are located in Table 6 and 9, respectively. Mean cell size was greater for OP rats than C rats, which in turn was greater than OR rats at week 1. Similar results were also found after week 3.

Insulin-stimulated Glucose Metabolism

Insulin-stimulated glucose metabolism was measured in pooled EPI cells of OP, OR, or C rats after 1 or 3 weeks of a dietary challenge. When comparing lipogenic responses (specifically fatty acid synthesis) between baseline and diet-challenged rats, EPI cells of baseline chow-fed rats were more responsiveness (5.0 ± 0.1-fold increase) to the maximum insulin concentration than cells of OP, OR, and C rats at 1 week (0.7 ± 0.1, 0.4 ± 0.03, and 2.7 ± 0.2, respectively). Short-term (1 week) and long-term (3 weeks) exposure to diets relatively high in fat (MHF = 32% fat and LF = 11% fat) compared with standard rat chow (approximately 5% kcal as fat) reduced the capacity of EPI cells to synthesize lipids from glucose in response to insulin (data not shown).

The remainder of this section will be subdivided into the different products of glucose metabolism measured in the experiments. Moreover, data from weeks 1 or 3 weeks were compared among groups in separate sections. Due to the age-related

Table 11. *Energy intake data of obesity-prone (OP), obesity-resistant (OR), & control (C) rats after 3 weeks on a moderately high fat (OP & OR) or low-fat diet (C)*

Variable	OP (n = 6)	OR (n = 6)	C (n = 5)
Energy intake (kcal)	1503.0 ± 30.5[a]	1330.4 ± 36.0[b]	1389.6 ± 17.0[b]
Feed efficiency (g/kcal)	0.048 ± 0.003[a]	0.042 ± 0.001[b]	0.043 ± 0.002[b]
REC* (kcal/gBW)	5.24 ± 0.06[a]	5.00 ± 0.05[b]	5.10 ± 0.04[ab]

* Relative energy consumption
Data are presented as mean ± SE
Different letters within row denote significant differences ($p < 0.05$ or less)

reductions in adipocyte responsiveness to insulin regardless of diet, week 3 experiments were performed with the basal and maximal insulin concentration conditions only. The first section will focus on the conversion of glucose into lipids (triglycerides). The second and third sections will address the two components of triglycerides; fatty acids and glyceride-glycerol. The last section will contain carbon dioxide (CO_2) production (via glucose oxidation) data from week 1 only.

Week 1 Data

(^{14}C)-Glucose Incorporation into Total Lipids (Triglycerides)

Absolute rates of basal glucose incorporation into total lipids did not differ among groups after 1 week on their respective diets (Figure 19). In the presence of insulin, moderately high fat feeding significantly reduced absolute rates of glucose incorporation into total lipids for EPI of both OP and OR rats compared to C rats (Figure 19). To examine the effects of insulin on glucose incorporation into lipids more directly, basal rates were subtracted from insulin-stimulated glucose incorporation rates (relative rates). Data were also expressed as percent increase over basal (i.e., insulin-stimulated minus basal/ basal *100%) to control for basal responses. As expected, relative rates and percent increase over basal data were still increased for C when compared with OP and OR (Figures 20 and 21). When comparing the responses of OP and OR EPI cells, relative rates of glucose conversion to lipids were increased at the submaximal doses of insulin (0.1 and 1.0 nM) for OP rats vs. OR rats (Figure 20). At the maximal concentration (3.5 nM), relative rates of glucose incorporation into lipids tended to be increased for OP vs. OR ($p = 0.073$). When expressed as percent increase over basal, EPI cells of OP rats more responsive to insulin than those of OR rats (Figure 21).

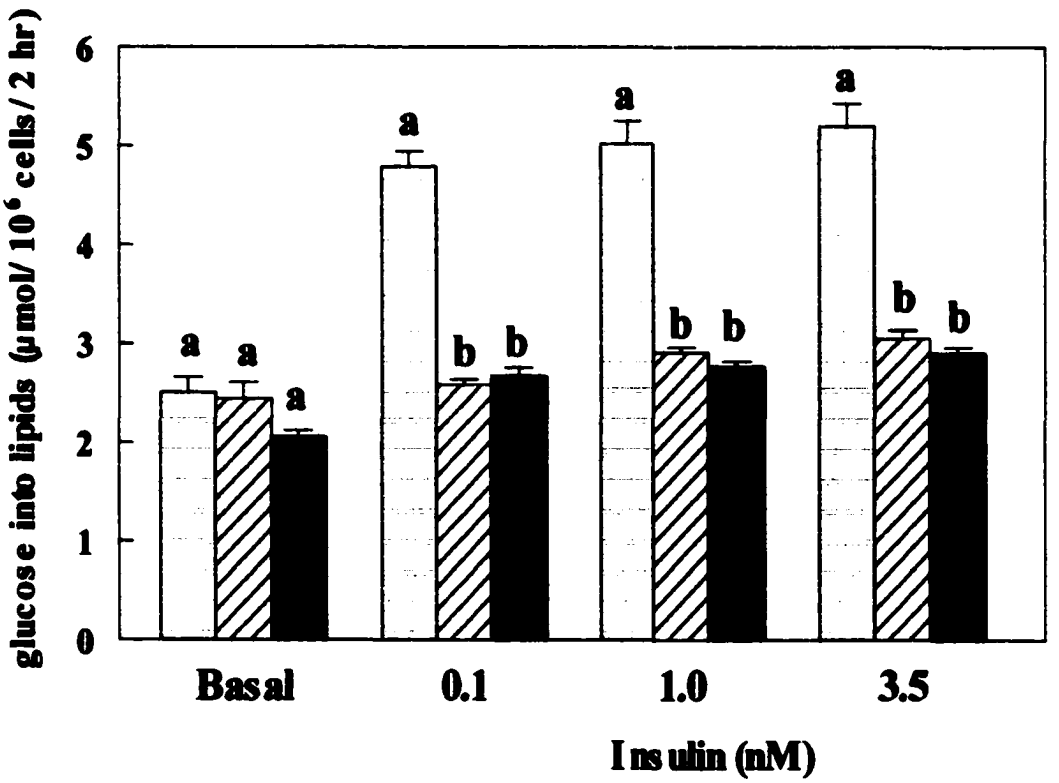

Fig. 19. Basal and insulin-stimulated rates of (^{14}C)-glucose incorporation into total lipids of epididymal adipocytes from obesity-prone (black bars), obesity-resistant (striped bars), & control (gray bars) rats after 1 week on a moderately high fat (OP & OR) or low-fat diet (C). Different letters within specific concentration denote significant differences ($p < 0.05$ or less). Data are presented as mean ± SE.

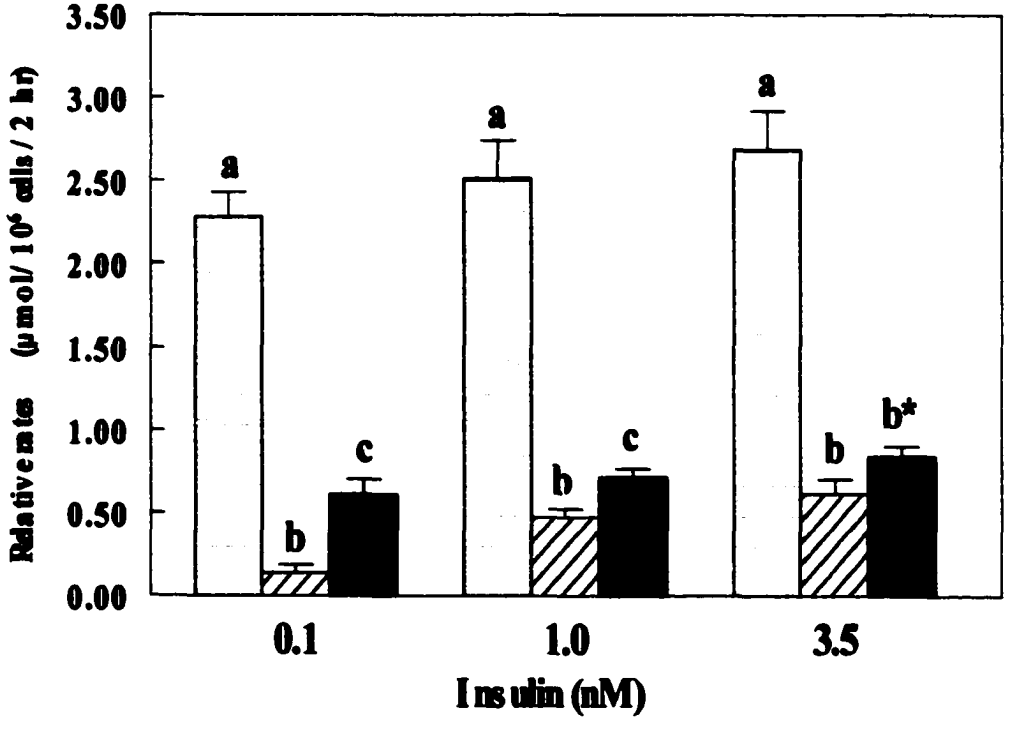

Fig. 20. Relative rates of insulin-stimulated (minus basal) (^{14}C)-glucose incorporation into total lipids of epididymal adipocytes from obesity-prone (black bars), obesity-resistant (striped bars), & control (gray bars) rats after 1 week on a moderately high fat (OP & OR) or low-fat diet (C). Different letters within specific concentration denote significant differences ($p < 0.05$ or less). * $p = 0.07$; OP > OR. Data are presented as mean ± SE.

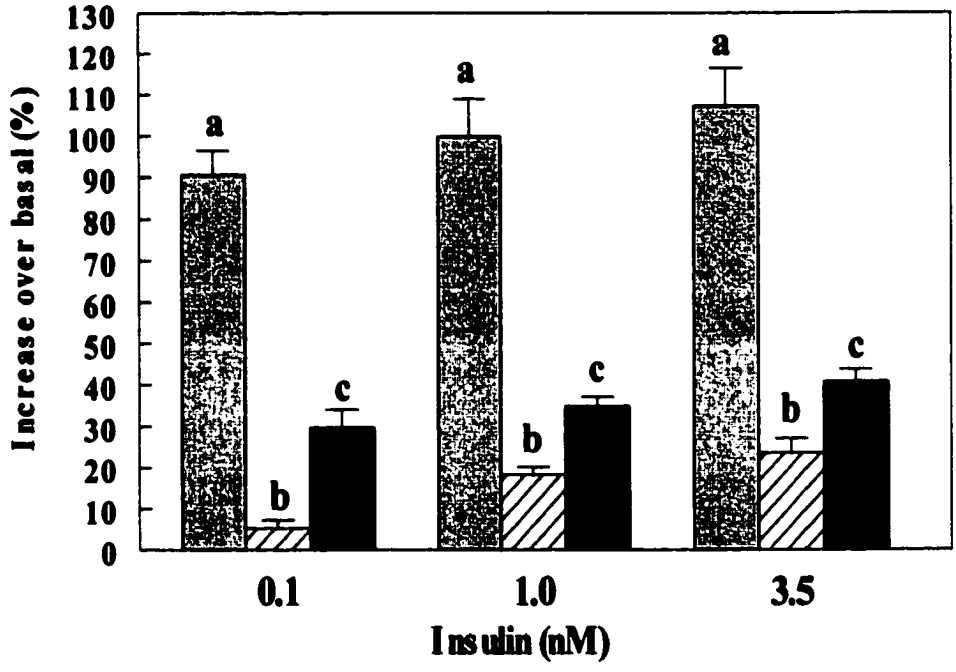

Fig. 21. Insulin-stimulated (^{14}C)-glucose incorporation into total lipids expressed as percent increase over basal in epididymal adipocytes from obesity-prone (black bars), obesity-resistant (striped bars), & control (gray bars) rats after 1 week on a moderately high fat (OP & OR) or low-fat diet (C). Different letters within specific concentration denote significant differences ($p < 0.05$ or less). Data are presented as mean ± SE.

(^{14}C)-Glucose Incorporation into Fatty Acids

Basal glucose incorporation into fatty acids was significantly different among groups on their respective diets (Figure 22). Insulin-stimulated glucose incorporation into fatty acids was reduced for EPI of OP and OR rats compared to C rats, whereas the insulin response was greater for OP rats than OR rats (Figure 22). Relative rates and percent increase over basal data were still increased for C when compared with OP and OR (Figures 23 and 24). When comparing the responses of OP and OR EPI cells, relative rates of glucose conversion to fatty acids were increased at all insulin concentrations for OP rats vs. OR rats (Figure 23). When expressed as percent increase over basal, EPI cells of OP rats were more responsive to insulin than those of OR rats (Figure 24).

(^{14}C)-Glucose Incorporation into Glyceride-glycerol

Basal glucose incorporation into glyceride-glycerol did not differ among groups on their respective diets (Figure 25). MHF feeding significantly affected insulin-stimulated glucose incorporation into glyceride-glycerol for EPI cells of OP and OR rats compared to C rats (Figure 25). Relative rates and percent increase over basal data were still increased for C when compared with OP and OR (Figures 26 and 27). When comparing the responses of OP and OR EPI cells, relative rates of glucose conversion to glyceride-glycerol were only increased at the lowest concentration of insulin (0.1 nM) for OP rats vs. OR rats (Figure 26). At the other concentrations, relative rates of glucose incorporation into glyceride-glycerol tended to be increased for OP vs. OR, but these differences were not significant. When expressed as percent increase over basal, the insulin effects were greater in EPI cells of OP rats than in those of OR rats (Figure 27).

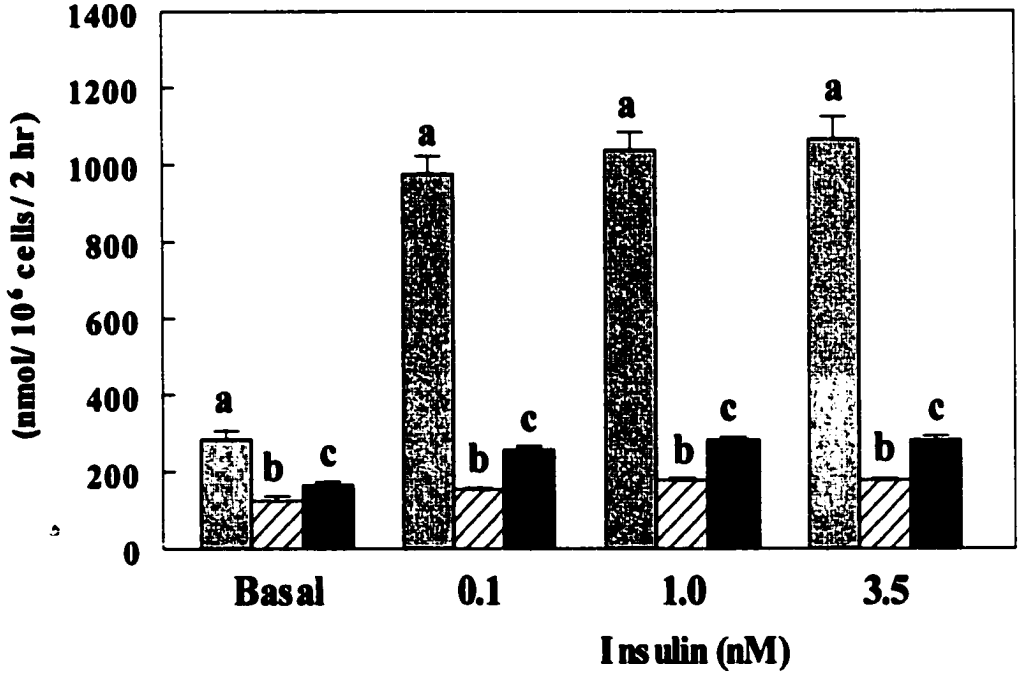

Fig. 22. Basal and insulin-stimulated rates of (^{14}C)-glucose incorporation into fatty acid moieties of lipids in epididymal adipocytes from obesity-prone (black bars), obesity-resistant (striped bars), & control (gray bars) rats after 1 week on a moderately high fat (OP & OR) or low-fat diet (C). Different letters within specific concentration denote significant differences ($p < 0.05$ or less). Data are presented as mean ± SE.

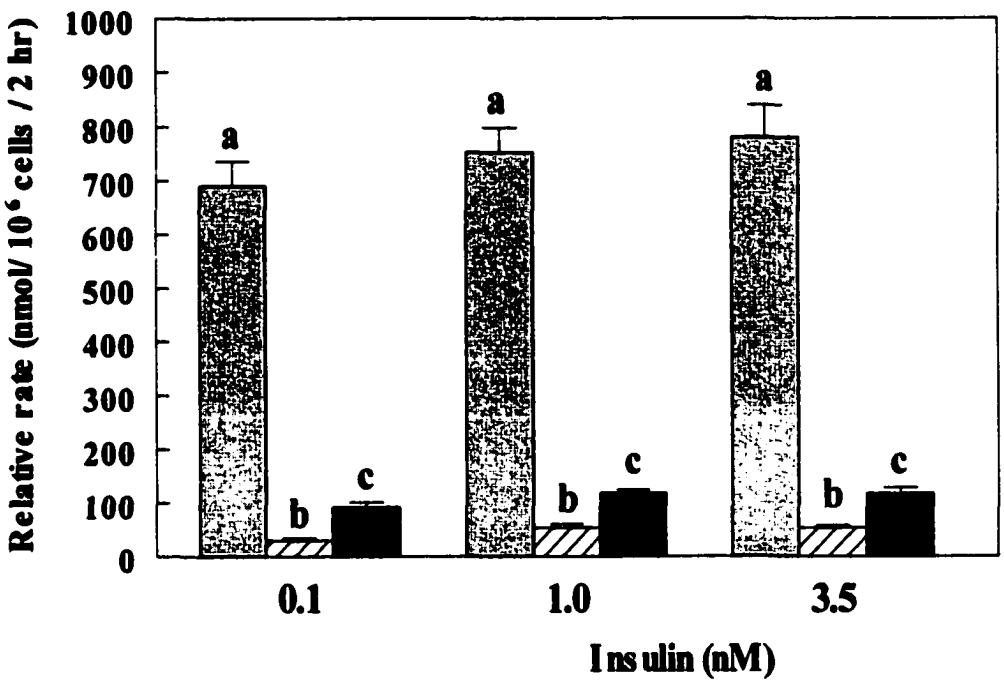

Fig. 23. Relative rates of insulin-stimulated (minus basal) (^{14}C)-glucose incorporation into fatty acid moieties of lipids in epididymal adipocytes from obesity-prone (black bars), obesity-resistant (striped bars), & control (gray bars) rats after 1 week on a moderately high fat (OP & OR) or low-fat diet (C). Different letters within specific concentration denote significant differences ($p < 0.05$ or less). Data are presented as mean ± SE.

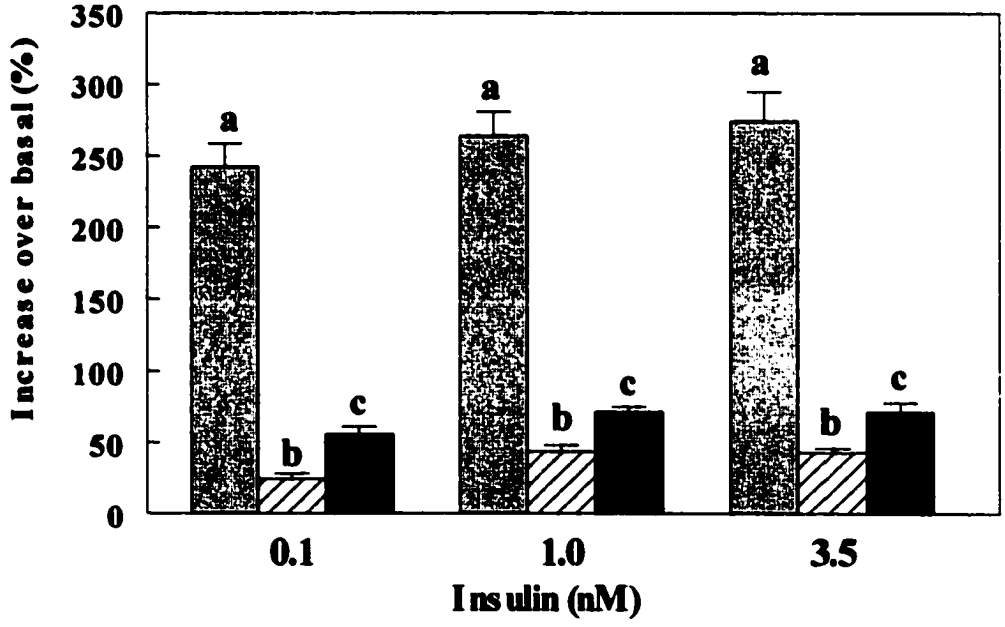

Fig. 24. Insulin-stimulated (^{14}C)-glucose incorporation into fatty acid moieties of lipids expressed as percent increase over basal in epididymal adipocytes from obesity-prone (black bars), obesity-resistant (striped bars), & control (gray bars) rats after 1 week on a moderately high fat (OP & OR) or low-fat diet (C). Different letters within specific concentration denote significant differences ($p < 0.05$ or less). Data are presented as mean ± SE.

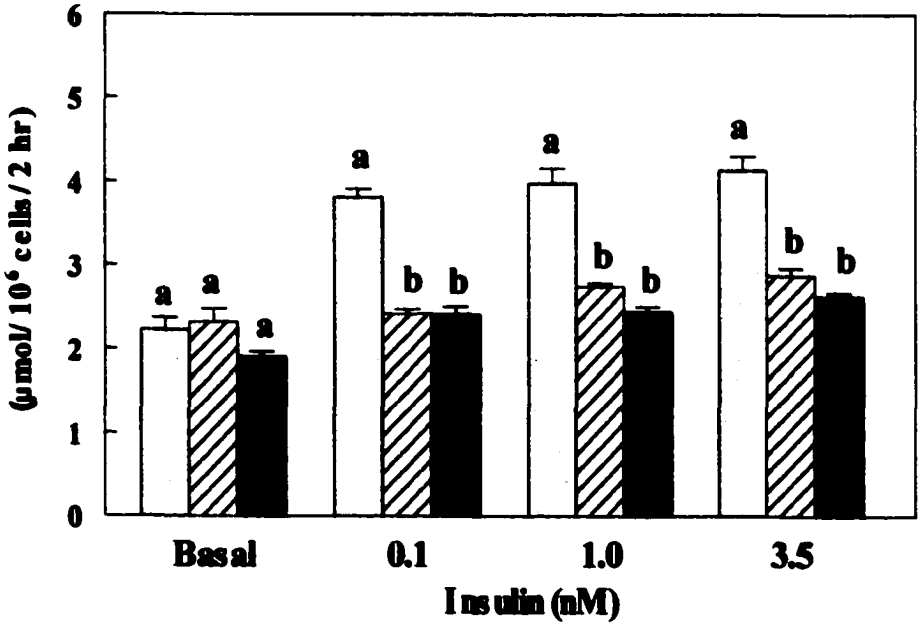

Fig. 25. Basal and insulin-stimulated rates of (^{14}C)-glucose incorporation into glyceride-glycerol moiety of lipids in epididymal adipocytes from obesity-prone (black bars), obesity-resistant (striped bars), & control (gray bars) rats after 1 week on a moderately high fat (OP & OR) or low-fat diet (C). Different letters within specific concentration denote significant differences ($p < 0.05$ or less). Data are presented as mean ± SE.

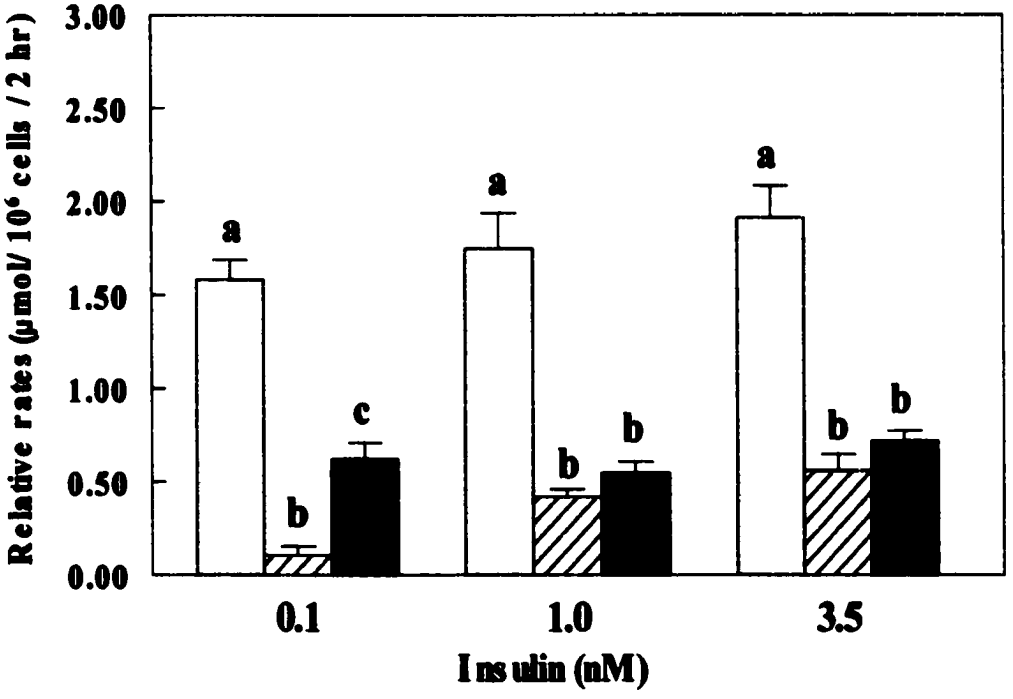

Fig. 26. Relative rates of insulin-stimulated (minus basal) (^{14}C)-glucose incorporation into glyceride-glycerol moiety of lipids in epididymal adipocytes from obesity-prone (black bars), obesity-resistant (striped bars), & control (gray bars) rats after 1 week on a moderately high fat (OP & OR) or low-fat diet (C). Different letters within specific concentration denote significant differences ($p < 0.05$ or less). Data are presented as mean ± SE.

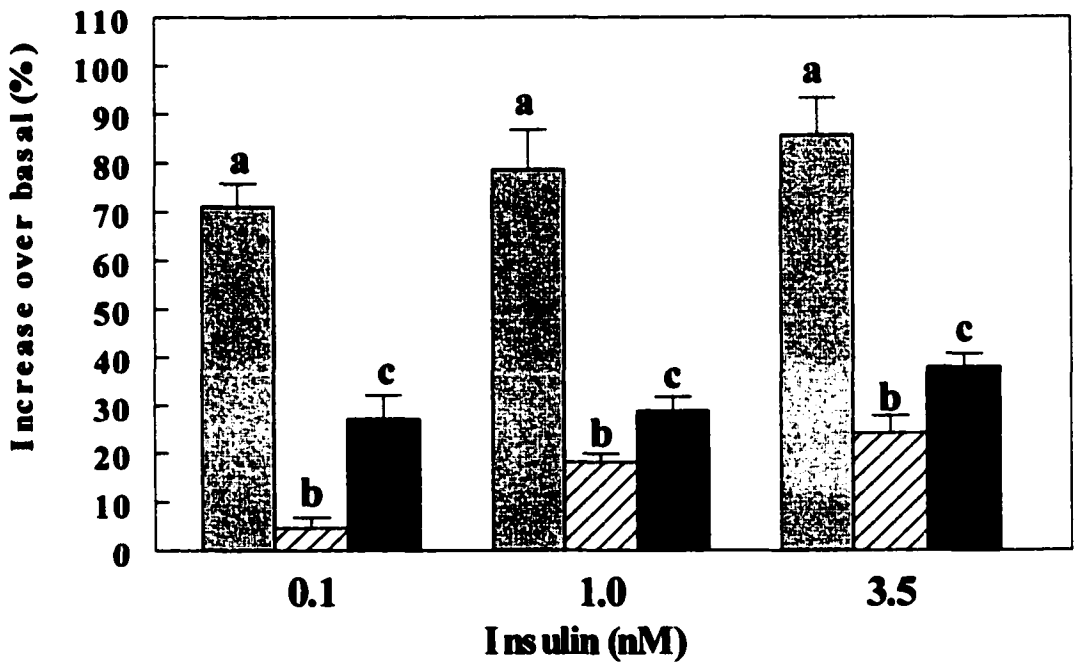

Fig. 27. Insulin-stimulated (^{14}C)-glucose incorporation into glycerol-glyceride moiety of lipids expressed as percent increase over basal in epididymal adipocytes from obesity-prone (black bars), obesity-resistant (striped bars), & control (gray bars) rats after 1 week on a moderately high fat (OP & OR) or low-fat diet (C). Different letters within specific concentration denote significant differences ($p < 0.05$ or less). Data are presented as mean ± SE.

(^{14}C)-Glucose Oxidation (Carbon Dioxide Production)

Absolute rates of basal glucose incorporation into total lipids did not differ among groups after 1 week on their respective diets (Figure 28). Insulin enhanced glucose oxidation for all groups but it was significantly increased in EPI cells of C rats compared to OP and OR rats, which did not differ (Figure 28). The direct effects of insulin were evaluated after subtracting insulin-stimulated from basal glucose incorporation rates. Relative rates and percent increase over basal data were still increased for C when compared with OP and OR, which again were not different (Figures 29 and 30).

Week 3 Data

(^{14}C)-Glucose Incorporation into Total Lipids (Triglycerides)

Absolute basal glucose incorporation into total lipids was significantly different among the groups after 3 week on their respective diets (Table 12). In the presence of the maximal insulin concentration, EPI cells of C rats had increased absolute rates of glucose incorporation into total lipids than those of OP rats, which in turn was greater than OR rats. Relative rates, as well as, percent increase over basal data were increased in EPI cells for C rats when compared with OP and OR rats. OP rats were still more responsive to the lipogenic effects of a maximal insulin dose when compared with OR rats.

(^{14}C)-Glucose Incorporation into Fatty Acids

Similar to data from week 1, basal glucose incorporation into fatty acids were significantly different among the groups after 3 week on their respective diets (Table 12). With a maximal concentration for insulin, EPI cells of C rats had increased absolute rates of glucose incorporation into fatty acids than those of OP rats, which in turn was greater than OR rats. EPI cells for C rats also had augmented relative rates and percent increase

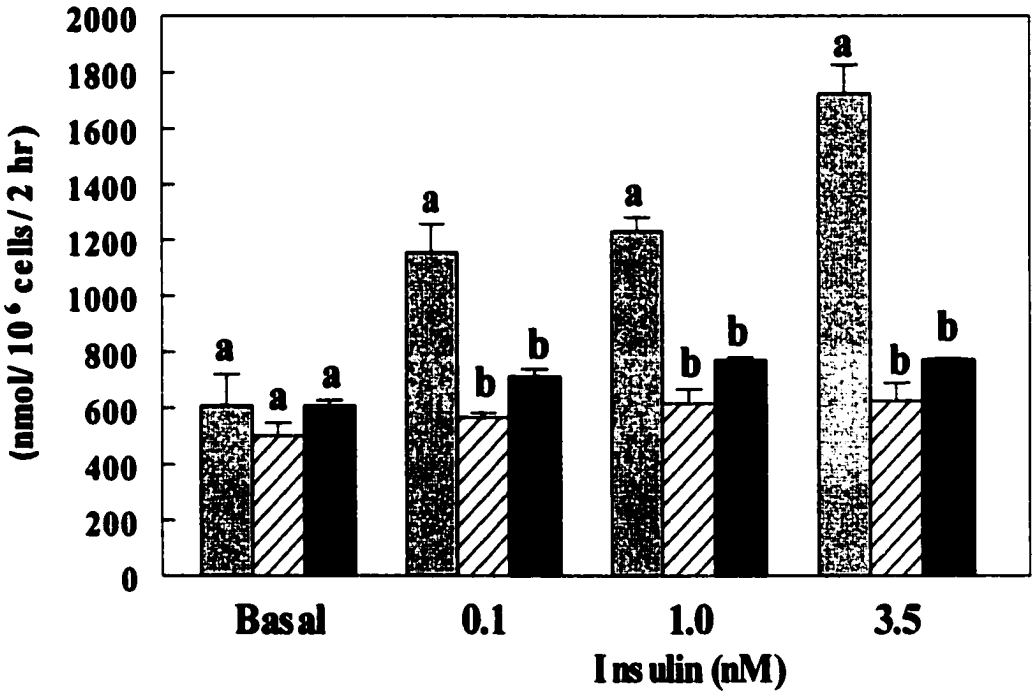

Fig. 28. Basal and insulin-stimulated rates of (^{14}C)-glucose oxidation via CO_2 production in epididymal adipocytes from obesity-prone (black bars), obesity-resistant (striped bars), & control (gray bars) rats after 1 week on a moderately high fat (OP & OR) or low-fat diet (C). Different letters within specific concentration denote significant differences ($p < 0.05$ or less). Data are presented as mean ± SE.

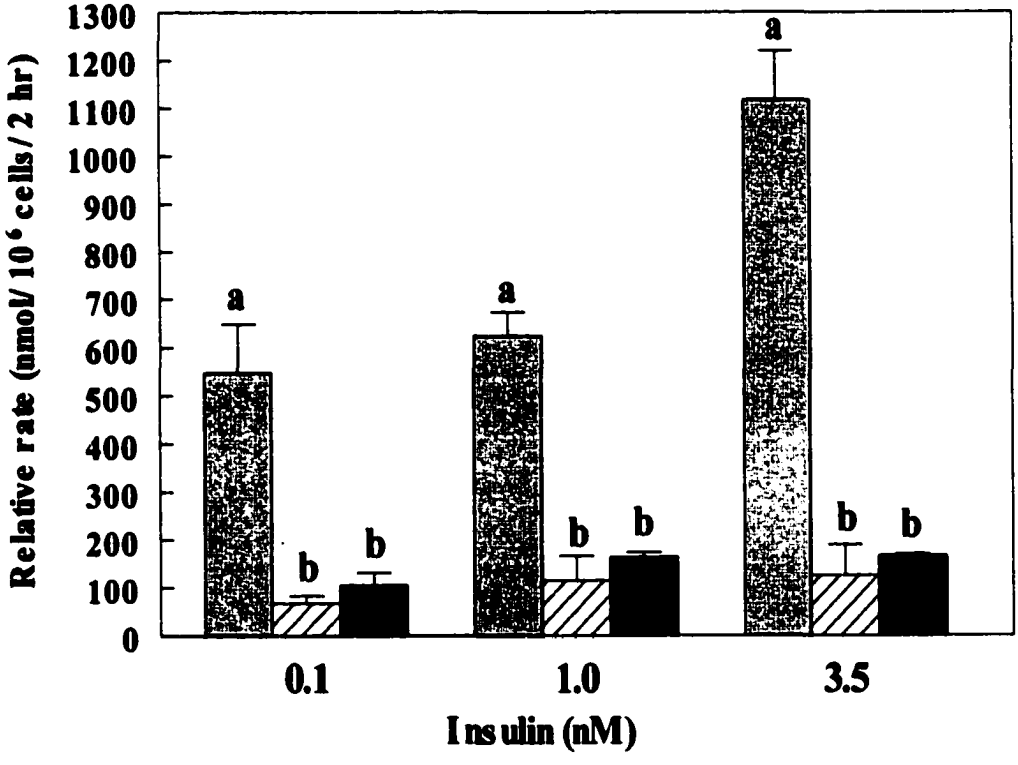

Fig. 29. Relative rates of insulin-stimulated (minus basal) (^{14}C)-glucose oxidation via CO_2 production by epididymal adipocytes from obesity-prone (black bars), obesity-resistant (striped bars), & control (gray bars) rats after 1 week on a moderately high fat (OP & OR) or low-fat diet (C). Different letters within specific concentration denote significant differences ($p < 0.05$ or less). Data are presented as mean ± SE.

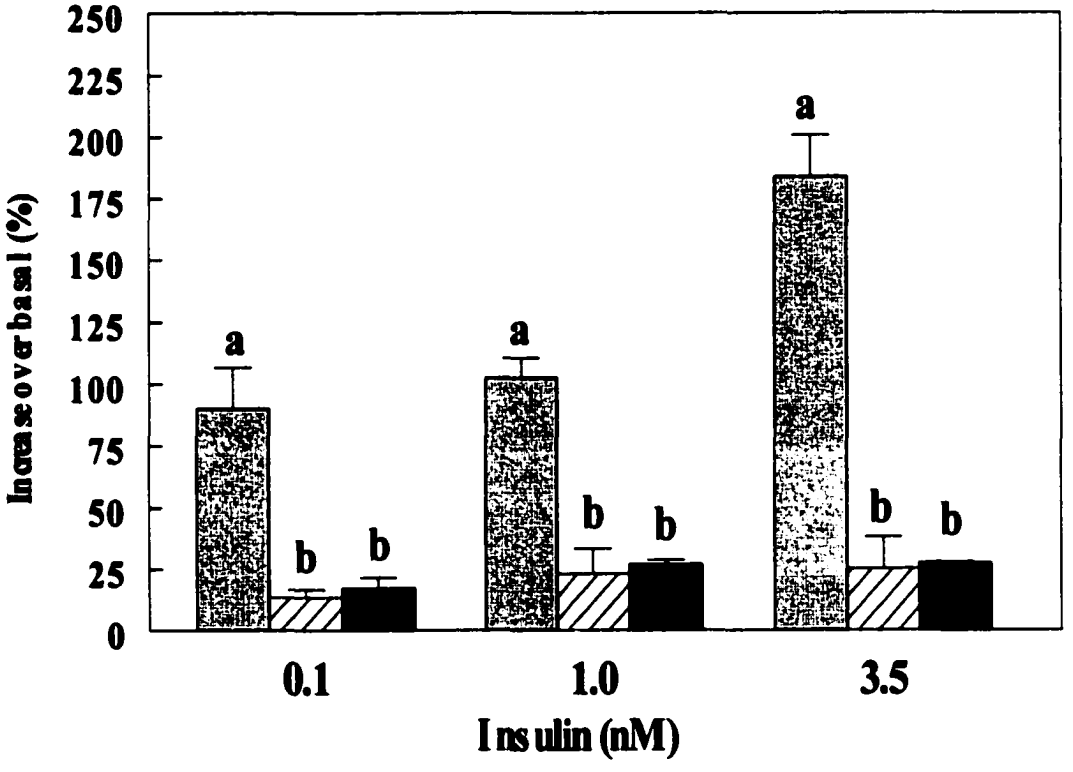

Fig. 30. Insulin-stimulated (^{14}C)-glucose oxidation via CO_2 production expressed as percent increase over basal in epididymal adipocytes from obesity-prone (black bars), obesity-resistant (striped bars), & control (gray bars) rats after 1 week on a moderately high fat (OP & OR) or low-fat diet (C). Different letters within specific concentration denote significant differences ($p < 0.05$ or less). Data are presented as mean ± SE.

Table 12. *(^{14}C)-glucose conversion to total lipids (fatty acids and glyceride-glycerol) in epididymal adipocytes of obesity-prone (OP), obesity-resistant (OR), and control (C) rats after consuming either a moderately high fat (OP & OR) or low fat diet (C) for 3 weeks*

Condition	C	OP	OR
Total lipids (μmol/10^6 cells/2 hr)			
Basal	2.34 ± 0.05^a	1.91 ± 0.06^b	1.54 ± 0.03^c
Insulin (3.5 nM)	3.88 ± 0.11^a	2.83 ± 0.03^b	2.19 ± 0.02^c
Insulin minus basal (μmol/10^6 cells/2 hr)	1.54 ± 0.11^a	0.92 ± 0.03^b	0.65 ± 0.02^c
Increase over basal (%)	65.7 ± 4.6^a	48.1 ± 1.6^b	42.1 ± 1.0^c
Fatty acids (nmol/10^6 cells/2 hr)			
Basal	87 ± 2^a	50 ± 4^b	26 ± 1^c
Insulin (3.5 nM)	307 ± 8^a	126 ± 4^b	52 ± 1^c
Insulin minus basal (nmol/10^6 cells/2 hr)	220 ± 8^a	76 ± 4^b	26 ± 1^c
Increase over basal (%)	253.2 ± 8.8^a	152.1 ± 7.7^b	99.4 ± 4.8^c
Glyceride-glycerol (μmol/10^6 cells/2 hr)			
Basal	2.26 ± 0.05^a	1.86 ± 0.06^b	1.51 ± 0.03^c
Insulin (3.5 nM)	3.63 ± 0.11^a	2.70 ± 0.02^b	2.13 ± 0.02^c
Insulin minus basal (μmol/10^6 cells/2 hr)	1.37 ± 0.11^a	0.85 ± 0.03^b	0.62 ± 0.02^c
Increase over basal (%)	60.8 ± 5.1^a	45.5 ± 1.5^b	$41.2 \pm 1.3^{b*}$

Data are presented as mean ± SE.
Different letters within a row denote significant differences ($p < 0.05$ or less).
OP vs. OR ($p = 0.07$)

over basal data when compared with OP and OR. OP rats were still more responsive to the lipogenic effects of insulin when compared with OR rats.

(^{14}C)-Glucose Incorporation into Glyceride-glycerol

Absolute rates of basal glucose incorporation into glyceride-glycerol were different among groups after 3 week on their respective diets (Table 12). There were reductions in absolute rates of glucose incorporation into glyceride-glycerol for EPI of OP and OR rats compared to C rats with insulin. Relative rates and percent increase over basal data were greater for C when compared with OP and OR. Comparisons between OP and OR EPI cell responses revealed that relative rate of glucose conversion to glyceride-glycerol was increased for OP rats vs. OR rats, but these differences approached significance when expressed as percent increase over basal ($p = 0.07$).

FAS mRNA Expression

FAS mRNA expression appeared to be lower in EPI fat of both OP and OR rats compared with C rats (Figure 31). When expressed as a percent of control, FAS mRNA expression was 50% higher in EPI fat of OP than that of OR rats.

DISCUSSION

Previously it has been reported that high fat feeding alters insulin-stimulated glucose metabolism in rat adipocytes compared with rats fed a low fat diet (52, 102, 112, 150), which was confirmed in the present study. However, we have reported differential body weight responses in rats consuming the same moderately high fat (MHF) diet (40, 100). These rats are classified as obesity-prone (OP) and obesity-resistant (OR) based on body weight gain. Therefore, we compared insulin-stimulated glucose metabolism in adipocytes OP and OR fed a MHF diet to account for the divergent responses to this diet

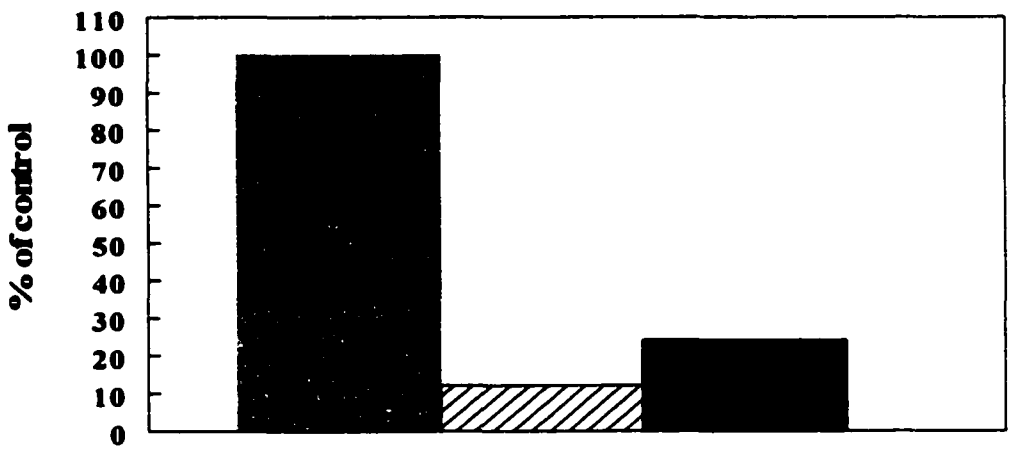

Fig. 31. FAS/β-actin ratios expressed as percent of controls (C = 100%) in epididymal fat tissues from obesity-prone (black bars), obesity-resistant (striped bars), & control (gray bars) rats after 1 week on a moderately high fat (OP & OR) or low-fat diet (C).

in the present study. We also included a low fat-fed group as a dietary control (C). Marked physiologic adaptations to a MHF diet were observed within one week of starting the diet in OP and OR rats. Plasma leptin concentrations and visceral adiposity were increased in OP rats vs. OR rats after 1 week, whereas the values in low fat-fed rats were non-significantly lower vs. OP rats. Furthermore, plasma leptin and body fat differences were more pronounced among groups after 3 weeks. Plasma insulin concentrations did not differ among groups at both time points. This was not consistent with other researchers that have found hyperinsulinemia or impaired glucose tolerance in OP rats after short-term exposure to hypercaloric diets (106, 107). We have also noted that long-term exposure to a MHF diet (14 wk) produced hyperinsulinemia in OP rats (100). Since glucose tolerance tests were not done, it cannot be ruled that OP rats did not have impaired glucose tolerance (reflective of insulin sensitivity in skeletal muscle) in the present study. Possibly the short-term dietary challenge was not sufficient to produce hyperinsulinemia in the present OP rats.

Cell size in EPI fat was also increased in OP rats vs. OR and C rats. OR cell size was also less than C cell size, suggesting that OR rats had reduced cell sizes prior to the diet or were able to protect their fat cells from increased dietary lipids. Moreover, the C group consists of rats that would diverge into OP and OR groups upon MHF challenge. This may have contributed to the cell size findings. These cell size differences were present after 1 and 3 weeks of the dietary challenge. Therefore, OP rats appeared to respond to increased dietary fats by increasing lipid storage and body fatness. These data suggested that although moderately high fat feeding reduced lipogenic responses to

insulin in adipocytes, diet-induced responses could be different in adipocytes from OP and OR rats.

High fat feeding affects insulin-stimulated glucose metabolism and cell size in adipocytes (102, 149). Insulin sensitivity is reduced in larger cells (37, 38, 73, 102, 113). One cell size-related mechanism for reduced lipogenesis is the increased basal lipolysis in larger adipocytes (38). As a result of elevated lipid breakdown, augmented intracellular non-esterified fatty acid concentrations shift glucose metabolism from fatty acid synthesis to glyceride-glycerol synthesis for re-esterification, thus reducing the capacity of the adipocyte to utilize glucose for lipogenesis (37, 59, 102). Therefore, the following sections will address the effects of MHF and LF feeding and cell size on basal and insulin-stimulated glucose metabolism.

Basal Effects

In agreement with previous studies, we found that MHF feeding altered basal lipogenic responses in adipocytes when compared with LF feeding. After 1 week, basal fatty acid synthesis was reduced in fat cells of MHF-fed rats compared with those of LF-fed rats. In adipocytes, the reduction in fatty acid synthesis for rats fed moderately high fat diet was related increased dietary fats (32% vs. 11% kcal as fat). In contrast, basal glucose oxidation (i.e., CO_2 production) and glyceride-glycerol production were not affected by MHF feeding. Since glucose was converted mainly into the glyceride-glycerol moiety of lipids, it was not surprising that basal lipid synthesis was not different between high and low fat groups. The changes in basal glucose metabolism in isolated EPI adipocytes were similar to the 1-week data reported by Lavau et al. (102), except that basal CO_2 production was not different among groups in the present study. They

reported that basal fatty acid synthesis and CO_2 production were reduced and production of glyceride-glycerol was unaffected with one week of high fat feeding. Ip et al (81) also noted a reduction in basal CO_2 production with high fat feeding. The discordant CO_2 findings may be related to the fat content of the diet. Their diets were composed of approximately 70% kcal as fat and our diet contained 32% kcal as fat, thus basal activity of glucose oxidative enzymes may be compromised by higher fat content. Moreover, basal fatty acid and glyceride-glycerol synthesis were further reduced in MHF fed rats compared with LF fed rats after 3 weeks. This suggested that longer term feeding affected glucose metabolism more than a 1-week dietary challenge.

Furthermore, this was the first study to appreciate the divergent body weight and fat responses to a MHF diet and to evaluate glucose metabolism in adipocytes of rats rendered OP and OR after exposure to this diet. In addition to diet-related effects, there were also within diet group (MHF-fed OP and OR rats) responses. EPI fat cells of OP rats converted more glucose into fatty acids than those of OR rats at basal conditions, whereas glucose oxidation and glyceride-glycerol (also total lipids) production did not differ after 1 week. These findings were independent of substrate availability because glucose uptake did not differ between EPI cells of OP and OR rats (see Chapter V). In contrast, EPI cells of OP rats had increased basal fatty acid and glyceride-glycerol synthesis after 3 weeks when compared with those of OR rats. This suggested that MHF feeding was less detrimental to basal lipogenic capacity within OP rat adipocytes.

Insulin Effects

Similar to the results found with glucose uptake, the metabolic responses to insulin were also depressed in adipocytes of rats fed either a MHF, LF, or chow diet (i.e.,

baseline rats). This demonstrated that even small changes in dietary fat content (32% vs. 11% vs. 5%, respectively) altered glucose metabolism in adipocytes. Insulin regulates various metabolic pathways within adipocytes via binding to its receptor and activating second messenger systems. Insulin stimulates uptake of glucose and fatty acids into adipocytes and skeletal muscle cells. Glucose oxidation is increased in order to facilitate lipid synthesis and storage within adipocytes. Lipolysis is also inhibited in adipocytes by insulin, thus highlighting the anabolic nature of this hormone. We found that insulin enhanced glucose metabolism for all groups, but metabolic responses (CO_2 production and fatty acid and glyceride-glycerol synthesis) were compromised in adipocytes of moderately high fat fed rats after 1 and 3 weeks. Furthermore, these group differences were independent of cell size because OR rats had the smallest cell size, but insulin-stimulated glucose metabolism appeared to be most affected in these cells. Moreover, the glucose metabolism data were adjusted for both cell size and cell number by the nature of the normalization method (e.g., umol/mg lipid * mg lipid/10^6 cells). Mechanisms other than cell size may have contributed to the differences among groups.

Moreover, marked differences were found in the lipogenic responses to insulin between EPI cells of OP and OR rats. The main finding with insulin stimulation was that glucose conversion into fatty acids was elevated nearly two-fold in EPI cells of OP vs. OR rats. Additionally, the production of glyceride-glycerol was also enhanced by insulin, but group differences were not present at all concentrations. Glucose oxidation did not differ between groups after 1 week. After 3 weeks of a dietary challenge, differences in insulin-stimulated lipogenesis were more pronounced between OP and OR rats. Glucose oxidation was not measured at week 3. Again, these findings were

independent of substrate availability because insulin-stimulated glucose uptake did not differ between EPI cells of OP and OR rats. Equivalent glucose uptake and differential metabolic responses imply channeling of glucose into different metabolic pathways within adipocytes of OP and OR rats. Glycogen and lactate are also products of glucose metabolism in adipocytes (73, 123), but were not measured in the present study. Group differences cannot be ruled out for these variables. EPI adipocytes of OP rats had an increased capacity to convert glucose to triglycerides (both glyceride-glycerol and fatty acid moieties) in response to insulin, thus increasing lipid storage and adiposity. Elevated triglyceride synthesis within OP adipocytes may have contributed to the augmented plasma and lipoprotein triglycerides concentrations found within OP rats. Triglycerides and free fatty acids are known to alter liver, specifically gluconeogenesis and skeletal muscle metabolism (141). Alternatively, EPI adipocytes of OR rats may have metabolized glucose to less metabolically detrimental products (e.g., glycogen and lactate). The differential lipogenic response to insulin is similar to findings from other animal models of obesity. Hypothalamic obesity was associated with increased lipogenesis *in vivo* 1 week post-lesions via increased insulin secretion and sensitivity (128, 129). Young obese Zucker rats have augmented lipogenic enzyme activity in adipose tissues with marked insulin resistance in skeletal muscles (22, 69). Elevated lipogenic capacity in Zucker rats appears to be a genetic effect because FAS activity is increased in stromal cells and preadipocytes in obese Zucker rats (22). Moreover, obese Zucker rats consume more calories *ad libitum* than lean Zucker rats (169). Increased caloric intake is associated with elevated lipogenic enzyme activities in fat tissues of obese Zucker rats (18a). Therefore, differences in adipocyte lipogenic capacity between

OP and OR rats may be related to the increased caloric consumption noted in OP rats in the present study. In summary, EPI fat cells of OP rats appeared to be more responsive to the lipogenic properties of insulin, specifically fatty acid synthesis at both time points, but whether this difference is primary or secondary to increased adiposity or caloric intake needs to be determined.

In the present study, lipogenic enzyme activity in fat cells appeared to be depressed with MHF feeding, but the reduction was greater for OR rats than for OP rats. In support of this finding, fatty acid synthase (FAS) mRNA expression was greater in EPI fat of OP rats compared to OR rats after 1 week on a MHF diet (50% greater). Caution must be taken when interpreting these results because these data were based on pooled adipose tissues. Pooled tissue and RNA technically yielded only a data point for each group. Due to lack of tissues, FAS mRNA data were not determined for week 3 rats. This seemed, however, to be in good agreement with the finding that the activity of lipogenic enzymes was reduced to roughly 13% of controls in EPI cells of high fat fed rats (102).

FAS is a key lipogenic enzyme that catalyzes the biosynthesis of long chain fatty acids from acetyl CoA precursors and is present in hepatocytes and adipocytes. FAS gene expression is regulated at the transcriptional level by hormonal and nutritional controls, which indicates that enzyme activity is related to mRNA expression (16). Glucose and insulin regulate FAS expression and activity *in vivo* and *in vitro*, in addition to dietary fats (22, 56, 90, 102, 122, 149). In the present study, non-fasting plasma insulin, glucose, and FFA concentrations did not differ among OP, OR, and C rats after 1 and 3 weeks on their respective diets. This implied that these factors were not

responsible for the apparent differences in insulin-stimulated glucose metabolism and FAS mRNA expression. However, plasma and very low-density lipoprotein triglycerides tended to be increased after 1 week and were significantly elevated after 3 weeks in OP rats compared with OR and C rats. An earlier study reported higher rates of fatty acid synthesis in isolated hepatocytes of diet-induced obese rats (126). These data suggested that FAS activity was greater in hepatocytes of OP rats compared with OR and C rats. Reduced suppression of FAS mRNA expression by dietary fats may have accounted for the increased triglyceride production in hepatocytes and adipocytes of OP rats compared with those of OR rats.

In summary, insulin responsiveness was reduced in adipocytes of rats fed a moderately high fat diet compared with low fat-fed rats. Epididymal adipocytes of OP rats appeared to be less affected by increased dietary fats than OR rats. The enhanced insulin effect might promote excess fat accretion noted in fat cells of OP rats at weeks 1 and 3 of a dietary challenge, ultimately resulting in the obese state. Altered regulation of FAS mRNA expression in response to a moderately high fat diet could play a role in the increased adiposity of OP rats. Increased insulin sensitivity at the onset of a dietary challenge might result in augmented visceral adiposity and triglyceride synthesis, which may have contributed to the altered metabolic state within OP rats.

CHAPTER VII

SUMMARY

In this dissertation, we attempted to understand and determine primary and secondary peripheral causes of excess fat accretion noted in obesity-prone (OP) rats upon introduction of a moderately high fat diet. Excess fat accretion was found in visceral fat depots within these OP rats. Adipose tissue is composed of adipocytes that are under hormonal control. These hormones control the major processes occurring in adipocytes including lipid mobilization (lipolysis) and lipogenesis. Lipolysis is stimulated mainly by catecholamines and secondarily by growth hormone. Lipogenesis is principally controlled by insulin. Adrenal cortex, thyroid, and sex steroid hormones also influence these processes but their roles are permissive and not primary.

In the first study, we investigated whether *in vivo* lipolysis was a causative factor in obesity development, as suggested by earlier researchers. We measured *in vivo* lipolysis by two distinct methods, whole body via plasma challenge and local via microdialysis. After the lipolytic studies, we challenged rats with a moderately high fat (MHF) diet. OP and OR (obesity-resistant) groups were determined based on body weight gain. Regardless of method, we found no differences in isoproterenol-stimulated glycerol release between OP and OR rats prior to a dietary challenge. This suggested that lipid mobilization was not a causative factor in obesity development in these rats.

In the second experiment, we addressed the role of increased adiposity on lipolytic responsiveness utilizing an *in vitro* approach. We evaluated lipolysis in isolated adipocytes of inbred OP and OR rats. These inbred rats were bred for obesity-prone or

obesity-resistant traits from an outbred Sprague-Dawley stock. These rats also enabled us to evaluate lipolytic responses from various depots without placing them on a MHF diet to determine groupings. We found that although OP and OR rats have similar body weights at 8 wk of age, OP rats have more visceral fatness than OR rats. Moreover, regional and group differences were found for basal and hormone-stimulated lipolysis. There were also differential responses to hormones or drugs. Altogether, we found that OP rats had reduced lipolytic responsiveness in visceral adipocytes but not subcutaneous adipocytes when compared to OR rats. Excess visceral adiposity in inbred OP rats may be the result of reduced responsiveness to lipolytic agonists or vice versa.

In studies 3 through 5, we explored the role of insulin on glucose uptake and metabolism with the premise that increased insulin sensitivity may have promoted excess substrate availability for lipid production or increased lipogenesis within adipocytes of OP rats. We investigated these processes (e.g., glucose uptake and lipogenesis) in isolated adipocytes of outbred rats identified as OP or OR after 1 and 3 weeks on a MHF diet. We also included low fat fed rats to examine dietary influences on insulin action. We found that MHF-feeding impaired insulin-stimulated glucose uptake and lipogenesis in adipocytes of OP and OR rats when compared to those of low-fat fed rats. The main interest in these studies was comparisons between OP and OR rats. OP rats were heavier and fatter than OR rats during the early stages of the dietary challenge. We noted that there were no group differences in insulin-stimulated glucose uptake, suggesting MHF feeding impaired this process equally between groups. The major finding in this set of experiments is that isolated adipocytes of OP rats are more proficient in synthesizing fatty acids from glucose (lipogenesis) than those of OR rats during the early stages of a

dietary challenge. Moreover, this finding may be related to the apparent difference in fatty acid synthase mRNA expression between OP and OR rats. The increased lipogenic responses to insulin in OP rats may have contributed to the increased visceral adiposity in these rats, but assigning causation should be done with caution.

If we combine the above results, it may be speculated that altered lipolysis was not a causative factor for excess adiposity in OP rats. Moreover, increased insulin sensitivity and responsiveness (via increased lipid synthesis) promoted excess fat accretion in OP rats. As obesity developed, adipocytes of OP rats became less responsive to lipolytic agents and exacerbated visceral fatness. Conversely, OR rats were protected from obesity by a combination of increased hormone-stimulated lipolysis and reduced sensitivity to the lipogenic effects of insulin. Either way, increased visceral fatness was associated with other metabolic perturbations found in OP rats.

In adipocytes and hepatocytes, increased insulin sensitivity in OP rats appeared to elevate visceral adiposity and plasma and VLDL triglycerides when compared with OR rats in the present study. These increases were present after one and three weeks on the MHF diet in our study. Levin et al. (106) also found that OP rats were hyperinsulinemic after only two weeks on a high energy diet compared with OR rats, suggesting insulin resistance in skeletal muscles and liver. We did not find differences in plasma insulin concentrations in the present study. We have, however, reported hyperinsulinemia in OP rats after 14 weeks on a MHF diet (100). Impaired glucose tolerance was also noted in OP after three months on the high energy diet (109), indicating insulin resistance at both the skeletal muscle and adipose tissue level. Therefore, initially increased insulin responsiveness in adipose tissues is lost as the obese state further develops within the OP

rat. It is the increased visceral adiposity and insulin resistance that are associated with other perturbations noted with the metabolic syndrome, dyslipidemia and hypertension (91, 130, 134). Our previous findings showed that OP rats developed symptoms of the metabolic syndrome during exposure to a moderately high fat diet (40, 100).

These symptoms were also noted in humans with growth hormone deficiency (83). Previously, Lauterio and colleagues (98, 101) found that growth hormone secretion was significantly reduced in OP rats vs. OR rats before and after the onset of obesity. Recently, we infused OP rats continuously with growth hormone (200 μg/day – dose approximates difference between OP and OR rats) via mini-osmotic pump for 4 weeks and examined its effect on visceral adiposity and plasma profiles. GH-infused OP rats had significantly less visceral adiposity and plasma insulin and triglyceride concentrations than saline-infused OP rats (Davies and Lauterio, in preparation). Similar metabolic improvements were noted in GH-deficient humans receiving GH replacement therapy (84). These data indicated that increased insulin sensitivity in adipose tissue and GH deficiency might contribute to obesity development in OP rats.

In summary the current experiments were able to:

1. Demonstrate that male Sprague-Dawley rats diverge into two distinct populations based on body weight gain after exposure to a moderately high fat (MHF) diet for 14 weeks. These populations were termed obesity-prone (OP) and obesity-resistant (OR).
2. Determine that *in vivo* lipolytic responsiveness, as assessed by two separate approaches, is not causative factor for obesity susceptibility or obesity resistance in male Sprague-Dawley rats (outbred).
3. Establish that OP rats are more efficient in storing energy as body weight than OR rats throughout the dietary challenge. In contrast, relative rates of body weight gain (i.e., growth) and food consumption are only increased in OP rats during the first two weeks of the dietary challenge. These data suggest that there is a dynamic phase and a static phase in response to the MHF diet. The dynamic phase is associated with marked hyperphagia and body weight accumulation.
4. Demonstrate that OP rats are hyperinsulinemic and hyperleptinemic compared to OR rats after a 14 week dietary challenge. In addition, relative leptin secretion (plasma leptin concentration per fat pad weight) is also increased in OP rats. Together these results indicate that OP rats develop apparent leptin and insulin resistance.
5. Compare *in vitro* responses to various lipolytic agents (e.g., isoproterenol, forskolin, and growth hormone (GH) + dexamethasone) in adipocytes from male Sprague-Dawley rats bred for either OP or OR traits without the confounding

effects of the MHF diet. Inbred OP rats are fatter than inbred OR rats even though both groups have similar body weights at 8 weeks of age.

6. Determine that visceral adipocytes of OP rats are less responsive (i.e., fold increase over basal) to lipolytic agents than those of OR rats. Subcutaneous adipocytes do not differ in responses to these agents.
7. Demonstrate that GH alone does not stimulate lipolysis in adipocytes regardless of group or visceral fat pad. In contrast, GH plus dexamethasone is only able to stimulate lipolysis in visceral adipocytes of OR rats. These data suggest that adipocytes of inbred OP rats are not responsive to lipolytic properties of GH.
8. Confirm that short-term exposure to MHF diet drastically reduces insulin-stimulated glucose uptake and metabolism in isolated adipocytes of OP and OR rats when compared to adipocytes of rats fed a low fat diet.
9. Establish that insulin-stimulated glucose metabolism is different in epididymal adipocytes of OP and OR rats after 1 and 3 weeks on a MHF diet. Moreover, adipocytes of OP rats are more responsive to the lipogenic actions of insulin, specifically fatty acid synthesis at weeks 1 and 3. Glucose oxidation and glyceride-glycerol synthesis are similar between OP and OR rats at week 1.
10. Demonstrate that fatty acid synthase mRNA expression is lower in epididymal fat of OP and OR rats compared with control rats. The level of mRNA expression in epididymal fat of OP rats is 50% greater than that of OR rats. These data support the *in vitro* lipogenic results and also characterize differential responses to MHF feeding in OP and OR rats.

11. Determine that plasma and VLDL triglyceride concentrations tend to be increased in OP rats compared with OR rats after 1 week and significantly elevated after 3 weeks. Therefore, hepatic lipogenic capacity may be augmented in OP rats compared with OR rats

12. Demonstrate that OP rats consume and store more energy as fat, as indicated by the elevated visceral fatness and plasma leptin concentrations after 1 and 3 weeks on the MHF diet.

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PUBLICATIONS

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