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INTERACTIONS OF GLUCOCORTICOID, DIET, AND ENVIRONMENTAL TEMPERATURE IN REGULATION OF ENERGY METABOLISM IN OB/OB MICE

By

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ABSTRACT

INTERACTIONS OF GLUCOCORTICOID, DIET, AND ENVIRONMENTAL TEMPERATURE IN REGULATION OF ENERGY METABOLISM IN OB/OB MICE

By

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Adrenalectomy arrests development of obesity in ob/ob mice fed high-carbohydrate stock diets and housed at 20-25°C partly by stimulating brown adipose tissue (BAT) thermogenesis. However, adrenalectomy fails to prevent obesity in ob/ob mice fed the high-fat diet. Therefore, one objective of my research was to determine whether diet composition (high-fat, high-glucose or high-starch) interacts with glucocorticoid to influence energy balance and BAT metabolism in ob/ob mice. A second objective was to determine whether adrenalectomy would also prevent the development of obesity and stimulate BAT thermogenesis in ob/ob mice housed at 35°C where BAT thermoregulatory heat production is not needed.

Ob/ob mice fed a high-fat diet developed gross obesity even though consumption of the high-fat diet stimulated BAT metabolism, as assessed by rates of norepinephrine turnover in BAT, GDP binding to BAT mitochondria, and GDP-inhibitable mitochondrial swelling. Also, ob/ob mice housed at 35°C retained dietary energy more efficiently than lean mice even though BAT thermogenic activity was equally low in ob/ob and lean mice. These results suggest that BAT plays a minimal role in development of obesity in ob/ob mice fed a high-fat diet, or in ob/ob mice housed in a warm environment.

Adrenalectomy failed to arrest the development of obesity or to influence BAT metabolism in ob/ob mice fed high-fat or high-glucose diets. However, adrenalectomy markedly decreased metabolic efficiency and enhanced BAT metabolism in ob/ob mice fed a high-starch diet and housed at 25'C or 35'C. The enhanced BAT metabolism in adrenalectomized ob/ob mice housed at 25'C was associated with increased sympathetic nervous system activity and stimulation of the GDP-binding protein without an increase in mitochondrial mass. But at 35'C, enhanced BAT metabolism was associated with increase in both sympathetic nervous system activity and mitochondrial mass, without specific stimulation of the mitochondrial GDP-binding protein. These results suggest that adrenalectomy-induced reductions in energetic efficiency in ob/ob mice are linked to stimulation of BAT metabolism.

The observations that high energetic efficiency is often, but not always, linked to low BAT metabolism, and removal of the adrenals often, but not always, prevents further development of obesity in ob/ob mice indicate that these factors contribute to the obesity characteristic of ob/ob mice, but are not likely the origin of the primary defect in ob/ob mice.

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CHAPTER 1. REVIEW OF LITERATURE

1.1. INTRODUCTION AND DISSERTATION RESEARCH OBJECTIVES

Obesity is a major nutrition related disorder in the western world affecting up to 30 to 40 % of the middle-aged American population. Many of these obese people are at an increased risk of disease and early death. Increased incidences of disease such as coronary heart disease, hypertension, diabetes and gallstones in the obese adult stress the need to identify the factors which contribute to obesity.

Basically, obesity is a problem of energy imbalance. Inappropriately high energy intake or low energy expenditure produces excess energy, which lead to increased fat storage and gain in body weight. However, among laymen it is widely accepted that some persons remain lean for years despite eating large amounts of food. Other individuals become obese on a normal food intake and fail to lose weight in spite of strenuous efforts to limit intake.

In a recent review Bray (9) summarized that genetic factors are important in making an individual resistant to those factors in the environment which would tend to enhance obesity. Also, the susceptibility of particular strains of animals and ethnic groups of humans to become obese supports the fact that genetic factors contribute to the development of obesity. The great difficulty in identifying the factors responsible for the development of obesity in man has led to considerable interest in animal models and a wide range of such models are now used in experimental studies on energy metabolism (12). These animal models are divided into those in which obesity is transmitted genetically, such as obese ob/ob mice, diabetic-obese db/db mice and Zucker or fatty fa/fa rats, and those in

which obesity is induced experimentally, such as lesioning of the ventromedial hypothalamus, the administration of goldthioglucose (GTG) or monosodium glutamate and, in some strains, feeding a high-fat diet. My research has focused on genetically obese ob/ob mice as an experimental animal. I will, therefore focus on genetically obese rodents.

Results of early studies showed that obesity could develop in genetically obese mice and rats in the absence of hyperphagia, and indicated that these obese mutants retained dietary energy with a greater efficiency than normal by reduced energy expenditure (1,9,71). The metabolic basis for this high efficiency of energy retention has been actively pursued for several decades and several mechanisms have been proposed by which high metabolic efficiency might be achieved (52,84,87,144). Results from studies carried out during the last ten years with a number of different animal models of obesity have shown that the high metabolic efficiency of obese animals may be associated with defective control of the sympathetic nervous system (SNS) and with suppression of an important component of energy expenditure, namely, adaptive thermogenesis in brown adipose tissue (BAT) (35,36, 43,45,50,60,61,142,147).

The SNS is thought to play a prominent role in initiating and maintaining BAT thermogenesis. When housed at 20-26'C and fed stock diets, BAT SNS activity, as indicated by noreinephrine (NE) turnover, is approximately 50 % lower in ob/ob mice than in lean counterparts (60, 61,142,147). Low rates of NE turnover in BAT of ob/ob mice are observed before visual signs of gross obesity are evident, indicating that low SNS activity in BAT of ob/ob mice is not simply a secondary

consequence of obesity (61). SNS stimulation of BAT metabolism is also depressed in obese (fa/fa) rats (67,136). BAT of fa/fa rats receives less sympathetic innervation than BAT of lean controls and this is observed after 3-4 months of age, suggesting that low SNS activity in BAT of obese rats is secondary to their development of gross obesity. Thermogenic capacity of BAT, as assessed by GDP binding to BAT mitochondria is reduced in ob/ob mice and rats when they are housed below their thermoneutral zone (35,36,43,45,50).

Adrenal secretions, specifically corticosterone, have long been known to play an important role in controlling energy balance through the regulation of food intake and energy expenditure. In genetically obese mice and rats, higher plasma corticosterone levels are associated with hyperphagia and defective BAT thermogenesis (29,37,47,69,109, 110). Adrenalectomy reduces energy intake and energy retention, and it increases BAT thermogenic activity in obese rodents (29,48,50,71, 108,132). Administration of corticosterone to adrenalectomized obese animals reverses many of the effects of adrenalectomy; corticosterone administration causes an increase in efficiency of energy retention and a decrease in BAT thermogenic activity (29). The effects of glucocorticoids on BAT thermogenesis appear to be mediated by changes in SNS activity, since adrenalectomy increases the NE turnover rate and NE concentration in BAT (132).

However, the outcome of adrenalectomy is affected by the diet consumed by animals. In ob/ob mice, adrenalectomy reduced the efficiency of energy retention and energy density of body weight gain to values approximating those in lean mice when they were fed a nonpurified high-carbohydrate stock diet, but adrenalectomy failed to

alter these parameters in ob/ob mice fed a semipurified high-fat or high-glucose diet (37,146). The changes in energy efficiency and body composition in adrenalectomized ob/ob mice fed a high-carbohydrate nonpurified stock diet are associated with increased SNS activity (132) and increased thermogenic capacity (48,50,71) in BAT. Therefore, adrenalectomized ob/ob mice fed purified high-fat or high-carbohydrate glucose diets may have maintained a high efficiency of energy retention partly because of failure of adrenalectomy to activate BAT metabolism. However, data have not been published to support this possibility.

Environmental temperature is another factor that affects energy balance and BAT thermogenesis. Exposure to cold increases energy expenditure to maintain body temperature in experimental animals (25,126,147), and the low capacity for thermoregulatory thermogenesis is claimed to be the primary factor for the development of obesity in ob/ob mice housed below thermoneutral temperature (43,45). When ob/ob and lean mice are housed in a warm environment (30-35'C) to eliminate the need for thermoregulatory thermogenesis, BAT thermogenic activity is equally low in both ob/ob and lean mice, but ob/ob mice still retain dietary energy more efficiently than lean mice (99,126). This result suggests that factors other than BAT contribute to the high efficiency of energy retention in ob/ob mice. Effects of adrenalectomy on energy balance and BAT metabolism have not been investigated in ob/ob mice housed at a thermoneutral temperature.

Therefore, the purpose of my research is to examine whether diet composition (high-fat and high-carbohydrate) and warm environmental temperature (35'C) interact with glucocorticoids to influence energy

balance and BAT metabolism in ob/ob mice. The hypothesis was that consumption of a high-fat or a high-glucose diet, and exposure to a warm environment, would interfere with activation of BAT metabolism in adrenalectomized ob/ob mice, and cause the adrenalectomized ob/ob mice to maintain high efficiency of energy retention.

1.2. ENERGY BALANCE IN GENETICALLY OBESE RODENTS

The first law of thermodynamics states that within the total system, energy is neither lost nor gained. This principle of energy conservation also holds for biological energy transformation. Whole animal energy balance can be formulated as energy intake = energy expenditure + energy storage. While energy intake is solely represented by metabolizable energy of the foods consumed, energy expenditure is the sum of 3 main components: basal metabolic rate (BMR), the energy cost of physical activity, and thermogenesis.

1.2.1. Energy Intake

The overall control of energy intake is integrated in the central nervous system. The hypothalamus is an important control center within the brain: destruction of the ventromedial hypothalamus is followed by hyperphagia and obesity, and damage to the lateral hypothalamus reduces food intake (10,64,94). It has been generally assumed that energy intake is the major controlling factor in energy balance regulation, and that a defective appetite control (i.e. hyperphagia) is the primary cause of obesity in man and experimental animals. Indeed, an increase in energy intake by genetically obese

(ob/ob) mice and rats (fa/fa) has been reported by a number of investigators (1,9,67,71). The regulation of food intake in adult ob/ob mice is abnormal, showing incomplete compensation of caloric intake in response to dilution of the diet with indigestible substances (92), but overconsumption of calories in response to a high-fat diet (31). However, the lateral and ventromedial hypothalamic centers regulating energy intake appear intact in ob/ob mice. Food intake of preobese ob/ob mice is very close to normal at this stage (69) and several studies indicated that during the suckling period energy intake is similar in ob/ob and lean siblings even though excess body fat deposition and body weight gain has already begun (69,128). This suggests that low energy expenditure rather than hyperphagia is the primary cause of the initial excessive fat deposition in preobese ob/ob mice.

1.2.2. Energy Expenditure

Results of early studies show that obesity could develop in genetically obese (ob/ob) mice and rats (fa/fa) in the absence of hyperphagia, and indicate that these mutants expend less energy and retain dietary energy with a greater efficiency than normal in order to accumulate more fat (1,9,71). Pair-feeding studies with several different obese mutants (fa/fa rat and ob/ob mice) have confirmed that a common feature of genetically obese rodents is the ability to gain excess body fat on an intake no greater than that of lean littermates (1,71). When obese mutants are fed ad libitum their energy gain is even higher than in the pair-fed condition indicating that hyperphagia also contributes significantly to development of obesity in these

animals. Energy expenditure per unit body weight, measured by oxygen consumption, is decreased in preobese ob/ob mice at 14-21 days of age, before the visual signs of obesity develop (58), and also in adult ob/ob mice (129). Body temperature of ob/ob mice is likewise depressed at ambient temperatures of 20-25'C (128,133). Indeed, the decreased oxygen consumption and the hypothermia resulting from the hypometabolism appear as two of the earliest detectable defects in the ob/ob mice. Similar alterations are evident in fa/fa rats (57). Thus, it appears that in obese mutants, development of obesity is due to the high efficiency of energy retention caused by reduced energy expenditure, with hyperphagia only occurring at a later stage.

1.3. METABOLIC BASIS FOR HIGH ENERGY EFFICIENCY

The metabolic basis for this high efficiency of energy retention in obese rodents has been actively pursued for several decades, and several mechanisms have been proposed by which metabolic efficiency might be achieved.

i) Uncoupling of oxidative phosphorylation

The metabolism of metabolic fuels might be uncoupled from the formation of adenosine triphosphate (ATP) or other high energy phosphate bonds. Increased heat production in BAT falls into this mechanism. Nicholls (87) has suggested that BAT mitochondria possess a proton conductance pathway which allows protons to 'leak' back across the inner mitochondrial membrane without obligatory synthesis of ATP. Thus, respiration is uncoupled from phosphorylation, with a resultant increase in heat production (see chapter 1.3.1.1).

ii) Increased utilization of ATP

Increased utilization of ATP stimulates mitochondrial substrate oxidation with subsequent increase in heat production. For example, Horwitz (52) has proposed that utilization of ATP by the Na,K-ATPase enzyme contributes significantly to energy expenditure (see chapter 1.3.2). Also there might be differences in the rates at which ATP or other high energy phosphate bonds are metabolized by other enzymes. Newsholme (84) suggested that several substrate cycles are responsible for metabolic regulation and weight control (see chapter 1.3.3).

1.3.1. Brown Adipose Tissue

Although the thermogenic function of brown adipose tissue (BAT) has been known for over 20 years, only recently was it realized that the energy expended for thermogenesis in BAT can contribute substantially to total energy expenditure. The quantitative importance to thermogenesis was shown by Foster & Frydman (27) to be such that the BAT, which constitutes only 1-2 % of body weight of rats, accounts for 60 % or more of the rise in metabolic rate on maximal stimulation by cold acclimation for 4 wks, and up to 26 % of total body heat production under these conditions. Blood flow to BAT is capable of a 27-fold increase, and can account for up to 25 % of the cardiac output. These variations in BAT activity suggest that BAT could be of importance in energy balance and the etiology of obesity .

1.3.1.1. Mechanism of heat production in brown adipose tissue

The principal mechanism of heat production by BAT involves the uncoupling of oxidative phosphorylation. BAT mitochondria have a

unique proton conductance pathway that permits them to become reversibly uncoupled, and thus to oxidize both endogenous and exogenous substrates at an extremly high rate independent of the need to phosphorylate ADP (87). The mechanism involves a specific protein (32,000 D polypeptide) which is on the outer surface of the inner mitochondrial membrane and is variously known as thermogenin, uncoupling protein, nucleotide binding protein, and GDP-binding protein. The sequence of BAT thermogenesis begins with a stimulus, such as cold stress or diet, which activates the sympathetic nervous system (SNS) which densely innervates BAT (87). Norepinephrine (NE), released by the sympathetic nerve endings, binds to beta-adrenergic recepters on BAT plasma membranes and activates adenyl cyclase, leading to increased c-AMP concentration. The increased c-AMP stimulates a hormone-sensitive lipase and accelerates subsequent lipolysis in the BAT cells. The released free fatty acids are used as fuel for increased mitochondrial oxidation. Fatty acids released during lipolysis stimulate the unique proton conductance pathway while purine nucleotides inhibit this pathway. The exact mechanisms for activation and inhibition of the pathway are yet to be established.

1.3.1.2. Assessment of thermogenesis

The only quantitative method for assessment of BAT thermogenesis in intact animals is measurement of its blood flow, with radioactive microspheres, and of the A-V difference in oxygen tension across the various BAT depots (27). Foster and Frydman (27) introduced this method and demonstrated that BAT could account for 60 % of the calorigenic response of the NE stimulus in cold acclimated rat.

However, this method have several disadvantages. First, it needs several cannulations for introduction of microsphere markers and withdrawal of arterial or venous blood. Second, BAT depots are distributed at many different sites, and the differently located masses of BAT may not exhibit a uniform increase in blood flow with increase in metabolic rate.

Indirect measures of BAT thermogenesis include the assessment of the increase in whole body metabolic rate in response to NE. This approach assumes that the increases in metabolic rate are caused by increased BAT metabolism, an assumption that is not entirely valid. Measurement of NE turnover in BAT provides information about the activity of the sympathetic nervous system, which is the key regulator of the BAT thermogenesis, in the tissue. It is a better indicator of sympathetic tone than are changes in concentration of NE in BAT, which may remain constant or even decrease when the sympathetic nervous system is activated (14). It also provides more direct information than does measurements of plasma or urinary NE. One approach to measuring NE turnover has been to follow the decline in NE content of innervated organs after injection of a drug, such as a-methyl-p-tyrosine, to block synthesis of NE (14). Another approach has been to measure the decline in ^{3}H-NE in innervated organs after injection of ^{3}H-NE (14,141). I found that the results from these two methods were comparable (see chapter 2.3).

Several in vitro methods have been used to give a qualitative index of thermogenic state of BAT. The ability of the 32,000 D protein to bind purine nucleotides, such as GDP, provides a means of assessing the capacity of BAT thermogenesis (87). The extent of

binding depends both on the concentration of the uncoupling protein and on the accessibility of the sites to externally added nucleotides. Binding is low in mitochondria from quiescent BAT, and is rapidly increased when the tissue is acutely stimulated by activation of its sympathetic nerves or by administration of NE. A rapid increase in binding, in the absence of changes in the amount of the protein, is associated with ultrastructural changes in the mitochondria and occurs during acute cold exposure (123). It has been demonstrated that such an increase can be observed even in the presence of the protein synthesis inhibitor cycloheximide (25), and is thus not due to the synthesis of new 32,000 D protein. This increase in GDP binding is interpreted as an "unmasking" of sites already present in the membrane. The fuctional significance of this "unmasking" is not entirely clear, but it serves as a useful index of the thermogenic activity of the tissue.

Nedergard and Cannon (81) have suggested that unmasking of GDP binding sites could result from mitochondrial swelling, as indicated by changes in the volume of the mitochondrial matrix. The significantly increased permeability of the inner membrane to H+ (or OH-) and Cl- occurs via the 32,000 D protein, and can be monitored by measuring the rate of GDP-inhibitable swelling (18). Sundin reported that swelling correlated with GDP binding in mitochondria from cold adapted rats (121). However, Swick and Swick (122) were unable to demonstrate any change in GDP binding associated with mitochondrial swelling. Recently they reported that Mg++ participates in the activation of GDP binding sites, and proposed a covalent modification as a mechanism of unmasking of GDP binding sites (123). In addition,

I found that incubation of isolated BAT mitochondria with Mg++ enhanced the binding of ^{3}H-GDP (see chapter 4.3).

Some measurements of the amount of BAT present in an animal may be inaccurate because of difficulty in recognizing the tissue visually. The wet weight of BAT is not an accurate measure of metabolic capacity, serving only as a very rough index of amount of lipid stored in the tissue. Measurement of total protein, DNA, or a mitochondrial marker enzyme such as cytochrome oxidase provides a better index of the amount, but not of the metabolic activity of the tissue. The recent development of an immunoassay for the measurement of amount of 32,000 D protein (97) provides a more direct means of identifying BAT thermogenic capacity than above methods, but this method does not provide an indication of the extent of "masking" of the protein present when the animal was killed.

1.3.1.3. BAT thermogenesis in obese rodents

Several studies with a number of different animal models of obesity have shown that the high metabolic efficiency of obese animals is associated with defective control of the sympathetic nervous system and adaptive thermogenesis in BAT. For example, measurements of blood flow and the A-V difference in oxygen across interscapular BAT in ob/ob and lean mice before and after injection of NE showed that the smaller metabolic response of ob/ob mice to NE was almost totally accounted for by a smaller response of BAT (27). It was also calculated that the diminished capacity for BAT thermogenesis in ob/ob mice could account for their lower energy expenditure and the consequent sparing of energy, which along with hyperphagia, would

promote the development of obesity.

The sympathetic nervous system is thought to be a key regulator of BAT thermogenesis, and SNS activity indicated by NE turnover is 50 % lower in BAT of ob/ob mice than in leans when they are housed at 20-26'C and fed a stock diet (60,61,142,147). Thermogenic activity assessed by GDP binding to BAT mitochondria is also reduced in ob/ob mice when they are housed below their thermoneutral zone (35,36,43,45,50). Low rates of NE turnover and GDP binding in BAT of ob/ob mice are observed before visual signs of gross obesity are evident, indicating that these defects are not simply a secondary consequence of obesity (61).

One of the abnormalities observed in ob/ob mice is the failure to maintain body temperature upon exposure to a cold environment (73). Exposure of animals to cold activates the SNS. When ob/ob mice are acutely or chronically exposed to cold (4'C or 14'C), NE turnover in BAT increases markedly (60,61,142,147). Thus, the SNS of ob/ob mice has the inherent ability to respond when severely challenged. However, these mice fail to respond with increased BAT thermogenesis at 4'C. This failure to activate nonshivering thermogenesis in BAT leads the animal to be sensitive to cold so that ob/ob mice die within a few hours when exposed to 4'C (73,128,129). Ob/ob mice can, however, survive exposure to less severe cold (14'C) (45,60). Although body temperature is still lower in ob/ob mice than in leans after 10-20 days at 14'C, BAT has been shown to be activated in these mice (100).

It has been sugested that an impairment of the capacity for diet-induced thermogenesis contributes to the increase in efficiency

of energy retention and consequent obesity in obese rodents. The fact that the hyperphagia of genetically obese animals leads to the accelerated development of obesity clearly indicates that the diet-induced thermogenesis of these animals is limited. In an experiment using a cafeteria diet (animals are fed a variety of palatable human foods), lean rats were found to deposit only 4 % of their excess energy intake during overfeeding, suggesting that they have a very substantial capacity to dissipate energy by diet-induced thermogenesis. In contrast, fa/fa rats deposited 55 % of their extra energy intake on the cafeteria diet. Similar results have been shown with ob/ob and lean mice. For the 5 week post-weaning period, ob/ob mice converted 3-4 times more dietary energy to body energy than lean mice, even though ob/ob mice consumed only 20-40 % more energy (69). Therefore it appears that regulatory diet-induced thermogenesis is defective in the obese mutant. Thus, obese mutants are defective in both diet-induced thermogenesis and cold-induced nonshivering thermogenesis, and this double defect was thought to provide an explanation for the deficit in energy expenditure and the high metabolic efficiency of the obese rodents. However, as I found and will discuss in chapters 2,3, and 4, the situation is more complex than what first appears; diet composition and environmental temperature can influence BAT metabolism in ob/ob mice without necessarily causing parallel changes in efficiency of energy retention in these animals.

1.3.2. Na,K-ATPase

It is claimed that a large proportion of energy expenditure is

associated with the maintenance of electrolyte equilibrium across the cell membrane, particularly of sodium (55). Na,K-ATPase utilizes the energy released by hydrolysis of ATP, and is therefore distinct from the BAT proton conductance pathway which decreases the efficiency of ATP formation. The increased utilization of ATP stimulates mitochondrial substrate oxidation with subsequent increases in heat production. Horwitz has suggested that heat is produced in BAT by the sodium pump activity itself and by increased cellular respiration which is stimulated by a signal generated from the activity of the sodium pump (52). However, its contribution to thermogenesis is probably small. The first observation suggesting a possible link between Na,K-ATPase and obesity was the report in 1978 that ob/ob mice had lower Na,K-ATPase activity in homogenates of liver and skeletal muscle than lean counterparts (135). Several investigators confirmed this result (68,115). Because these genetically obese mice have a well defined abnormality in cellular thermogenesis, these observations provided a potential biochemical mediator at the cellular level for this thermogenic deficiency. Although alterations in Na,K-ATPase activity and/or a decrease in the enzyme units have been demonstrated in liver and muscle of ob/ob mice, others have failed to demonstrate any difference between ob/ob and lean mice in the number of Na,K-ATPase enzyme units in skeletal muscle (20) or in the enzyme activity in BAT (62). Other obese models such as GTG obese mice and genetically obese fa/fa rats do not appear to have a defective Na,K-ATPase activity (12,135). Therefore the lack of clear results reduces the likelihood that this mechanism is a common primary factor in the etiology of obesity. Furthermore, inhibition of Na,K-ATPase by

a minor fraction of the thermogenic effect is mediated by sodium pumping (19).

1.3.3. Substrate cycles

Substrate cycles, also referred to as futile cycles, are another mechanism that might be involved in heat production and modulation of energy efficiency. These cycles occur when there are 2 opposing metabolic pathways in the cell with seperate enzymes catalyzing forward and reverse reactions. The continual cycling of substrates through a series of synthetic and degradative reactions which have the same initial and final energy status (i.e.reversible) requires ATP and therefore involves an obligatory loss of energy as heat. A number of such thermogenic substrate cycles have been suggested;

i) Glycolytic pathways

glucose to glucose-6-phosphate cycle

fructose-6-phosphate to fructose-1,6-diphosphate cycle

pyruvate to phosphoenolpyruvate cycle

Newsholme proposed that the maximum rate of heat production by the phosphofructokinase/fructose-1,6-bisphosphatase substrate cycle in human muscle could account for 50 % of the daily caloric intake (84). Then, it is possible that genetics could predetermine the activity of fructose diphosphatase which would, in turn, affect the rate of substrate cycling. Also the biochemical factors that control the activity of both phosphofructokinase and fructose diphosphatase could be genetically affected. However, there is currently not enough data to support this hypothesis. One study has shown that phosphofructokinase is less responsive to norepinephrine stimulation

in genetically obese rats than normal rats (93).

ii) Triglyceride (TG) hydrolysis-reesterification cycle

This involves the hydrolysis of adipose tissue TG to FFA, followed by their reesterification with a-glycerophosphate and requires 7 mole of ATP per mole of TG cycled. In mouse adipose tissue, the rate of TG/FFA cycling is increased by feeding (15) which suggests an involvement of this mechanism in diet-induced thermogenesis, and this increase is inhibited by beta-adrenoreceptor antagonist.

The problem with the hypothesis that substrate cycles play an important role in thermogenesis is that the amount of heat produced from these cycles represents only a small percentage of the heat known to be produced by the animals (15). Also data obtained from ob/ob mice indicate that maximum activities of key enzymes related to several substrate cycles are increased rather than decreased, thus suggesting that hormonal control of these futile metabolic pathways would be key if these cycles are to participate in the control of energy balance (85).

1.3.4. Protein Turnover

Nitrogen balance studies have shown that, when given the same amount of dietary protein, ob/ob mice tend to deposit amino acid carbon skeletons in the form of fat, rather than muscle protein. This is in agreement with the enhanced energetic efficiency of obese animals, since there is a close relationship between body protein metabolism and the rate of energy expenditure (144). Pullar and Webster (95) utilized data from obese fa/fa and lean rats to calculate that 2.25 kcal of metabolizable energy is required to deposit 1.0 kcal

that 2.25 kcal of metabolizable energy is required to deposit 1.0 kcal of protein, and that 1.36 kcal metabolizable energy is required for the same amount of fat deposition. It is then clear that as obese rodents direct nutrients towards adipose tissue and away from lean tissue, this contributes to their high metabolic efficiency. Miller et al. (78) suggested that protein turnover may be an important cycle for the regulation of energy balance in mice and that this cycle is impaired in ob/ob mice. On the other hand, the fractional breakdown rate of skeletal muscle estimated from urinary excretion of 3-methylhistidine is greater in ob/ob mice than in leans, with estimates of similar fractional synthesis rates (131). Therefore, it is difficult at the present time to assess the importance of the protein turnover in the enhanced efficiency of energy retention observed in obese mice and rats.

1.4. NEURAL CONTROL OF THERMOGENESIS

1.4.1. Central Control

Although BAT has been established as an important effector of thermogenesis, little is known about the central control mechanisms involved. SNS is a key regulator of BAT. However, it is possible that the parasympathetic nervous system (PNS) also participates in this process.

The hypothalamus has long been considered to be the integrating center for control of food intake. Reciprocal modulations in the regulation of food intake and autonomic nervous system after lateral or ventromedial hypothalamic lesions are well documented. The

ventromedial hypothalamus (VMH) apparently acts as an integrator for energy balance regulation; affecting satiety (10,11), activation of sympathetic nervous activity to BAT (51), and inhibition of parasympathetic nervous activity. Lesions of the VMH generally produce hyperphagia, hyperinsulinemia (10), and reduce sympathetic nervous system activity (10,111). VMH-lesioned animals become obese even when their intake is restricted to that of controls, indicating increased metabolic efficiency in the lesioned animals, and in rats lesioned shortly after weaning obesity can develop in the absence of any hyperphagia (10).

In contrast to hyperphagia and obesity which is induced by destruction of the ventromedial hypothalamus, destruction of the lateral hypothalamus (LH) produces large decreases in food intake and increases in metabolic rate (10). LH lesion-induced thermogenesis seems to be mediated, in part, by increased activity of the sympathetic nervous system since norepinephrine turnover and urinary catecholamines are elevated after the lesion (10,138).

Other hypothalamic areas can also influence food intake and BAT thermogenesis and participate in energy balance regulation. Lesioning of paraventricular nucleus (PVN) results in hyperphagia and obesity (3,65), but data on the effect of PVN lesions on sympathetic activity are contradictory. BAT norepinephrine turnover and sympathetic firing rates in rats with PVN lesions were similar to control rats (111). Yoshimatsu (139), on the other hand, found a decrease in sympathetic activity in the splanchnic nerve of rats with PVN lesions. Therefore, PVN lesion-induced obesity may not result from alterations in the autonomic nervous system.

Genetically obese (ob/ob) mice have reduced brain weights and cortical brain volumes compared to lean mice; morphometric analyses of ob/ob mice brains reveal a significant decrease in soma cross sectional areas of individual neurons in certain brain regions including the VMH, which is indicative of a hypothalamic dysfunction in this genetic obesity syndrome (5).

1.4.2. Peripheral Control

1.4.2.1. Sympathetic control

The sympathetic origin of a large fraction of the neural afferents to the BAT was established by the finding of a high tissue catecholamine content and by application of histochemical fluorescence technique. Thermogenic responses of BAT are principally controlled by its sympathetic innervation (17). NE and epinephrine act directly on the brown adipocyte to activate the proton conductance pathway and possibly other thermogenic systems. A physiological role for NE has been established for BAT thermogenesis during cold exposure, overfeeding, and arousal from hibernation (103). The findings that concentrations of exogenous NE required to cause significant thermogenesis are far in excess of normal circulating levels of NE support the importance of local release of NE from sympathetic nerve endings in close proximity to the brown adipocyte (100).

Both beta- and alpha-adrenoreceptors have been shown to be present in BAT and both receptors are required for a maximum response in vivo (28). However, beta- rather than alpha-adrenoreceptors play the dominant role in mediating NE-stimulated thermogenesis in the whole animal, BAT in vivo (103) and brown adipocytes in vitro (79).

Neverthless, experiments both in vivo and in vitro suggest that alpha-adrenoreceptor stimulation has a significant influence (34). As much as 20 % of the effect of NE on the respiration of hamster brown adipocytes in vitro may be due to stimulation of alpha-adrenoreceptors of the alpha 1-subtype (79). Unlike beta-adrenoreceptor mediated thermogenesis, this effect does not appear to be due to a c-AMP mediated activation of the proton conductance pathway. It has been proposed that alpha-adrenoreceptors participate in NE-induced turnover of phosphatidylinositol (79), gating of calcium channels, initial electrical changes in the plasma membrane (34), stimulation of Na,K-ATPase (111) and stimulation of T4 5'-deiodinase (119). The exact function of these changes is not yet clear and they play a relatively minor role in the thermogenic response of isolated cells to NE (79).

Sympathetic nervous system activity in genetically obese mice and rats has been widely studied. NE turnover has been measured in BAT, heart, liver and pancreas of these animals. When housed at 20-26'C and fed stock diets, NE turnover in BAT is approximately 50 % lower in ob/ob mice than in leans (60,62,142,147). This lowered SNS activity has been demonstrated even at an early age, before obesity is visually evident (61). The low NE turnover in BAT of ob/ob mice is not a consequence of generalized depression of SNS activity because NE turnovers in other organs of ob/ob mice are essentially comparable to those reported for leans (61). SNS stimulation of BAT metabolism in fa/fa rats is also depressed as it is in ob/ob mice (67,136).

Exposure of animals to cold activates the SNS. When genetically obese mice or rats are acutely exposed to cold, NE turnover in BAT and

several other organs increases markedly (see chapter 1.7). Diet is another factor that is capable of influencing the activity of the SNS in animals (see chapter 1.6). When ob/ob mice are fed a cafeteria diet, NE turnover in BAT increases to the level of leans (44). Based on these data one can conclude that genetically obese mice and rats have generally comparable capacity for SNS activity to lean counterparts, and that the primary defect may be in mechanisms controlling norepinephrine release from sympathetic nerve endings to BAT in obese mice and rats.

1.4.2.2. Parasympathetic control

Little is known about the influence of the parasympathetic nervous system (PNS) on energy balance and thermogenesis, although an increased parasympathetic activity has been implicated in the development of obesity. Powley and Opsahl (94) were the first to report that subdiaphragmatic vagotomy completely reverses the hyperphagia and obesity produced by VMH lesions in the rat. Several investigators replicated this finding and further observed that vagotomy prior to the VMH lesions or knife cuts prevents lesion-induced hyperphagia and weight gain (53). Also, vagotomy increases oxygen consumption and blood pressure in cold acclimated rats. The hyperinsulinemia that characterises obesity has been largely attributed to an overactive parasympathetic vagal activity, and subdiaphragmatic vagotomy or transplantation of pancreatic beta-cells attenuates the VMH-induced hyperinsulinemia (53). Genetically obese fa/fa rats fail to increase oxygen consumption after food, but this response can be completely restored to normal by atropine treatment,

indicating high parasympathetic activity may also be responsible for the defective thermogenesis and increased fat deposition in these mutants (102). However, conflicting results have also been obtained. Some researchers have shown that vagotomy produces only a partial attenuation or no effect on VMH obesity, and in genetically obese fa/fa rat, vagotomy does not reverse the obesity (90). Therefore, the lack of consistent results makes the importance of parasympathetic control in the etiology of obesity unclear.

1.5. HORMONAL CONTROL OF THERMOGENESIS

1.5.1. Insulin

Insulin has an important role in the regulation of both energy balance and BAT thermogenesis. Injection of insulin is followed by an increase in food intake. The effect is dose-dependent and will produce obesity (64). There are two distinct roles of insulin in control of BAT. First, insulin acts directly to modulate glucose metabolism and second, insulin acts centrally to regulate BAT SNS activity. Insulin promotes glucose utilization and lipogenesis in BAT (82), and Schackney and Joel (113) found glucose uptake and fatty acid synthesis are stimulated in BAT slices by exogenous insulin. The possibility that the action of insulin is directly on the hypothalamus is supported by the observations of Debons et al. (29). They showed that gold thioglucose does not injure the VMH of hyperglycemic diabetic animals. Parenteral administration of insulin to such diabetic animals lowers blood glucose and restores the sensitivity of the VMH to the destructive consequences of treatment with gold

thioglucose. Furthermore, intrahypothalamic injection of insulin to diabetic animals restores its sensitivity to the destructive effects of gold thioglucose. This suggests that insulin might act directly on the ventromedial nucleus.

Several studies have shown a requirement for insulin in cold and diet induced thermogenesis in BAT (101). BAT thermogenesis is suppressed in insulin-deficient diabetic rats and in insulin-resistant diabetic mice, such as ob/ob mice and db/db mice (35,45). Both GDP binding and the content of the 32,000 D protein were reduced in diabetic rats compared to normo-insulinemic controls, and were increased in hyperinsulinemic rats compared to controls. Severely diabetic rats could not sustain the increased metabolic rate needed for survival in a cold environment. Development of insulin resistance in BAT of ob/ob mice also has important consequences for thermogenesis. At 4 wks of age, before insulin resistance develops, the ob/ob mouse shows the normal increase in GDP binding on acute exposure to cold. However, by 5 wks of age, when insulin resistence has developed in BAT, the response to cold is greatly blunted (76). The reversal of insulin resistance by administering an oral hypoglycemic agent (ciglitazone) to ob/ob mice leads to the complete restoration of the acute increase in GDP binding in response to cold (76).

1.5.2. Glucocorticoids

Adrenal function has long been known to be implicated in the regulation of food intake and body weight in man (47) and experimental animals (8). Prolonged administration of glucocorticoids causes

obesity in mice and rats, and elevated plasma corticosteroids and adrenal hypertrophy in genetically obese rodents have been claimed to be associated with hyperphagia and obesity (29,37,47,69,109,110). Adrenalectomy reduces food intake and body weight gain in rats (4) and in genetically obese rodents (29,37,71,108), and subsequent administration of adrenal glucocorticoids rapidly reverses this effect (29).

Glucocorticoids have also been implicated in the control of energy expenditure. Corticosteroid treatment and excessive amounts of glucocorticoids in genetically obese rodents reduces the activity of the thermogenic pathway in BAT from mice and rats, and adrenalectomy increases energy expenditure in these animals (29,48-50). Several laboratories have demonstrated increased activity of the SNS as measured by increased norepinephrine turnover in BAT, as well as increased binding of GDP to BAT mitochondria following adrenalectomy of genetically obese rodents (48,50,71,132). Adrenalectomy also lowers plasma insulin concentrations (145), removes the insulin resistance in muscle (91), and restores the activity of the sodium pump to normal (115). Thus, glucocorticoids reciprocally modulate both energy intake and energy expenditure with high levels of glucocorticoid promoting positive energy balance and low levels facilitating negative energy balance.

Bray (9) proposed mechanisms through which adrenal hormones could induce hyperphagia by interfering with the hypothalamic hunger-satiety mechanism. Also, recently it has been suggested that the effect of glucocorticoids may be modulated by the corticotropin releasing factor (CRF) in the PVN. After adrenalectomy, the negative feedback signal

produced by gluococorticoids is absent, thus CRF will be released into the hypothalamic portal circulation to stimulate adrenocorticotropic hormone (ACTH) output from the pituitary. This increased CRF may also serve as the principal stimulus for the reduction of food intake and for the increased sympathetic activity following adrenalectomy. Indeed, CRF injected into the ventricle of the normal rat and fa/fa rat decreases food intake, and body weight gain (2,98), and increases circulating levels of epinephrine and norepinephrine (16) and BAT thermogenic activity assessed by GDP binding to mitochondria (2).

Glucocorticoids also are known to have a general action on protein metabolism in skeletal muscle opposite to that of insulin, producing a catabolic rather than an anabolic response. Loss of body weight, marked atrophy of certain skeletal muscles (125), and decreased rates of muscle protein synthesis (96,114,117) after glucocorticoid administration have been consistently observed. The role of protein degradation in the loss of skeletal muscle protein after glucocorticoid administration is less certain, with some studies showing no change in the degradative rate (117) and some showing increases (125). Adrenalectomy, which has been shown to increase muscle mass in ob/ob and db/db mice to normal (114), returns protein synthesis rates in muscles of db/db mice to those of lean counterparts. Although the classic experiments of Long (70) demonstrating the effects of adrenalectomy and steroid treatment on protein and carbohydrate metabolism in vivo have led to the general view that the net stimulation of protein catabolism in muscle by glucocorticoids serves to make amino acids available for gluconeogenesis in liver, the site of action of glucocorticoids on

muscle protein turnover is presently unknown. However, an impairment in peptide-chain initiation has been suggested to be responsible for the decrease in protein synthesis observed in glucocorticoid-treated animals (96).

1.5.3. Thyroid Hormone

Thyroid hormones exert potent effects on metabolic rate. Circulating triiodothyronine (T3) levels are elevated in cold exposure and hyperphagic animals. Thyroid hormone is required for the thermogenic response of BAT to NE (41). However, since only permissive amounts of thyroxine are sufficient for normal thermogenic response of BAT in thyroidectomized rats, thyroid hormone probably does not exert a direct role in these changes (130). Moreover, provision of excess exogenous thyroid hormone may result in suppression of BAT thermogenic function in intact animals (104). The effects of thyroid hormones on BAT thermogenesis are complicated by the interactions between thyroid hormones and catecholamines. These two systems appear to act in concert to promote thermogenesis whilst also modulating the activity of each other (41). There are reports of thyroid hormones modulating beta-adrenergic receptor number in many tissues, including BAT (121). These complex interactions are further complicated by the recent report that BAT converts T4 to T3 (66) and that the 5'-deiodinase which catalizes this conversion is stimulated by NE (119). It has been shown that thyroid hormones sensitize BAT to NE (39), and it has been proposed that the reduction of the thyroid hormone levels during a fast reduces the response of BAT to NE (95). However, most studies show that thyroid hormones suppress BAT

thermogenesis by stimulating thermogenesis in other tissues and thereby indirectly suppressing sympathetic activity because of the lowered requirement for BAT thermogenesis (89).

The low metabolic rate of the ob/ob mice has suggested that it might be hypothyroid. However, ob/ob mice appear to have no major hypothalamic-pituitary-thyroid abnormality and the T3 level in ob/ob mice blood is normal or above normal for most of its life (11). It is, however, possible that some tissues of the ob/ob mice may fail to respond to the T3 present in blood. Hillgartner suggested T3 availability to target tissue is impaired in ob/ob mice (40). These mice have an exaggerated increase in metabolic rate in response to a dose of thyroid hormone that is without effect in lean mice (46,68), suggesting a partial resistance to the effect of endogenous T3. Moreover, treatment of ob/ob mice with thyroid hormone permits a normal thermogenic response to injected NE and of its BAT to cold exposure (46). Since the only role for T3 in BAT function and growth appears to be a permissive one that allows the acute thermogenic effect of NE on the tissue (130), the effect of thyroid hormone on BAT of the ob/ob mice is probably due to improved responsiveness to BAT.

1.6. DIET COMPOSITION AND THERMOGENESIS

The composition of the diet consumed by animals affects energy balance and BAT thermogenesis. Thermogenesis can be activated by overconsumption of either carbohydrate or fat, and is particularly sensitive to protein deficiency (44,49,59,77,105,140-143). The refined sugars, sucrose and glucose, stimulate sympathetic nervous

system in experimental animals and man. In the rat, ad libitum access to dilute solutions of sucrose increases norepinephrine turnover rate in a variety of tissues including heart, liver, pancreas, and kidney (140,141). Also Tappy et al. (124) demonstrated that fructose ingestion in man elicits significant increases in thermogenesis despite low plasma insulin levels and suggests that the stimulation of thermogenesis after carbohydrate ingestion is not dependent on increases in the insulin per se.

Rats (105,143) and mice (44) overfed a cafeteria diet have increased SNS activity and BAT thermogenic activity. Since cafeteria diets are usually high in fat content, fat might have a stimulatory effect on the sympathetic nervous system. Indeed, feeding purified high fat diets rich in polyunsaturated fatty acids lead to an activation of thermogenesis in BAT (77). Rothwell et al. (107) examined the effects of a cafeteria diet with varying fat contents, and found that rats fed a high fat cafeteria diet showed the largest energy expenditure as compared to those fed a low fat diet. Also, Kuroshima et al. (63) observed that a high fat diet improved the cold tolerance of rats, and suggested that the high fat diet increased nonshivering thermogenesis.

Cafeteria diets low in protein produce particularly large increases in BAT activity (105). Purified low protein diets decrease energy efficiency and increase energy expenditure by increasing metabolic rate with similar or even slightly lower energy intake than controls (59,106). The reduced energy efficiency has been associated with increased sympathetic activation of BAT (59,106), increased concentration of triiodothronine (T3), and thyroxine (106). Therefore

both the amount of energy consumed and the composition of the diet affect energy balance and energy expenditure.

1.7. ENVIRONMENTAL TEMPERATURE AND THERMOGENESIS

Environmental temperature is another factor that affects energy balance and BAT thermogenesis. Voluntary energy intake and body weight regulation are highly dependent on environmental temperature and closely related to thermoregulation. Brobeck (13) suggested that energy intake was largely determined by the requirement to produce heat for maintenance of body temperature. It is now obvious that intake control is subject to many diverse influences, but Brobeck's thermostatic hypothesis does suggest that various relations could exist between energy balance regulation and thermoregulation. Acclimation to cold is associated with hyperphagia and a large increase in the capacity of animals to respond to NE by increasing metabolic rate. The large metabolic response is known to occur primarily in BAT (27), and development of the enhanced response is associated with increased SNS activity of the tissue (60,61, 141,143,147), mitochondrial mass (25,126), and an activation of the proton conductance pathway (126)

When ob/ob mice are acutely or chronically exposed to cold, SNS activity determined by NE turnover rate in BAT and several other organs (heart, pancreas, lung and skeletal muscle) increases markedly (60,61,142,147) suggesting normal stimulatory function of SNS in this condition. However, these mice are unable to fully and rapidly activate BAT thermogenesis in response to increased SNS activity.

This leads to hypothermia during acute cold exposure (43,45).

Rats and mice housed under common laboratory conditions at 20-25'C are mildly cold exposed. Exposure to environmental temperatures within the thermoneutral range for rats (28-30'C) and mice (33-35'C) reduces energy intake and energy expenditure to the minimum consistent with maintenance requirements and thermoregulation (99,126). This reduced energy expenditure is associated with reduced BAT SNS activity and BAT thermogenic activity. Obese (ob/ob) mice housed at 33-35'C still retain dietary energy with a higher efficiency than lean counterparts, and develop obesity even though their SNS activity and thermogenic activity of BAT are equally low (99,126). These results indicate that factors other than defective BAT thermoregulatory thermogenesis must be involved in the etiology of high metabolic efficiency of these obese animals. I have explored the effects of warm environment and adrenalectomy on energy balance and BAT metabolism in ob/ob mice (chapter 4).

CHAPTER 2. EFFECTS OF A HIGH-FAT DIET AND ADRENALECTOMY ON ENERGY BALANCE AND BROWN ADIPOSE TISSUE METABOLISM IN OB/OB MICE

2.1. INTRODUCTION

Genetically obese (ob/ob) mice deposit excess fat even when energy intake is limited to that of lean littermates (1). Considerable evidence has recently accumulated to suggest that this increase in efficiency of energy retention is related at least in part to defective thermogenic capacity of brown adipose tissue (BAT) (42,127). Thermogenesis in BAT results from uncoupling of oxidative phosphorylation via a unique mitochondrial proton conductance pathway (87). The activity of the proton conductance pathway, as assessed by measurement of radiolabelled GDP binding to BAT mitochondria, is reduced in ob/ob mice (36,43,45,48). Overall regulation of BAT thermogenesis is controlled principally by the sympathetic nervous system (87), and sympathetic nervous system activity, as reflected by norepinephrine (NE) turnover in BAT, is also diminished in BAT of ob/ob mice (60-62,132,142,147). Reduced thermogenic capacity of BAT (36) and diminished sympathetic stimulation of the tissue (60) are among the earliest detectable abnormalities associated with development of obesity in ob/ob mice.

Adrenalectomy is the only manipulation identified thus far that will normalize efficiency of energy retention and prevent development of obesity in ob/ob mice (37,108,120). However, the effectiveness of adrenalectomy is diet-dependent. Adrenalectomy prevents development of obesity in ob/ob mice fed a high-carbohydrate stock diet, whereas obesity continues to develop in adrenalectomized ob/ob mice fed a high-fat semipurified diet (37,120). The changes in energy efficiency and body composition in adrenalectomized ob/ob mice fed a high-

carbohydrate stock diet are associated with increased thermogenic capacity (48) and increased sympathetic nervous system activity in BAT (132). BAT metabolism has not been investigated in adrenalectomized ob/ob mice fed a high-fat diet. In the present study I thus examined the possibility that consumption of a high-fat semipurified diet interferes with stimulation of BAT metabolism in adrenalectomized ob/ob mice.

2.2. MATERIALS AND METHODS

Animals

Female obese (ob/ob) and lean (ob/+ or +/+) mice were obtained from our breeding colony of C57 BL/6J-ob/+ mice. They were weaned at 3 week of age, housed at 23-25'C in solid-bottom plastic cages with wood shavings for bedding, and fed a high-carbohydrate stock diet (Wayne Lab-Blox, Continental Grain company, Chicago, IL). Water was available ad libitum. Room lights were on from 0700 to 1900 h daily. At 3.5 week of age, ob/ob and lean pairs were separated from their littermates and housed individually. At 4 week of age mice were bilaterally adrenalectomized (ADX), or sham operated (SHAM), through dorsal incisions while under light ether anesthesia. ADX mice received 0.9% NaCl solution to drink after surgery.

All mice were fed a high-fat semipurified diet ad libitum after surgery unless otherwise indicated. The high-fat diet contained (g/100g): 29.46 casein, 0.45 methionine, 16.20 glucose, 7.36 corn oil, 32.56 tallow, 7.36 cellulose, 5.15 mineral mix (7), 1.47 vitamin mix (7), and 0.29 choline chloride. The diet provided 22, 12 and 66% of

metabolizable energy, respectively, as protein, carbohydrate and fat and contained 5.41 kcal metabolizable energy per gram. Food intake and body weight were recorded twice weekly during the 3-week feeding period.

Four days before the end of the feeding trials, blood sample was obtained from the tail of each mouse between 0900 and 1000 h for determination of plasma corticosterone concentrations. Plasma was diluted 1:9 with borate buffer (0.5 M, pH 8.0) and incubated for 30 min. at 60'C to denature coticosterone-binding proteins. Ethanol was then added to precipitate proteins and extract corticosterone. After drying an aliquot of the ethanol extract, the radioimmunoassay was conducted with antiserum from Endocrine Sciences, tarzana, CA. The lower limit of detection with this assay was 0.15 ug corticosterone/dl plasma. Adrenalectomized mice with nondetectable plasma corticosterone in this assay were assigned a value of 0.15 ug corticosterone/dl. Adrenalectomized mice with plasma corticosterone concentrations >lug/dl were excluded from the study; approx. 15% of the adrenalectomized mice were excluded on this basis. Plasma corticosterone concentrations averaged 0.47 ± 0.03 ug/dl in adrenalectomized ob/ob mice and 0.58 ± 0.04 ug/dl in adrenalectomized lean mice. Randomly selected sham ob/ob and lean mice had 11.7 ± 2.4 (n=18) and 5.7 ± 0.9 (n=18) ug corticosterone/dl plasma, respectively.

Experimental Design

Four groups of mice (SHAM and ADX ob/ob, SHAM and ADX lean) were fed the high-fat diet from 4 to 7 week of age. Measurements included energy intake, body energy gain, and rates of NE turnover in BAT and

heart. BAT metabolism was further evaluated in additional SHAM and ADX mice that were either maintained at 23-25'C or exposed to 4'C for 2 h immediately before death to examine their ability to withstand acute cold stress. Parameters measured included body temperature at 23-25'C, BAT weight, BAT protein content, cytochrome c oxidase activity, GDP binding to BAT mitochondria, and rates of GDP-inhibitable BAT mitochondrial swelling. In the last trial lean and ob/ob mice (unoperated) fed the high-fat diet or stock diet from 4 to 7 week of age were placed at 4'C, and rectal temperatures were recorded after 0,30,60,90 and 120 min of cold exposure.

Body Energy Determination

Mice were killed by cervical dislocation and the carcasses were frozen. To measure body energy, carcasses were thawed and softened for 1 h in an autoclave, homogenized in water and oven dried at 45'C. Body energy content was determined by combustion of an aliquot of the dried homogenate in a bomb calorimeter (Parr Instruments, Moline,IL). Body energy values for 10 littermate pairs of lean and ob/ob mice killed at 4 week of age were used to develop regression equations to predict the initial body energy of mice killed at 7 week of age. Body energy gain was calculated as the difference between measured body energy at 7 week of age and the predicted body energy at 4 week of age. Energy density of the tissue gain was calculated as kcal gained/g body weight gained. Energy efficiency was calculated as kcal gained/kcal metabolizable energy consumed during the 3-week feeding period.

Norepinephrine (NE) Turnover

Rates of NE turnover were assesed by two methods (60,132). In the first method, mice at the end of the 3-week feeding trial were injected with levo-(ring 2,5,6-^{3}H) NE (48.4 Ci/mmol, New England Nuclear, Boston, MA)in saline (0.3 ml; i.p.) between 0900 and 1000 h. Each unanesthetized lean mouse received 250 uCi ^{3}H-NE/kg body weight. ob/ob mice received the same total amount of isotope. Mice were killed by cervical dislocation 1,3,5 or 8 h after ^{3}H-NE injection. BAT depots (interscapular and subscapular) were rapidly removed, combined, and frozen. Hearts were also removed and frozen. The decreases in specific activity of ^{3}H-NE within BAT and heart were used to assess NE turnover. In the second method, mice were injected with 300 mg of a-methyl-p-tyrosine/kg body weight (in 0.3 ml saline; i.p.) between 0900 and 1000 h to block synthesis of NE. These animals were killed 0,2,4 or 6 h after injection by cervical dislocation and the decreases in NE content within BAT and heart were used to assess NE turnover.

To measure NE content in tissues, samples were homogenized in ice-cold 0.4 N perchloric acid containing 5 mM EDTA, 10 mM sodium metabisulfate and dihydroxybenzylamine as an internal standard. After removal of protein by centrifugation, NE and dihydroxybenzylamine were adsorbed onto acid-washed alumina and 0.5 M Tris-HCl buffer (pH 8.6) was added. After shaking for 20 min, the buffer was removed by aspiration. The alumina was washed once with cold water and the wash then removed as completely as possible by aspiration. NE was eluted from alumina with 0.1 N perchloric acid. An aliquot of the perchloric acid eluate was injected into a high performance liquid chromatography

system, and NE was measured with an electrochemical detector (LC-4 amperometric detector, Bioanalytical Systems, West Lafayette, IN) as previously described (60). To measure ^{3}H-NE specific activity (method 1), ^{3}H-NE was collected from the column and counted in a liquid scintillation counter. Linear regressions, calculated by the method of least squares, were used to describe the decline in specific activity of ^{3}H-NE (method 1) or the decline in content of NE (method 2) in each organ. Fractional rates (k) of NE turnover were calculated from semilogarithmic plots of ^{3}H-NE specific activity (method 1) or NE content (method 2). NE turnover rates were calculated as the product of fractional rates of turnover (k) and either total NE per organ for each mouse (method 1), or the total NE per organ for each mouse at the time 0 point (method 2).

BAT cytochrome c oxidase, GDP binding and Mitochondrial Swelling

Mice were killed by cervical dislocation between 0900 and 1000 h and interscapular and subscapular BAT depots were rapidly removed, combined, weighed, and placed in ice cold sucrose buffer. For preparation of mitochondria, BAT from two mice in the same treatment group was pooled. Mitochondria were isolated in buffer containing 250 mM sucrose and 5 mM K-TES (pH 7.2) by the procedure of Cannon et al. (18). Protein content of mitochondrial preparations and BAT homogenates (after extraction of lipids with acetone-petroleum ether) was measured by a modified Lowry method (6,72).

Cytochrome c oxidase activity in BAT homogenates and in isolated mitochondria were measured spectrophotometrically at 25'C (134). Cytochrome c was reduced by excess sodium hydrosulfite and passed

through a Sephadex G-10 column previously washed with ascorbate and equilibrated with potassium phosphate buffer (pH 7.0) containing diethylenetriamine pentaacetic acid. Recovery of mitochondrial cytochrome c oxidase from BAT homogenates was determined and used for the calculation of total mitochondrial protein and GDP binding per BAT depot.

The binding of ^{3}H-GDP to BAT mitochondria was determined by the method described by Nicholls (86) with slight modifications. In brief, mitochondria (0.5-1 mg mitochondrial protein/ml) were incubated in a media containing 100 mM sucrose, 20 mM K-TES, 1 mM EDTA, 2 uM rotenone, 100 uM potassium atractyloside, 2.5 x 10^{6} dpm/ml ^{3}H-GDP (New England Nuclear; 10.2 Ci/mmol) and unlabled GDP (10 uM). ^{14}C-sucrose, 5.55 x 10^{5} dpm/ml, (New England Nuclear; 673 mCi/mmol) was included to calculate the volume of media trapped in the final mitochondrial pellet. Nonspecific binding was assessed from the binding of ^{3}H-GDP in the presence of excess unlabeled GDP (200 uM). Incubations were conducted at 25'C for 10 min. After incubation, the tubes were quickly centrifuged, the supernatent removed and the mitochondrial pellet was subsequently dissolved (Beckman tissue solubilizer-450) and counted in a liquid scintillation counter.

The rate of chloride-induced, GDP-inhibitable BAT mitochondrial swelling has been proposed as a measure of proton conductance (121). Chloride ion permeability was measured spectrophotometrically as the swelling of BAT mitochondria in isotonic KCl media (88). Mitochondria (0.2-0.4 mg protein/ml), obtained only from mice housed at 23-25'C, were added to a media consisting of 100 mM KCl, 5 mM TES, 5 uM rotenone, 0.5 uM valinomycin, pH 7.2. The rate of BAT mitochondrial

swelling was monitored in the presence or absence of 0.1 mM GDP by the decrease in absorbance at 420 nm in a recording spectrophotometer (Gilford Instrument Lab. Inc. Oberlin, OH) at 25'C during the initial 30 s. The reaction was initiated by addition of mitochondria to the media.

Body Temperature

Rectal temperatures of mice were measured with a telethermometer (Yellow Springs, OH) inserted 1.5 cm into the rectum. Care was taken to cause minimal disturbance before the measurements, and all measurements were recorded when temperature reading reached a plateau.

Statistics

Data were subjected to two- or three-way factorial analysis of variance, and statistical comparisons among treatment means were made with the Bonferroni-t test (33).

2.3. RESULTS

During the 3 week after surgery, SHAM ob/ob mice consumed 20% more energy, gained 2.5 times more body weight, and 5 times more energy than SHAM lean mice (Figure 2.1). Adrenalectomy reduced energy intake of ob/ob mice so that it was similar to that of their lean littermates, but weight gain and energy gain of ADX ob/ob mice still remained severalfold higher than in lean mice. These parameters in lean mice were unaffected by adrenalectomy (Figure 2.1).

Energy density of tissue gain (kcal gained/g body weight gained)

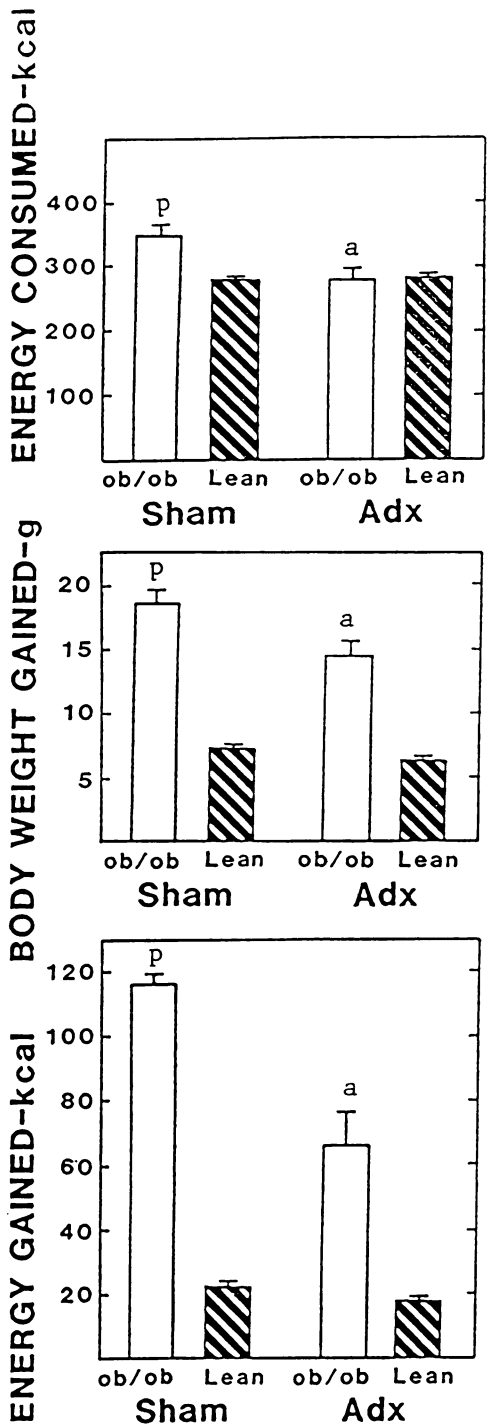


Fig 2.1 Energy consumption, body weight gain and energy gain of sham-operated (Sham) and adrenalectomized (Adx) ob/ob and lean mice fed a high-fat diet for 3 wks. Initial body energy values (ob/ob, 36 kcal/mouse and lean, 18 kcal/mouse) were estimated from linear regression equations relating body energy to body weight of mice killed at the time of surgery. Each bar represents the mean±SE for 7-12 mice. a, significant effect ($P<0.05$) of adrenalectomy within phenotype. p, significant effect ($P<0.05$) of phenotype within same surgical group.

and efficiency of energy retention(kcal gained/kcal consumed x 100) in SHAM ob/ob mice were two and four times higher, respectively, than in lean mice (Figure 2.2). Adrenalectomy caused only slight decreases in energy density of tissue gain and efficiency of energy retention (15 and 17 %, respectively) in ob/ob mice; these parameters remained two and four times higher in ADX ob/ob mice than the lean mice (Figure 2.2). These results parallel earlier reports (37,120), which showed that consumption of a high-fat diet prevents the normalization of body composition and energy efficiency that occurs when ob/ob mice fed a high carbohydrate-stock diet are adrenalectomized.

BAT and heart weights and NE content for mice fed the high-fat diet from 4 to 7 week of age are presented in Table 2.1. BAT depots of ob/ob mice weighed more than 2.5 times those of lean mice, although these depots contained approximately the same amount of NE as BAT of lean mice. Removal of the adrenals slightly decreased (19 %) the weight of BAT in ob/ob mice without influencing NE content. Heart weight and NE content were generally comparable in ob/ob and lean mice; adrenalectomy did not affect these parameters (Table 2.1)

Fractional rates (k) of NE turnover , assessed by injection of ^{3}H-NE, were similar in BAT of SHAM ob/ob mice and lean mice (Figure 2.3). This finding was unexpected because numerous previous reports have shown that fractional rates of NE turnover in BAT of ob/ob mice are approximately 50 % lower than rates in BAT of lean mice (60-62,132,142,147). But these latter mice had been fed high-carbohydrate stock diets, rather than a high-fat diet. A second method for assessing NE turnover was employed to independently verify that fractional rates of NE turnover are not depressed in ob/ob mice

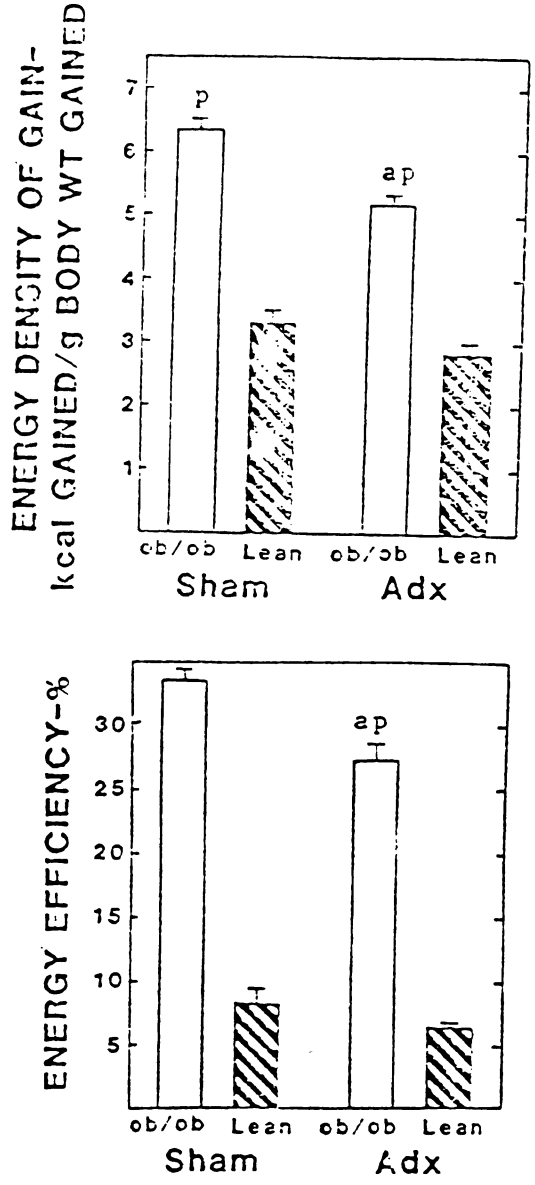


Fig 2.2 Energy density of tissue gain (an indicator of relativeproportions of lean and fat gain) and efficiency cfenergy gain (kcal gained/kcal consumed x 100) in mice fed a high fat diet for 3 wks. Each bar represents the mean±SE for 7-12 mice. a, significant effect ($P<0.05$) of adrenalectomy within phenotype. p, significant effect ($P<0.05$) of phenotype within same surgical group.

TABLE 2.1 Organ weights and norepinephrine content of mice fed a high-fat diet.

	SHAM		ADX	
	ob/ob	Lean	ob/ob	Lean
Method 1				
BAT				
Wt - mg	377±18[p]	133±5	301±21[ap]	119±4
NE - ng	251±11[p]	219±8	245±11[p]	217±10
HEART				
Wt - mg	139±7[p]	108±4	114±3[a]	106±4
NE - ng	107±5	96±5	101±4	92±3
Method 2				
BAT				
Wt - mg	342±10[p]	139±3	281±11[ap]	121±3
NE - ng	195±9	207±17	181±6	159±6
HEART				
Wt - mg	118±2	113±2	117±3	115±3
NE - ng	68±2	71±3	78±9	76±7

Each value represents mean+SE for 40-65 mice for tissue weight and 20-40 mice for NE content. In method 1 NE turnover was assessed after injection of ^{3}H-NE whereas a-methyl-p-tyrosine was used to assess NE turnover in method 2. Mice had been fed a high fat diet for 3 wks. BAT represents combined interscapular and subscapular brown adipose tissue depots. a, significant effect ($P<0.05$) of adrenalectomy within phenotype. p, significant effect ($P<0.05$) of phenotype within same surgical group.

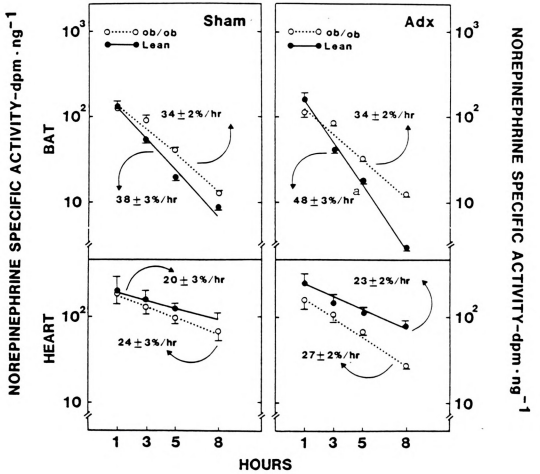


Fig 2.3 Norepinephrine specific activity after injection of ^{3}H-norepinephrine in mice fed a high-fat diet for 3 wks. Points represent means±SE for 10-13 mice killed 1,3,5 or 8 hours after H-NE injection. Numbers in each panel represent the fractional rates of NE turnover (k)±SE calculated from the slopes (b) of each regression line (k = b/0.434). BAT represents pooled interscapular and subscapular depots. a, significant effect ($P<0.05$) of adrenalectomy within phenotype.

fed the high-fat diet. Results after injection of a-methyl-p-tyrosine confirmed the findings with ^{3}H-NE (Figure 2.4). Adrenalectomy failed to accelerate fractional rates of NE turnover in BAT of ob/ob mice. Fractional rates of NE turnover were increased in ADX lean mice as measured by method 1 but not as measured by method 2.

Fractional rates of NE turnover measured in heart by method 1 were unaffected by phenotype or adrenalectomy (Figure 2.3). As estimated by method 2, fractional rates of NE turnover were slower in hearts of SHAM ob/ob mice than in hearts of lean mice, and adrenalectomy accelerated the fractional rates of NE turnover in hearts of ob/ob mice to equal rates in lean mice (Figure 2.4).

Calculated rates of NE turnover (NE content x k) in BAT of SHAM ob/ob mice were similar to those of lean mice as assessed by H-NE disappearance (Figure 2.5). As assessed by the a-methyl-p-tyrosine method, calculated rates of NE turnover were lower in SHAM ob/ob mice than in lean mice because SHAM ob/ob mice had a slightly slower fractional rates of NE turnover and slightly less NE in BAT. Adrenalectomy failed to influence calculated rates of NE turnover in BAT of ob/ob mice (Figure 2.5). Rates of NE turnover in heart were unaffected by phenotype or adrenalectomy, except for the higher rate of NE turnover, assessed by the a-methyl-p-tyrosine method, in hearts of ADX ob/ob mice than in hearts of SHAM ob/ob mice.

The essentially normal rates of NE turnover observed in BAT of ob/ob mice fed the high-fat diet suggest that the tissue may also exhibit a normal thermogenic capacity, in contrast to the low thermogenic capacity of BAT reported in ob/ob mice fed high-carbohydrate stock diets (36,43,45,48). Several parameters of BAT

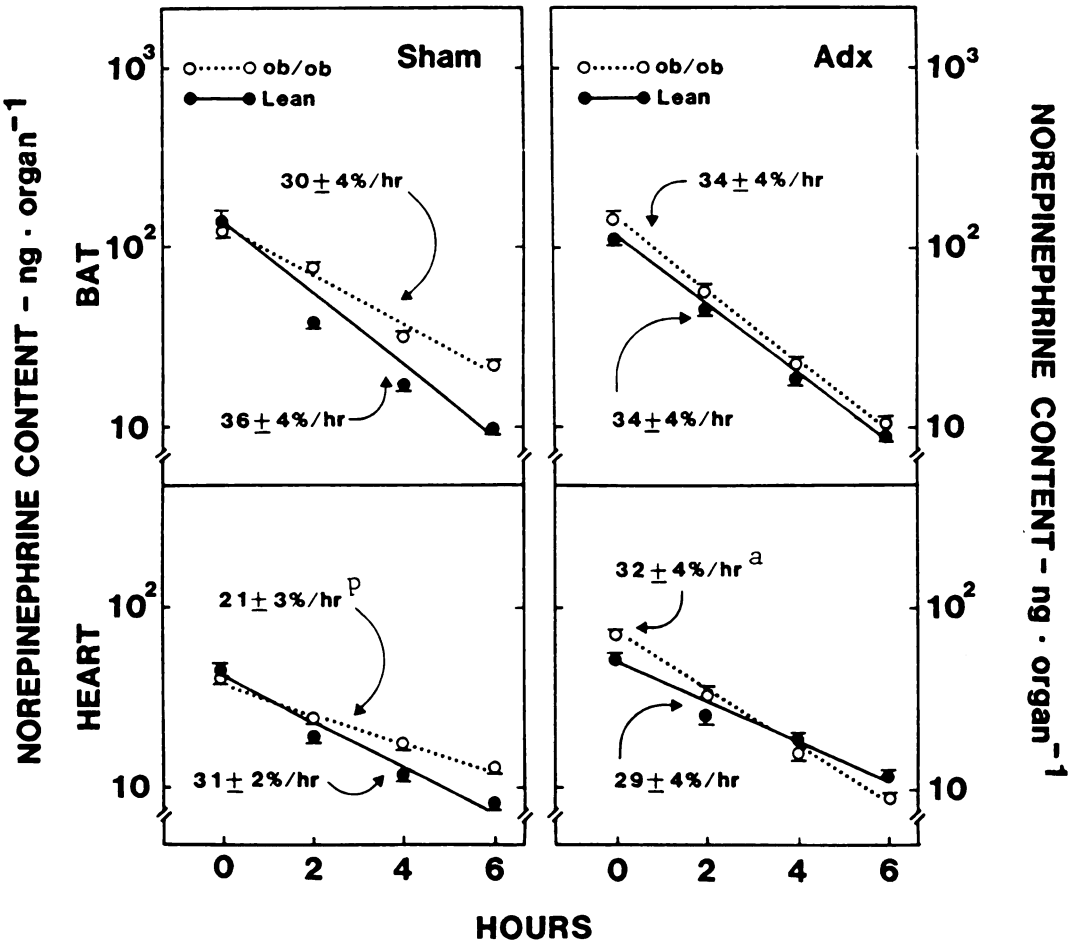


Fig 2.4 Norepinephrine content after injection of a-methyl-p-tyrosine in mice fed a high fat diet for 3 wks. Points represent means±SE for NE content in 7-16 mice killed 0,2,4 or 6 hours after a-methyl-p-tyrosine injection. Numbers in each panel represent the fractional rates of NE turnover (k)±SE caculated as in Fig 2.3. a, significant effect ($P<0.05$) of adrenalectomy within phenotype. p, significant effect ($P<0.05$) of phenotype within same surgical group.

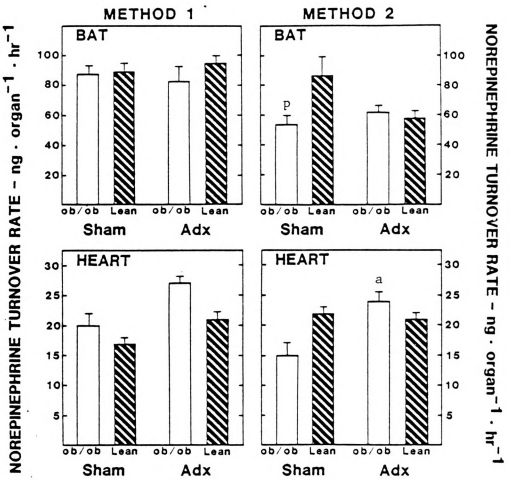


Fig 2.5 Calculated rates of norepinephrine turnover in mice fed a high-fat diet for 3 wks. In method 1 ^{3}H-NE was employed to estimate NE turnover and in method 2 a-methyl-p-tyrosine was used. Each bar represents the Mean+SE for 20-45 mice. NE turnover rates were calculated as the product of k (Fig 2.3 and 2.4) and NE content (table 1). a, significant effect ($P<0.05$) of adrenalectomy within phenotype. p, significant effect ($P<0.05$) of phenotype within same surgical group.

metabolism pertaining to BAT thermogenesis in ob/ob mice fed the high-fat diet were therefore examined. Because acute cold exposure has been shown to unmask GDP binding sites in BAT mitochondria of lean mice fed high-carbohydrate stock diets, but not in ob/ob mice (43,45), effects of acute cold exposure on BAT in mice fed the high-fat diet were also evaluated.

BAT weight and protein content, mitochondrial protein content, and BAT cytchrome c oxidase activity from mice maintained at 23-25'C and fed the high-fat diet for 3 week were unaffected by phenotype, adrenalectomy or acute cold exposure (2 h at 4'C), except that cold-exposed ob/ob mice had more BAT protein and a higher cytochrome c oxidase activity than lean counterparts (Table 2.2 and Figure 2.6). Likewise, GDP binding to BAT mitochondria, an indicator of the activity of the proton conductance pathway in BAT mitochondria, was unaffected by phenotype, adrenalectomy or acute cold exposure (Figure 2.6). The only exception was that adrenalectomized lean mice housed at 23'C had higher GDP binding per miligram mitochondrial protein than the other groups housed at 23'C.

The effectiveness of GDP as an inhibitor of potassium chloride-induced mitochondrial swelling was used as a further assessment of the thermogenic capacity of BAT (121,122). In agreement with results of the GDP binding studies, GDP-inhibitable swelling was unaffected by phenotype or adrenalectomy (Table 2.3). Thus, all parameters of BAT function measured in the present study (i.e., NE turnover, cytochrome c oxidase activity, GDP binding to mitochondria and GDP-inhibitable swelling of mitochondria) suggested that ob/ob mice fed a high-fat diet have a normal BAT function that is not altered in response to

TABLE 2.2 BAT weight and protein content of mice maintained at 23-25'C or acutely exposed to cold

	SHAM		ADX	
	ob/ob	Lean	ob/ob	Lean
Maintained at 23-25'C				
Wt - mg	457±19[p]	157±8	276±23[ap]	129±6
total protein - mg	30±1	25±4	31±2	26±5
mito. protein - mg	14±2	11±1	16±2	12±1
Acutely exposed to cold (4'C)				
Wt - mg	514±26[p]	119±6	281±29[ap]	112±6
total protein - mg	35±2[p]	20±1	31±2[p]	21±1
mito. protein - mg	16±3	11±1	13±1	12±1

Each value represents mean+SE for 4-5 analyses, each obtained from BAT pooled from 2 mice that had been fed a high fat diet for 3 wks. Mice were maintained at 23-25'C for 3 wk; acutely cold-exposed mice were housed at 4'C for 2 hr before tissues were excised. a, significant effect ($P<0.05$) of adrenalectomy within phenotype. p, significant effect ($P<0.05$) of phenotype within same surgical group.

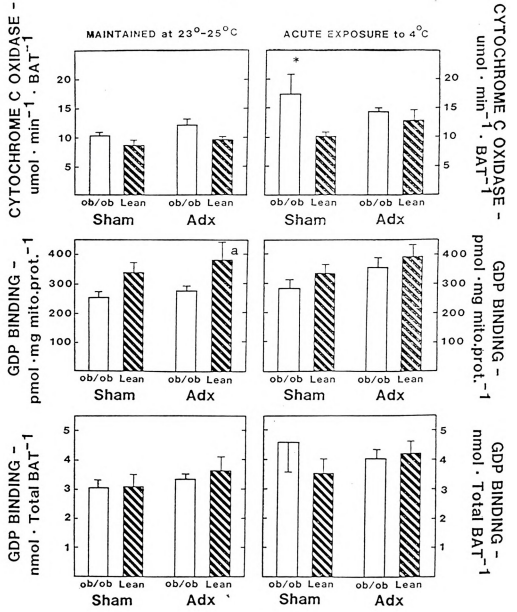


Fig 2.6 **Cytochrome c oxidase activity and GDP binding in BAT of mice fed a high-fat diet for 3 wks and maintained at 23-25'C or exposed to cold (4'C) for 2 hr.** Each bar represents mean±SE for 4-5 analyses, each obtained from pooled interscapular and subscapular BAT depots from 2 mice. Total GDP binding per BAT pad was calculated from the recovery of cytochrome c oxidase and GDP binding/mg mitochondrial protein. The percentage of total homogenate cytochrome c oxidase recovered in mitochondrial preparations in mice maintained at 23-25'C averaged 22.4±1.8, 20.5±1.7, 18.4±1.5, 18.1±1.4% in sham-ob/ob, sham-lean, Adx-ob/ob, and Adx-lean mice, respectively, and 21.0±1.7, 18.9±1.4, 18.1±1.6, 15.7±1.2% in mice acutely exposed to cold. a, significant effect ($P<0.05$) of adrenalectomy within phenotype. p, significant effect ($P<0.05$) of phenotype within same surgical group. The asterick (*) indicates a significant effect of acute cold exposure on cytochrome c oxidase activity in sham-ob/ob mice.

TABLE 2.3 Mitochondrial swelling

	SHAM		ADX	
	ob/ob	Lean	ob/ob	Lean
Condition				
- GDP	930±149	980±65	932±206	854±91
+ 0.1 mM GDP	501±48	490±63	444±82	510±28
GDP inhibition	429±102	490±13	487±133	417±100

Each value represents mean+SE for 4-5 analyses, each obtained from BAT pooled from 2 mice that had been fed a high fat diet for 3 wks. There were no significant differences between phenotypes or sham and adrenalectomized mice ($P<0.05$).

adrenalectomy. These results contrast with the low thermogenic activity of BAT in ob/ob mice fed high-carbohydrate stock diets (36,43,45,48), and with the marked response of BAT in these mice to adrenalectomy (48).

Improved functional capacity of BAT in ob/ob mice fed the high-fat diet would be expected to affect their cold intolerance. Ob/ob mice housed at 23-25'C and fed high-carbohydrate stock diets usually have rectal temperatures approximately 2'C lower than lean mice (58). ob/ob mice fed the high-fat diet maintained rectal temperatures approximately 1'C below that of lean mice throughout the day with no effect of adrenalectomy (Figure 2.7). Pronounced differences in cold tolerance were apparent when ob/ob mice fed the high-carbohydrate stock diet or the high-fat diet were exposed to 4'C. Ob/ob mice fed the stock diet exhibited characteristic hypothermia when exposed to cold; rectal temperature dropped to 25'C within 2 h (Figure 2.8). Ob/ob mice fed the high-fat diet were much more resistant to development of hypothermia; after an initial drop, rectal temperatures plateaued at 31-32'C. Lean mice decreased their rectal temperatures only slightly when exposed to 4'C.

2.4. DISCUSSION

Results of the present study demonstrate that BAT thermogenic activity in ob/ob mice fed a high-fat diet equals that observed in lean counterparts and that adrenalectomy fails to stimulate BAT metabolism in these animals. First, I will focus on the finding in control ob/ob mice and then on effects of adrenalectomy. Ob/ob mice

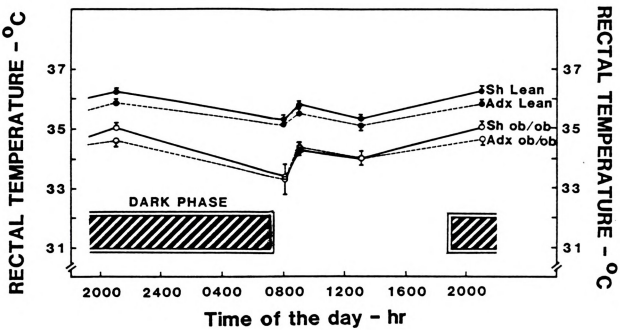


Fig. 2.7 Rectal temperatures of mice housed at 23-25'C and fed a high-fat diet. Dark phase of the day is shown by the cross-hatched bar. Points represent mean±SE for 15-19 mice. At all time points body temperatures of ob/ob mice were lower than in lean mice ($P<0.05$). Adrenalectomy had no significant effect on rectal temperatures ($P<0.05$).

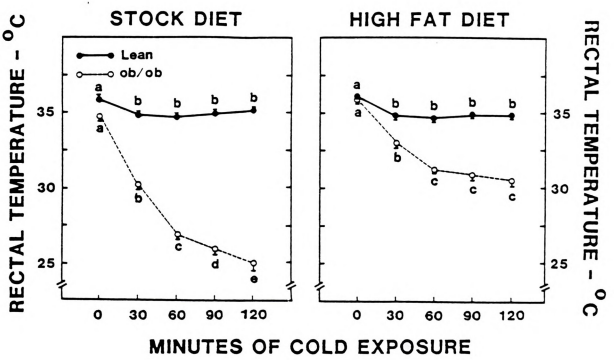


Fig. 2.8 Effects of acute cold exposure (4°C) on rectal temperatures of mice fed a stock or high-fat diet. Points represent mean±SE for 11 mice. Points on a line within a panel with different lettes are significantly different ($P<0.05$). ob/ob mice had lower ($P<0.05$) rectal temperature than lean mice at each time measured, except for ob/ob mice fed high-fat diet just prior to cold exposure.

fed the high-fat diet exhibited a very high efficiency of energy retention, as has been observed previously (37,69,120). Even when pair-fed a high-fat diet, ob/ob mice maintain a higher efficiency of energy retention than lean mice (69), indicating that metabolic efficiency is increased in these animals. As stated in the introduction, defective thermogenic capacity in BAT of ob/ob mice has been linked to this enhanced efficiency (42,127). Virtually all of these studies on BAT metabolism, however, were conducted in mice fed high-carbohydrate stock diets. Evidence from the present study and elsewhere (44) indicates that consumption of a high-fat diet can reverse the low thermogenic capacity of BAT in ob/ob mice without depressing their overall efficiency of energy retention.

Reversal of the low thermogenic capacity of BAT in ob/ob mice fed the high-fat diet is probably associated with stimulation of the sympathetic nervous system, as reflected by measurements of NE turnover by two independent methods (Figures 2.3, 2.4 and 2.5). Comparisons of NE turnover in BAT of mice fed a high-carbohydrate stock diet with those of the present study demonstrate that the high-fat diet selectively stimulated NE turnover in ob/ob mice with minimal effects in lean mice. Fractional rates of NE turnover in BAT of ob/ob mice fed a high-carbohydrate stock diet averaged 17 %/h (60-62) vs. 30-34 %/h when fed the high fat-diet (Figures 2.3 and 2.4). In lean mice, fractional rates of NE turnover in BAT averaged 32 %/h when fed a high-carbohydrate stock diet (60-62) and were only slightly higher (36-38 %/h) in the present study when fed a high-fat diet (Figures 2.3 and 2.4). Comparisons of NE turnover in ob/ob and lean mice fed other diets high in fat also show that NE turnover in

ob/ob mice is more responsive to these dietary manipulations than is NE turnover in BAT of lean mice (44).

Selective stimulation of NE turnover in BAT of ob/ob mice fed a high-fat diet may be a consequence of interactions among fat metabolism, environmental temperature, and phenotype. It is known that energy expenditure associated with deposition of body fat is greater when a high-carbohydrate diet is fed than when a high-fat diet is fed. This energy expenditure related to nutrient assimilation would contribute to maintenance of body temperature and reduce the requirement for thermoregulatory thermogenesis in animals housed below their thermoneutral temperature (approx. 33'C for mice). Thus, mice fed a high-fat diet and housed below their thermoneutral temperature, as was the case in the present study, would be expected to have a greater demand for thermoregulatory thermogenesis than when fed a high-carbohydrate diet. Mercer and Trayhurn (77) demonstrated such an adaptation to diet composition in mice housed at 4'C. ob/ob mice appear to require a lower environmental temperature to activate BAT thermogenesis than lean mice; rates of NE turnover in BAT of ob/ob mice are not increased until the environmental temperature is dropped below 25'C, whereas rates of NE turnover in BAT of lean mice increase approximately threefold when going from 33 to 25'C (60). Possibly, the low energy cost of converting dietary fat to body fat in ob/ob mice housed at 23-25'C was equivalent to a lowering of environmental temperature in ob/ob mice fed a high-carbohydrate diet and triggered an activation of the sympathetic nervous system.

Further evidence that dietary fat per se is not the sole factor contributing to the elevation in NE turnover in BAT of ob/ob mice is

the finding that ob/ob pups at 2 week of age consuming the high-fat milk produced by the mother have lower rates of NE turnover in BAT than lean counterparts (61). Because these pups were able to huddle together in a nest, the temperature in the near environment of individual pups was unknown and may have influenced the results. A systematic evaluation of the interaction between diet composition and environmental temperature in the regulation of BAT metabolism in ob/ob mice is needed to resolve this issue.

Regardless of the mechanisms responsible for activation of the sympathetic nervous system in BAT of ob/ob mice fed the high-fat diet, all parameters measured indicate that the tissue of ob/ob mice fed the high-fat diet had essentially the same thermogenic activity as BAT of lean mice. Cytochrome c oxidase activity, GDP binding to mitochondria, and GDP-inhibitable swelling of mitochondria were as high in BAT of ob/ob mice as in lean mice. Acute cold exposure failed to increase GDP binding to BAT mitochondria in either lean or ob/ob mice. This is probably related to the rather cool environment in which the mice were housed (23-25'C), and to the high-fat diet they were fed. GDP binding to BAT mitochondria was increased when lean mice (7 week of age) fed a high-carbohydrate stock diet and housed at 23-25'C were acutely exposed to cold; binding was 393 ± 32 pmol GDP/mg BAT mitochondrial protein at 23-25'C and 497 ± 18 pmol GDP/mg BAT mitochondrial protein after exposure to 4'C for 2 h ($P<0.05$). This demonstrates that diet composition exerted a role in the failure of acute cold to further increase GDP binding to BAT mitochondria in mice fed the high-fat diet. Ob/ob mice fed the high-fat diet maintained a slightly lower body temperature than lean mice, but exhibited a marked

improvement in cold tolerance, demonstrating that BAT was functionally active. These data question a major role for BAT in the high efficiency of energy retention in ob/ob mice fed a high-fat diet. BAT also appears to play a minimal role in the high efficiency of energy retention observed in ob/ob mice housed in a warm environment (34.5'C) (99).

Adrenalectomy was ineffective in blocking development of obesity in ob/ob mice fed the high-fat diet. Energy intake was essentially the only parameter of energy balance measured that was normalized (Figure 2.1), in agreement with earlier reports(37,120). This finding supports a role for adrenal secretions, likely glucocorticoids, in the hyperphagia of ob/ob mice (116) independent of diet composition. Effects of adrenalectomy on efficiency of energy retention in ob/ob mice are, however, dependent on diet composition. Removal of the adrenals failed to have a major effect on efficiency of energy retention in ob/ob mice fed the high-fat diet (Figure 2.2), whereas it did in ob/ob mice fed the high-carbohydrate stock diets (37,120).

My working hypothesis was that ADX ob/ob mice fed the high-fat diet maintained a high efficiency of energy retention in part at least because of failure of adrenalectomy to activate BAT metabolism, as occurs in ADX ob/ob mice fed a high-carbohydrate stock diet (48,132). Results of the present study negate this hypothesis: BAT metabolism was activated simply by feeding the high-fat diet. In this context the failure of adrenalectomy to change BAT metabolism in ob/ob mice fed the high-fat diet is entirely consistent with the lack of effect of adrenalectomy on efficiency of energy retention in these mice.

The metabolic basis for development of obesity in ob/ob fed a

high-fat diet remains to be resolved. Hyperphagia is a contributing factor, but cannot explain the high efficiency of energy retention in young ob/ob pups (69) or in the adrenalectomized ob/ob mice used in the present study where hyperphagia was absent (Figure 2.1). Subtle abnormalities in BAT metabolism, undetected by approaches used in the present study, may contribute to development of obesity in ob/ob mice fed the high-fat diet. A major role for BAT in ob/ob mice fed the high-fat diet must, however, be questioned.

It would also appear possible to discount the lower body temperature of ob/ob mice fed the high-fat diet as a major energy conservation mechanism. If lower body temperature was a major determinant of energy imbalance in these mice, then ob/ob mice consuming the high-fat diet (body temperature only about 1°C lower than in lean mice) should be less efficient in retaining body energy than ob/ob mice fed a high-carbohydrate stock diet (where body temperature is about 2°C lower than in lean mice). This is not the case (37,69). Furthermore, housing ob/ob mice at elevated environmental temperatures should minimize any difference in body temperature between ob/ob and lean mice, but it does not reduce the differences in metabolic efficiency (99). The eventual explanation for high metabolic efficiency in ob/ob mice will require consideration and understanding of the interaction between diet composition and neuroendocrine regulation in these mice.

CHAPTER 3. EFFECTS OF DIFFERENT TYPES OF DIETARY CARBOHYDRATE AND ADRENALECTOMY ON ENERGY BALANCE AND BROWN ADIPOSE TISSUE METABOLISM IN OB/OB MICE

3.1. INTRODUCTION

Adrenalectomy reduces the efficiency of energy retention (kcal body energy retained/kcal consumed) in obese (ob/ob) mice fed nonpurified high-starch diets to values similar to those of lean mice (37,108,120). But when a purified high-glucose diet is fed, adrenalectomy fails to arrest development of obesity in ob/ob mice (37,146). The critical diet component is type of carbohydrate, because in direct comparisons of energy balance in adrenalectomized ob/ob mice, those fed a high-starch purified diet exhibited suppressed efficiency of energy retention whereas those fed a high-glucose purified diet continued to develop gross obesity (146). The mechanism(s) involved in this diet-dependent response of ob/ob mice to adrenalectomy have not been ascertained.

The high efficiency of energy retention in ob/ob mice fed nonpurified high-starch diets is associated with low thermogenic activity in brown adipose tissue (BAT) (42,127). Adrenalectomy reduces the efficiency of energy retention in these mice to levels comparable to lean mice, and concomitantly increases sympathetic nervous system stimulation of their BAT (132) and the thermogenic activity of the tissue (48). Is the thermogenic activity of BAT in ob/ob mice fed a high-glucose purified diet comparable to that observed in ob/ob mice fed a high-starch purified diet? Does consumption of a high-glucose purified diet interfere with activation of BAT metabolism in adrenalectomized ob/ob mice? The present study was undertaken to address these questions. Mice (ob/ob and lean littermates) were adrenalectomized or sham-operated and fed purified

diets identical in composition except for the source of carbohydrate (starch or glucose). BAT metabolism was assessed by measurements of GDP binding toBAT mitochondria, mitochondrial swelling and rates of norepinephrine (NE) turnover. Effects of adrenalectomy and of consumption of the high-starch and high-glucose diets on plasma concentrations of glucose, insulin and thyroid hormones were also measured.

3.2. MATERIALS AND METHODS

Animals and Diets

Female obese (ob/ob) and lean (ob/+ or +/+) littermates obtained from our breeding colony (C57BL/6J-ob/+) were weaned at 3 weeks of age. They were housed in solid-bottom plastic cages with wood shavings for bedding in a room maintained at 23-25°C with a 12 h light-dark cycle (lights on at 0700 h). A high-starch nonpurified diet (Wayne Lab-Blox, Continental Grain, Chicago, IL) and water were available ad libitum. At 3 1/2 weeks of age, ob/ob and lean pairs were separated from littermates and housed individually. At 4 weeks of age, bilateral adrenalectomies were performed through dorsal incisions while mice were under ether anesthesia. Sham-operated mice were exposed to the same surgical procedure, except the adrenal glands were left intact. Adrenalectomized mice received 0.9 % NaCl solution to drink following surgery. Ten ob/ob and lean littermates were killed at 4 weeks of age to estimate initial body energy of the experimental mice.

Mice were fed one of two purified (high-starch or high-glucose) diets for 3 weeks following surgery. The high-glucose diet contained (g/100g) 65 glucose, 20 casein, 0.3 methionine, 5.0 corn oil, 3.5 mineral mixture (7), 1.0 vitamin mixture (7), 0.2 choline chloride, and 5.0 cellulose. The high-starch diet was formulated by replacing glucose, on an equal energy basis, with starch (65 g glucose replaced with 59.2 g starch). The calculated metabolizable energy value of the high-glucose and high-starch diets were 3.67 and 3.90 kcal/g, respectively. The diets provided 66, 22, and 12% of metabolizable energy as carbohydrate, protein, and fat, respectively. Food intake and body weights were recorded twice weekly.

Plasma Hormones and Glucose

Mice were killed by decapitation at the end of the 3 week feeding period and the blood was collected in a heparinized beaker and immediatly centrifuged to harvest plasma. All mice were killed between 0900-1000 h, except for mice used to measure NE turnover; they were killed at various time intervals between 0900-1800 h. Plasma corticosterone concentrations were determined by RIA (Endocrine Sciences, Tarzana, CA) with modifications as previously described (chapter 2). The lower limit of detection with this assay was 0.15 ug corticosterone/dl plasma. Adrenalectomized mice with nondetectable plasma corticosterone in this assay were assigned a value of 0.15 ug corticosterone/dl plasma. Only those adrenalectomized mice with plasma corticosterone concentrations below 1 ug/dl were included in the analyses. Plasma corticosterone concentrations averaged 0.26 ± 0.04 and 0.58 ± 0.04 ug/dl in adrenalectomized ob/ob and lean mice fed the

high-starch diet, respectively. Sham-operated ob/ob mice and lean mice fed the high-starch diet had values of 9.3±1.4 and 5.7±0.9 ug corticosterone/dl plasma, respectively. Adrenalectomized ob/ob and lean mice fed the high-glucose diet had 0.51±0.04 and 0.52±0.04 ug corticosterone/dl plasma, respectively. Sham-operated ob/ob and lean mice fed high-glucose diet had 11.5±2.3 and 4.9±1.0 ug corticosterone/dl, respectively. Plasma insulin concentrations were determined by RIA with anti-porcine insulin serum and rat insulin standard (Novo Research Laboratories, Bagsvaerd, Denmark). Plasma glucose concentrations were measured by the glucose oxidase/peroxidase method (Boerhinger-Mannheim, Indianapolis, IN). Plasma thyroxine and triiodothyronine were assayed by the method of Nejad et al. (83) using charcoal dextran to separate bound from free hormone.

BAT cytochrome c oxidase, GDP binding and Mitochondrial Swelling

Mice were killed between 0900 and 1000 h at the end of the 3 week feeding period. BAT (interscapular and subscapular pads) was rapidly removed, combined and weighed. BAT from two mice in the same treatment group was pooled and homogenized (5% wt/vol) in ice cold 250 mM sucrose buffer containing 5 mM K-TES (pH 7.2). Mitochondria were isolated from the homogenate as described by Cannon et al. (18). Protein content of the mitochondrial preparations and BAT homogenates [following extraction of lipids with acetone-petroleum ether (6)] was measured by a modified Lowry method (72). Cytochrome c oxidase activity and the binding of ^{3}H-GDP to BAT mitochondria were measured as previously described (chapter 2). The rate of acetate (or chloride)-induced GDP-inhibitible BAT mitochondrial swelling was

measured spectrophotometrically at 520 nm by incubating mitochondria (0.1 to 0.2 mg protein/ml) in a medium consisting of 100 mM K-acetate or KCl, 5 mM TES, 5 uM rotenone, 0.5 uM valinomycin, pH 7.2, as described before (chapter 2).

Norepinephrine (NE) Turnover

At the end of the 3 week feeding trial, L-(ring 2,5,6-^{3}H) NE (52.9 Ci/mmol, New England Nuclear) in 0.3 ml saline was injected (intraperitoneally) into unanesthetized mice between 0900-1000 h to measure rates of NE turnover. Each lean mouse received 250 uCi ^{3}H-NE/kg body weight and ob/ob mice received the same total amount of isotope as lean mice. Mice were decapitated 1,3,5 or 8 h after ^{3}H-NE injection. BAT (interscapular and subscapular pads) and hearts were immediately removed, frozen on dry ice and stored at -70'C until NE assays were performed (within 1 week). NE content, specific radioactivity, fractional rates (k) of NE turnover and calculated rates of NE turnover were determined as previously described (chapter 2). Briefly, tissues were homogenized in perchloric acid and NE was extracted onto acid-washed alumina. NE was eluted from the alumina and determined by HPLC with electrochemical detection (Bioanalytical Systems, West Lafayette, IN). NE was collected from the column and counted in a liquid scintillation counter to measure ^{3}H-NE specific radioactivity. Slopes (b) of linear regressions describing the disappearance of ^{3}H-labled NE from tissues were used to calculate the fractional turnover rates (k) of NE. Rates of NE turnover were calculated as the product of fractional turnover rate (k) for each experimental group and NE content of organs of individual animals.

Body Composition

Gastric contents were removed and carcasses were autoclaved for 1 h and then homogenized in an equal volume of water. Homogenates were dried at 50'C and combusted in a bomb calorimeter (Parr Instruments, Moline, IL). Gains in body energy were calculated as the difference between final and initial body energy, where initial body energy was estimated by using linear regression equations developed from body energy and body weights of the mice killed at 4 weeks of age. The linear regression equations used to predict initial body energy were $y = 4.86x - 31.55$ and $y = 1.61x - 1.86$ for ob/ob and lean mice, respectively, where y represents body energy and x represents body weight. Energy density of gain and efficiency of energy retention were calculated as kcal gained/g body weight gained and kcal gained/kcal metabolizable energy consumed during the 3 week feeding period, respectively.

Statistics

Data were subjected to 3 factorial analysis of variance (phenotype x surgery x diet) and statistical comparisons among treatments were made with the Bonferroni t-test (33). Data are presented as means $\pm$ SE and all significant effects are at the $P<0.05$ level.

3.3. RESULTS

Sham-operated ob/ob mice did not consume more energy than lean mice (Figure 3.1), contrary to what is usually observed in non-operated 4-7 week old ob/ob mice (69). Adrenalectomy reduced

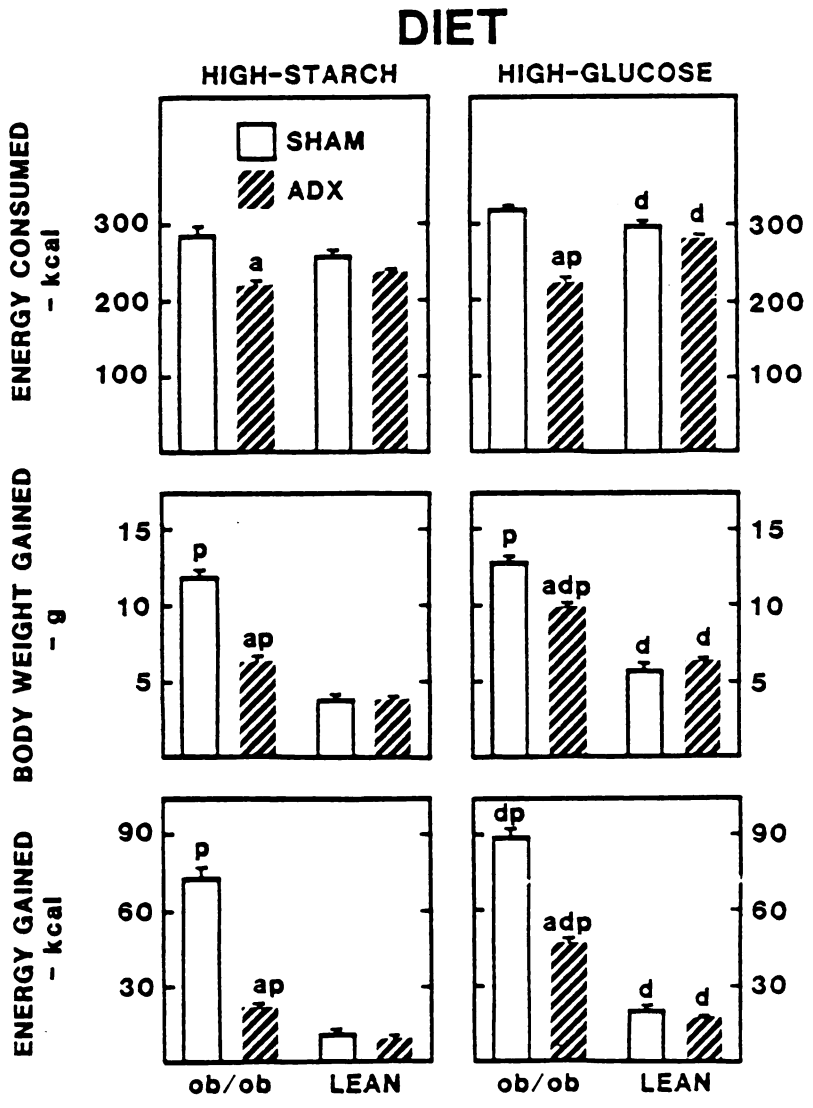


Fig. 3.1 Energy consumption, body weight gain and energy gain of mice fed high-starch or high-glucose diets. Each bar represents the mean±SE for 12-25 mice. Letter a indicates significant effect ($P<0.05$) of adrenalectomy within phenotype and diet treatment; p, significant effect of phenotype within surgical and diet treatment; d, significant effect of diet within surgical and phenotype treatment.

energy intake of ob/ob mice more than that of lean mice. Lean mice consumed less high-starch diet than high-glucose diet. Sham-operated ob/ob mice gained considerably more body weight and body energy than lean mice independent of diet consumed (Figure 3.1). Adrenalectomy reduced the weight gain and energy gain more in ob/ob mice fed the high-starch diet than in ob/ob mice fed the high-glucose diet.

Energy density of tissue gain (an indicator of relative proportions of fat and lean tissue gain) in sham-operated ob/ob mice was two times higher than in lean mice independent of diet consumed (Figure 3.2). Adrenalectomy lowered the density of gain in ob/ob mice fed the high-starch diet more than it lowered it in adrenalectomized ob/ob mice fed the high-glucose diet. Efficiency of energy retention paralleled responses of body weight gain and energy gain (Figure 3.2). Sham-operated ob/ob mice retained energy 4 to 5 times more efficiently than lean mice independent of diet fed. Adrenalectomy markedly decreased (approx. 62%) the efficiency of energy retention in ob/ob mice fed the high-starch diet but adrenalectomy caused only a slight decrease (approx. 25%) in efficiency of energy retention in ob/ob mice fed the high-glucose diet (Figure 3.2). These parameters in lean mice were unaffected by adrenalectomy

Brown adipose tissue (BAT) of sham-operated ob/ob mice weighed more than 4 times that of lean mice (Table 3.1). Adrenalectomy decreased the weight of BAT in ob/ob mice. BAT of ob/ob mice fed the high-starch diet weighed significantly less than that of mice fed the high-glucose diet. Protein content of BAT was higher in ob/ob mice than in lean mice independent of diet, and was unaffected by adrenalectomy. Adrenalectomy had no affect on these parameters in

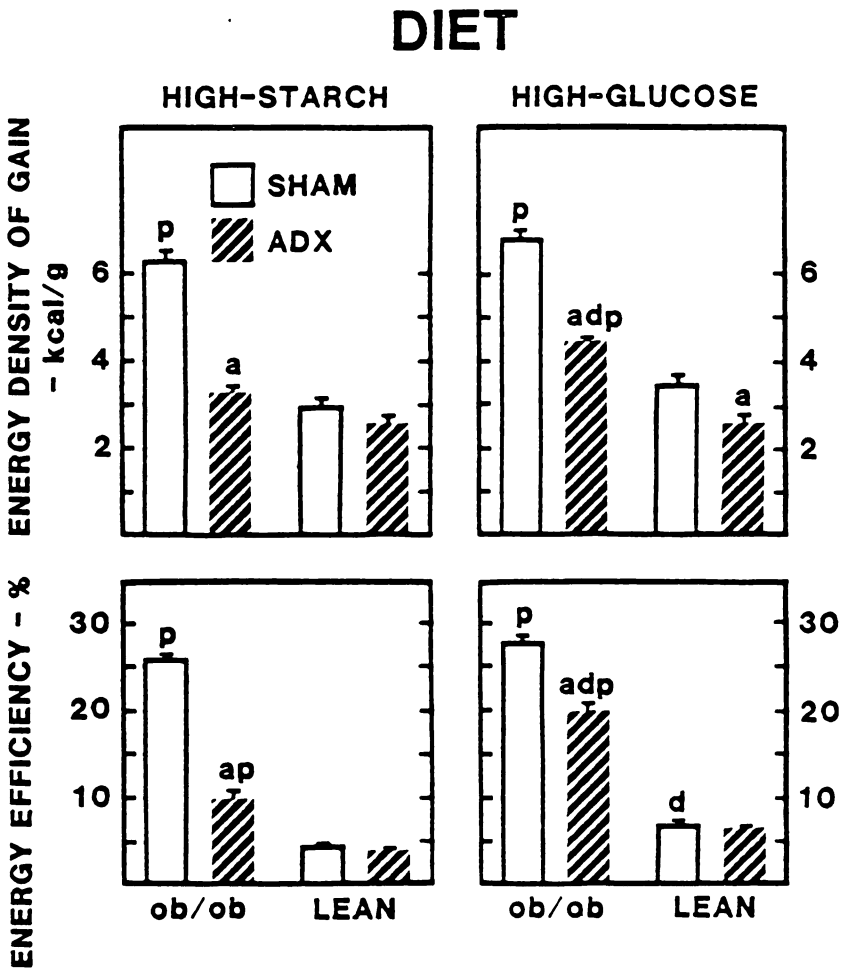


Fig. 3.2 Energy density of tissue gain and efficiency of energy retention in mice fed high-starch or high-glucose diets. Each bar represents the mean±SE for 12-25 mice. Letter a indicates significant effect ($P<0.05$) of adrenalectomy within phenotype and diet treatment; p, significant effect of phenotype within surgical and diet treatment; d, significant effect of diet within surgical and phenotype treatment.

TABLE 3.1 Organ weights, BAT protein, norepinephrine content and norepinephrine turnover rate (NETO).

	Ob/Ob		Lean	
	SHAM	ADX	SHAM	ADX
Starch diet				
BAT				
weight-mg	529±12^{p}	367±17^{ap}	138±4	109±5
protein-mg	35±2^{p}	36±1^{p}	22±1	23±1
NE-ng	202±6	226±7^{a}	184±5	161±6
NETO-ng/BAT/h	43±2^{p}	68±3^{ap}	68±3	57±3
Heart				
weight-mg	100±3	91±2	96±2	93±3
NE-ng	60±3	55±3	66±3	59±3
NETO-ng/Heart/h	10±1	12±1	18±1	14±1
Glucose diet				
BAT				
weight-mg	621±24^{dp}	450±21^{d}	145±6	106±4
protein-mg	36±3^{p}	37±2^{p}	23±0.3	21±1
NE-ng	177±6	220±11^{ap}	166±7	156±9
NETO-ng/BAT/h	33±2^{p}	43±3	51±3^{d}	54±4
HEART				
weight-mg	97±2	86±1	96±2	88±2
NE-ng	53±1	52±2	55±1	56±2
NETO-ng/Heart/h	5±0.1^{d}	9±0.2^{a}	8±0.2^{d}	9±1^{d}

Values represent means+SE for 22-32 mice for tissue weight , NE content and NETO (NE turnover rate), and 6-8 analyses for protein content, each obtained from a pooled interscapular and subscapular BAT pads from 2 mice. a, significant effect ($p<0.05$) of adrenalectomy within phenotype and diet treatment; p, significant effect ($p<0.05$) of phenotype within surgical and diet treatment; d, significant effect ($p<0.05$) of diet within surgical and phenotype treatment.

lean mice.

Cytochrome c oxidase activity, an indicator of mitochondrial oxidative capacity, was unaffected by phenotype, adrenalectomy or diet (Figure 3.3). Thermogenic activity of BAT was determined by measuring GDP binding to BAT mitochondria (Figure 3.3). GDP binding (per mg mitochondrial protein, or per total BAT pads) was lower in sham-operated ob/ob mice than in lean mice independent of diet. Adrenalectomy increased GDP binding in ob/ob mice fed the high-starch diet but failed to increase binding in ob/ob mice fed the high-glucose diet.

Because the rate of GDP-inhibitible mitochondrial swelling has been proposed as a measure of the proton conductance pathway (121, 122), swelling measurements were included to support the GDP binding data. The results were similar whether proton conductance (K-acetate) or chloride conductance (KCl) was measured. In agreement with results of the GDP binding studies, rates of GDP-inhibitible K-acetate or chloride swelling were lower in sham-operated ob/ob mice than in lean mice independent of diet, and were elevated by adrenalectomy in ob/ob mice fed the high-starch diet, but not in ob/ob mice fed the high-glucose diet (Figure 3.4).

Sympathetic nervous system stimulation of BAT metabolism was determined by measuring NE turnover. Fractional rates(k) of NE turnover in BAT of sham-operated ob/ob mice were much lower than in lean mice independent of diet (Figure 3.5). Adrenalectomy accelerated the fractional rates of NE turnover in BAT of ob/ob mice fed the high-starch diet, but failed to increas fractional rates of turnover in ob/ob mice fed the high-glucose diet. BAT of ob/ob mice contained

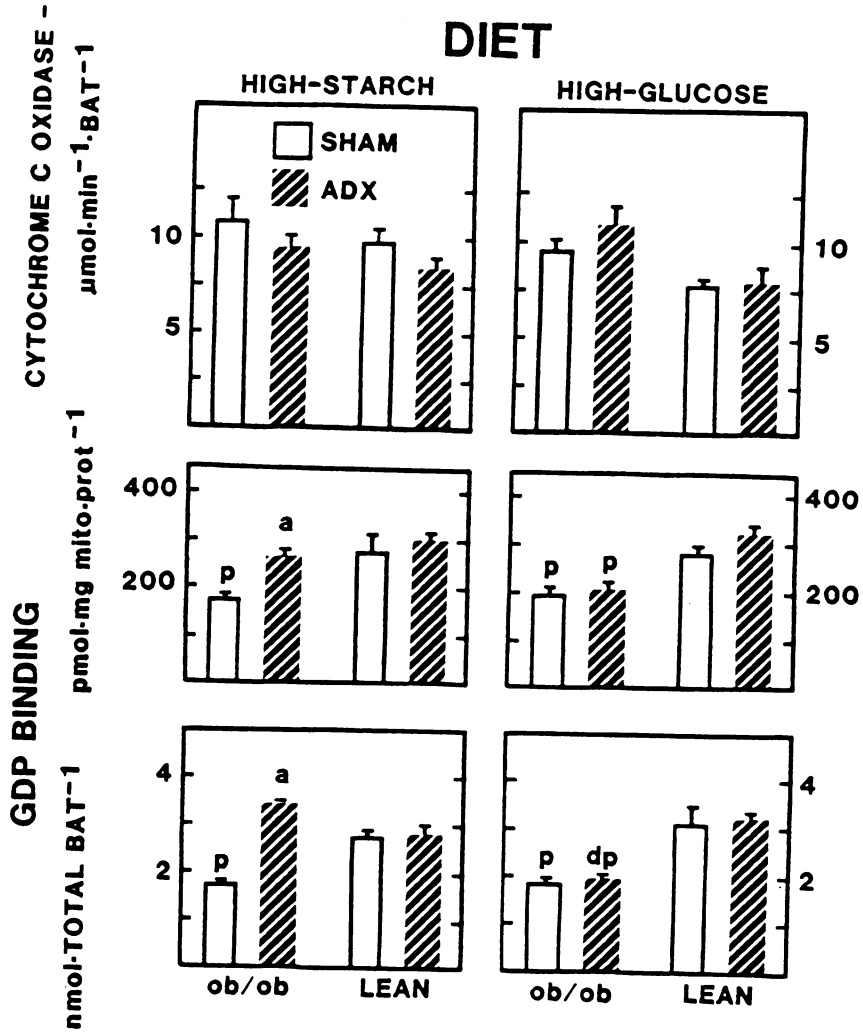


Fig. 3.3 Cytochrome c oxidase activity and GDP binding in brown adipose tissue (BAT) of mice fed high-starch or high-glucose diets. Each bar represents mean±SE for 6-8 analyses, each obtained from a pooled preparation of interscapular and subscapular BAT pads from 2 mice. Total GDP binding per BAT pad was calculated from the recovery of cytochrome c oxidase and GDP binding/mg mitochondrial protein. Recovery of cytochrome c oxidase activity was calculated from the percentage of total homogenate cytochrome c oxidase recovered in mitochondrial preparations. Cytochrome c oxidase recovery averaged 28.6±2.2, 22.0±1.6, 22.4±2.0, and 21.8±1.0 % in SHAM-ob/ob, SHAM-lean, ADX-ob/ob, and ADX-lean mice fed the starch diet, respectively, and 24.2±2.2, 17.2±0.9, 21.5±1.9, and 17.8±1.2 %, respectively, in mice fed the glucose diet. Letter a indicates significant effect ($P<0.05$) of adrenalectomy within phenotype and diet treatment; p, significant effect of phenotype within surgical and diet treatment; d, significant effect of diet within surgical and phenotype treatment.

Fig. 3.4 Acetate- or chloride-induced, GDP-inhibitible mitochondrial swelling in brown adipose tissue of mice fed high-starch or high-glucose diets. Each bar represents the mean+SE for 7-8 analyses, each obtained from interscapular and subscapular BAT pads pooled from 2 mice. Means are expressed in arbitary units as change in absorbance at 520 nm/mg mitochondrial protein/min at 25'C. Letter a indicates significant effect ($P<0.05$) of adrenalectomy within phenotype and diet treatment; p, significant effect of phenotype within surgical and diet treatment; d, significant effect of diet within surgical and phenotype treatment.

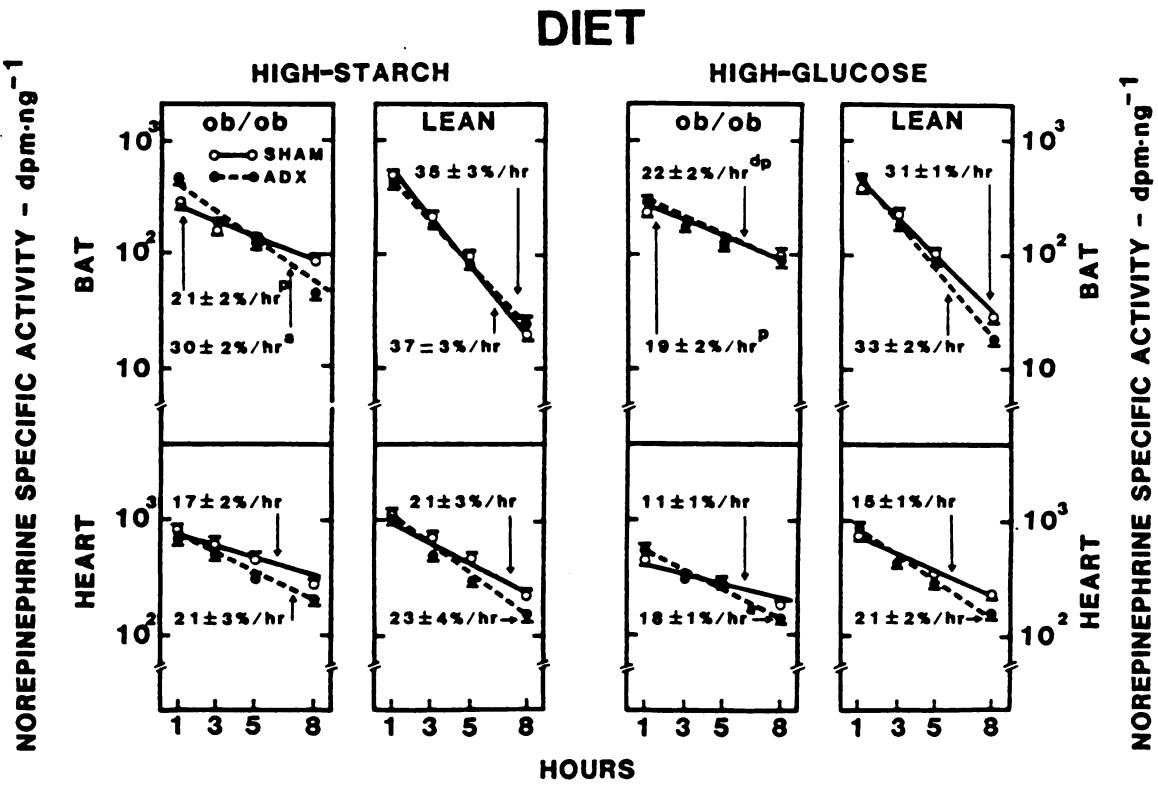


Fig. 3.5 Norepinephrine (NE) specific radioactivity of mice fed high-starch or high-glucose diets. Each point represents the mean+SE for 6-8 mice killed 1,3,5 or 8 hrs after ^{3}H-NE injection. Numbers in each panel represent the fractional rates of NE turnover (k)±SE calculated from the slopes (b) of each regression line (k = b/0.434). Letter a indicates significant effect ($P<0.05$) of adrenalectomy within phenotype and diet treatment; p, significant effect of phenotype within surgical and diet treatment; d, significant effect of diet within surgical and phenotype treatment.

approximately the same amount of NE as BAT of lean mice, and adrenalectomy increased the NE content of BAT in ob/ob mice (Table 3.1). Calculated rates of NE turnover (NE content x k) in BAT were approximately 40 % lower in sham-operated ob/ob mice than in lean mice independent of diet fed (Table 3.1). NE turnover was elevated by adrenalectomy in ob/ob mice fed the high-starch diet, but not in ob/ob mice fed the high-glucose diet.

In heart, fractional rates of NE turnover and calculated rates of NE turnover were similar in sham-operated ob/ob and lean mice independent of diet fed (Figure 3.5 and Table 3.1). Adrenalectomy increased the fractional and calculated rates of NE turnover in hearts of ob/ob mice fed the high-glucose diet but not in ob/ob mice fed the high-starch diet.

Concentrations of glucose in plasma of mice fed the high-starch diet were lower than those of mice fed the high-glucose diet, and were unaffected by phenotype. Plasma glucose concentrations of ob/ob mice fed the high-glucose diet were decreased 18 % by adrenalectomy (Figure 3.6).

Plasma insulin concentrations were more than 20 times higher in sham-operated ob/ob mice than in lean mice independent of diet (Figure 3.6). Adrenalectomy markedly decreased plasma insulin concentrations in ob/ob mice fed the high-starch diet. Plasma insulin concentrations were also suppressed in adrenalectomized ob/ob mice fed the high-glucose diet, but the values remained higher in adrenalectomized ob/ob mice fed the high-glucose diet than in adrenalectomized ob/ob mice fed the high-starch diet. Plasma insulin concentrations in lean mice were unaffected by surgery or diet.

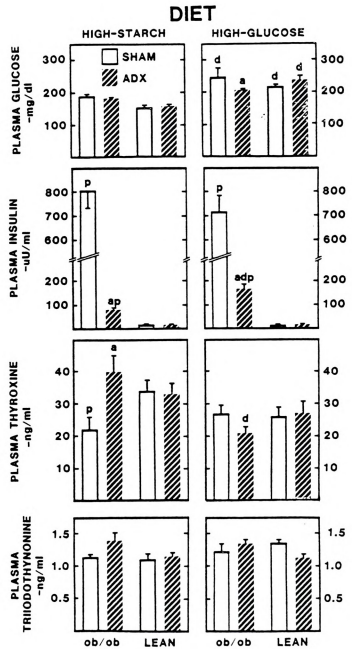


Fig. 3.6 Plasma glucose, insulin, thyroxine and triiodothyronine concentrations of mice fed high-starch or high-glucose diets. Each bar represents the mean±SE for 10-21 mice. Letter a indicates significant effect ($P<0.05$) of adrenalectomy within phenotype and diet treatment; p, significant effect of phenotype within surgical and diet treatment; d, significant effect of diet within surgical and phenotype treatment.

Plasma thyroxine concentrations were low in sham-operated ob/ob mice fed the high-starch diet and were elevated by adrenalectomy. No treatment effects on plasma thyroxine concentrations were evident in mice fed the high-glucose diet or on plasma triiodothyronine concentrations in mice fed either diet (Figure 3.6).

3.4. DISCUSSION

Results of the present study confirm earlier findings (37,120) that effects of adrenalectomy in ob/ob mice are diet dependent. Adrenalectomy prevented further development of obesity and stimulated BAT metabolism in ob/ob mice fed the high-starch purified diet, but not in ob/ob mice fed the high-glucose purified diet.

The sham-operated ob/ob mice were not hyperphagic; therefore, the high efficiency of energy retention in these mice was totally a consequence of enhanced metabolic efficiency. Defective thermogenic capacity in BAT has been proposed as one mechanism to enhance metabolic efficiency in ob/ob mice housed at temperatures below thermoneutrality (approx. 33'C) (35,42,43,60,127). Consistent with this, reduced GDP binding to BAT mitochondria and GDP-inhibitible mitochondrial swelling were observed in sham-operated ob/ob mice fed either the high-starch or the high-glucose diet. This reduced thermogenic activity of BAT in ob/ob mice is likely a result of low sympathetic nervous system stimulation of the tissue, as reflected by the slow rates of NE turnover observed in these mice independent of diet fed. Rates of NE turnover in BAT of these ob/ob and lean mice were comparable to those observed in mice fed stock diets (60).

Food intake was reduced in ob/ob mice after adrenalectomy as reported earlier (37,108,120,132). This supports a role for adrenal secretions in the control of food intake in ob/ob mice independent of diet (23,24,37,120). But effects of adrenalectomy on energy balance in ob/ob mice fed the high-starch diet cannot be entirely explained by their reduced energy intake, since pair feeding ob/ob mice with leans did not decrease efficiency of energy retention (69). Thus, adrenalectomy must increase energy expenditure per unit energy consumed in ob/ob mice fed the high-starch diet. One source of increased energy expenditure in the mice may be BAT, because GDP binding to BAT mitochondria, and the rate of GDP-inhibitible mitochondrial swelling were higher in adrenalectomized ob/ob mice fed a high-starch diet than in sham-operated ob/ob mice. Rates of NE turnover in BAT of adrenalectomized ob/ob mice fed the high-starch diet were likewise increased, indicating that the increased BAT thermogenic activity is closely associated with sympathetic nervous system stimulation of BAT (87). All these effects of adrenalectomy in ob/ob mice fed the purified high-starch diet mimic the reported responses to adrenalectomy in ob/ob mice fed nonpurified high-starch diets (48,132).

Consumption of the high-glucose purified diet diminished most of the effects of adrenalectomy in ob/ob mice. Neither energy balance nor BAT metabolism were markedly changed after adrenalectomy of ob/ob mice fed the high-glucose diet. These results contrast with the findings in adrenalectomized ob/ob mice fed the high-starch diet. Thus, the type of carbohydrate consumed plays an important role in regulating metabolic responses to adrenalectomy. The observation that

high-glucose diet can to some extent offset the effects of adrenalectomy on energy balance and BAT metabolism in ob/ob mice indicates that adrenal secretions per se cannot entirely explain the development of obesity in ob/ob mice. Therefore, some other factor that can be influenced by diet must participate in development of obesity in these mice.

Adrenalectomy decreases plasma insulin concentrations (30,91), restores insulin sensitivity to skeletal muscle (91) and improves glucose tolerance (37) of obese rodents fed nonpurified high-starch diets. In agreement with these findings, plasma insulin concentrations were markedly lowered in adrenalectomized ob/ob mice fed the purified high-starch diet. Adrenalectomized ob/ob mice fed the glucose diet continue to have an impaired glucose tolerance (37) and have a higher plasma insulin concentration than adrenalectomized ob/ob mice fed starch diet (Figure 3.6). These results parallel the changes in energy balance and BAT thermogenesis observed in adrenalectomized ob/ob mice fed diets varying in type of carbohydrate and suggest that the type of carbohydrate consumed by ob/ob mice may influence the outcome of adrenalectomy by affecting plasma insulin concentrations.

High concentrations of adrenal steroids have been shown to modify thyroid function by suppressing iodide uptake and turnover by the thyroid (54). In this regard York et al (137) reported that adrenalectomy reduced the half-life of thyroidal iodine in ob/ob mice to values comparable to those in lean mice. These mechanism may have contributed to the increase in plasma thyroxine concentration observed in adrenalectomized ob/ob mice fed starch diet (Figure 3.6). However,

other factors must be involved as well because adrenalectomy failed to increase plasma thyroxine concentrations in ob/ob mice fed glucose. The extent to which these diet-dependent effects of adrenalectomy on thyroid hormones contribute to the observed changes in energy balance in ob/ob mice awaits further investigation.

CHAPTER 4. EFFECTS OF WARM ENVIRONMENT AND ADRENALECTOMY ON ENERGY BALACE AND BROWN ADIPOSE TISSUE METABOLISM IN OB/OB MICE

4.1. INTRODUCTION

Adrenalectomy prevents development of obesity in ob/ob mice housed at 20-25'C and fed a high-starch diet partly by stimulating the low thermogenic activity of brown adipose tissue (chapter 3). When ob/ob and lean mice are housed in a warm environment (33-35'C) brown adipose tissue thermogenic activity is equally low in both groups of mice, but ob/ob mice still retain dietary energy more efficiently than lean mice (60,99). These results suggest that factors other than brown adipose tissue contribute to the high efficiency of energy retention in ob/ob mice. The present study was undertaken to determine if adrenalectomy would prevent development of obesity in ob/ob mice housed in a warm environment (35'C), and if the thermogenic activity of brown adipose tissue in adrenalectomized ob/ob mice would be stimulated under these conditions.

4.2. MATERIALS AND METHODS

Animals and Diets

Female C57BL/6J obese (ob/ob) and lean (ob/+ or +/+) littermates obtained from our breeding colony were weaned at 3 week of age and housed in plastic solid-bottom cages with wood shavings for bedding at 23-25'C with a 12 h light-dark cycle (lights on at 0700). A high-starch nonpurified diet (Wayne Lab-Blox, Continental Grain, Chicago, IL) and water were available ad libitum.

At 3.5 week of age, ob/ob and lean pairs were separated from littermates and housed one mouse per cage at 35'C for 3-4 days of

adaptation. At 4 week of age, bilateral adrenalectomy and sham surgery were conducted as previously described. After surgery, adrenalectomized mice received a 0.9 % NaCl solution ad libitum. Ten pair of ob/ob and lean mice were killed at 4 week of age to estimate initial hindlimb muscle weight and body energy content. All experimental mice were fed ad libitum a high-starch semipurified diet, except for one group of sham-operated ob/ob mice that was pair-fed to adrenalectomized ob/ob mice. The pair-fed ob/ob mice were given food at 1400 h daily. The diet contained (g/100 g) 20 casein, 0.3 methionine, 59.2 starch, 5.0 corn oil, 3.5 mineral mix (7), 1.0 vitamin mix (7), 0.2 choline chloride, and 5.0 cellulose. This diet provided 22, 66 and 12 % of metabolizable energy as protein, carbohydrate and fat, respectively. Food intake and body weights were recorded weekly.

Experimental Design

Five groups of mice (SHAM and ADX ob/ob, SHAM and ADX lean, and pair-fed SHAM ob/ob) were housed at 35'C and fed the high-starch diet from 4 to 8 weeks of age. Energy balance, hindlimb muscle weight, plasma hormones and glucose, and BAT metabolism were determined. Additional 4 week old mice (Sham and ADX ob/ob, and SHAM and ADX lean) were housed at 35'C for only 10 days to explore the early effects of a warm environment and adrenalectomy on BAT thermogenic activity, rates of norepinephrine turnover in BAT and heart, and plasma insulin and glucose concentrations.

Plasma Hormones and Glucose

At the end of the feeding period, mice were killed by decapitation between 0900-1000 h, except for animals used to measure norepinephrine turnover which were killed at various time intervals according to the schedule indicated in Figure 4.6. Plasma corticosterone concentrations were determined by RIA (Endocrine Science, Tarzana, CA) as previously described. The lower limit of detection with this assay was 0.15 ug corticosterone/dl plasma. Adrenalectomized mice with plasma corticosterone concentrations below limits of detection were assigned a value of 0.15 ug corticosterone/dl plasma. Only those adrenalectomized mice with plasma corticosterone concentrations below 1 ug/dl were included in the study. Plasma corticosterone concentrations averaged 0.6 ± 0.01 and 0.5 ± 0.04 ug/dl in adrenalectomized ob/ob and lean mice, repectively. Sham-operated ad lib-fed ob/ob, pair-fed ob/ob, and lean mice had values of 12.6 ± 1.4, 11.2 ± 1.0, and 10.4 ± 1.2 ug corticosterone/dl plasma, repectively, after the 4 wk experimental period. Plasma insulin, glucose, thyroxine, and triiodothyronine concentrations were measured as described before.

Hindlimb Muscle Weights and Energy Balance

Weights of muscles from both hindlimbs were recorded after stripping all of the muscles from the bones and removing all visible connective tissue and fat. All tissues were returned to the carcass. Gastric contents were removed, and carcasses were then autoclaved and homogenized in water. The homogenates were dried at 50'C, and aliquots of the dried homogenates were combusted in a bomb calorimeter. Body energy gain and efficiency of energy retention were calculated as

described before. Body protein was determined by a microkjeldahl method (118) on aliquots of carcass homogenate.

BAT cytochrome c oxidase, GDP binding, and Mitochondrial Swelling

Interscapular and subscapular brown adipose tissue (BAT) depots were rapidly removed after mice were killed. Tissues from two mice in the same treatment group were pooled and homogenized. Aliquots of the homogenate were obtained for estimation of protein content and cytochrome c oxidase activity. Mitochondria were isolated from the remaining homogenate for determination of protein content and cytochrome c oxidase activity as described before.

Binding of ^{3}H-GDP to BAT mitochondria was determined by the method described by Nicholls (86) with slight modifications. In brief, an aliquot of the mitochondrial suspension containing 0.25-0.5 mg of protein was added to 0.5 ml of incubation media (pH 7.1) containing 100 mM sucrose, 20 mM K-TES, 1 mM EDTA (not included when Mg++ was added, see below), 2 uM rotenone, 100 uM potassium atractyloside, 2.5 x 10^{6} dpm ^{3}H-GDP/ml incubation media (New England Nuclear;10.2 Ci/mmol) and unlabeled GDP (10 uM). ^{14}C-sucrose, 5.55 x 10^{5} dpm/ml incubation media (New England Nuclear; 673 mCi/mmol), was included to calculate the volume of media trapped in the final mitochondrial pellet. Nonspecific binding was assessed from the binding of ^{3}H-GDP in the presence of excess unlabeled GDP (200 uM). Incubations were conducted at 25'C for 10 min and the tubes were quickly centrifuged. The mitochondrial pellet was subsequently dissolved (BTS-450) and counted in a liquid scintillation counter.

The possibility of activation of GDP binding sites by covalent

modification was examined by incubating BAT mitochondria with Mg++ (123). The mitochondrial suspensions were incubated with or without 10 mM MgCl in an incubation media containing 250 mM sucrose and 5 mM K-TES (pH 7.2) at 37'C for 20 min. Pellets were washed twice with 0.2 mM EDTA to facilitate the removal of Mg++. These mitochondrial preparations were used for GDP binding assay as discussed above.

The rate of acetate-induced, GDP-inhibitable BAT mitochondrial swelling was measured spectrophotometrically at 520 nm by incubating mitochondria (0.1-0.2 mg mitochondrial protein/ml incubation media) in a media consisting of 100 mM K-acetate, 5 mM TES, 5 uM rotenone, and 0.5 uM valinomycin (pH 7.2) (88,122). The rate of BAT mitochondrial swelling was calculated as the difference in absorbance change between assays conducted with and without 0.1 mM GDP.

Norepinephrine (NE) Turnover

Rates of norepinephrine turnover were estimated in mice at the end of the 10 day feeding trial. Levo (ring-2,5,6-^{3}H) norepinephrine (40.8 Ci/mmol, New England Nuclear), diluted in 0.9% saline to provide a dose of 250 uCi ^{3}H/kg body wt., was injected (i.p.) in a volume of 0.2 ml between 0900 and 1000. Mice were killed 2,6,10,14,18, or 24 h after injection. BAT (interscapular and subscapular) depots and hearts were rapidly removed and frozen. Norepinephrine content and specific radioactivities in tissues were measured by high-performance liquid chromatography (HPLC) and electrochemical detection as previously described. Norepinephrine was collected from the column and counted in a liquid scintillation counter to measure ^{3}H-norepinephrine specific radioactivity. Slopes of linear regressions describing the

disappearance of ^{3}H-norepinephrine from tissues were used to calculate the fractional turnover rates of norepinephrine. Rates of norepinephrine turnover were calculated as the product of fractional turnover rate for each experimental group and norepinephrine content of organs of individual animals.

Statistics

Data (ad libitum fed SHAM and ADX ob/ob, and lean mice) were analyzed statistically by analysis of variance, followed by Bonferonni t-test (33). Comparisons between pair-fed ob/ob and ADX ob/ob, and between pair-fed ob/ob and ad libitum fed ob/ob mice, was conducted by t-test for paired data. All differences were considered significant at $P<0.05$.

4.3. RESULTS

Energy intake of ad libitum fed, sham-operated ob/ob mice housed at 35'C was 25-40 % higher than that of their lean littermates throughout the 4 week study; total intake was 30 % greater in ob/ob mice than in lean mice (Figure 4.1). Likewise, these sham-operated ob/ob mice gained more body weight and retained energy with a higher efficiency than lean mice (Figure 4.1). Adrenalectomy reduced energy intake, body weight gain, energy gain and efficiency of energy retention of ob/ob mice to values essentially comparable to lean counterparts (Figure 4.1). Effects of adrenalectomy on body energy gain and efficiency of energy retention in ob/ob mice were partially a consequence of reduced energy intake, because pair-fed, sham-operated

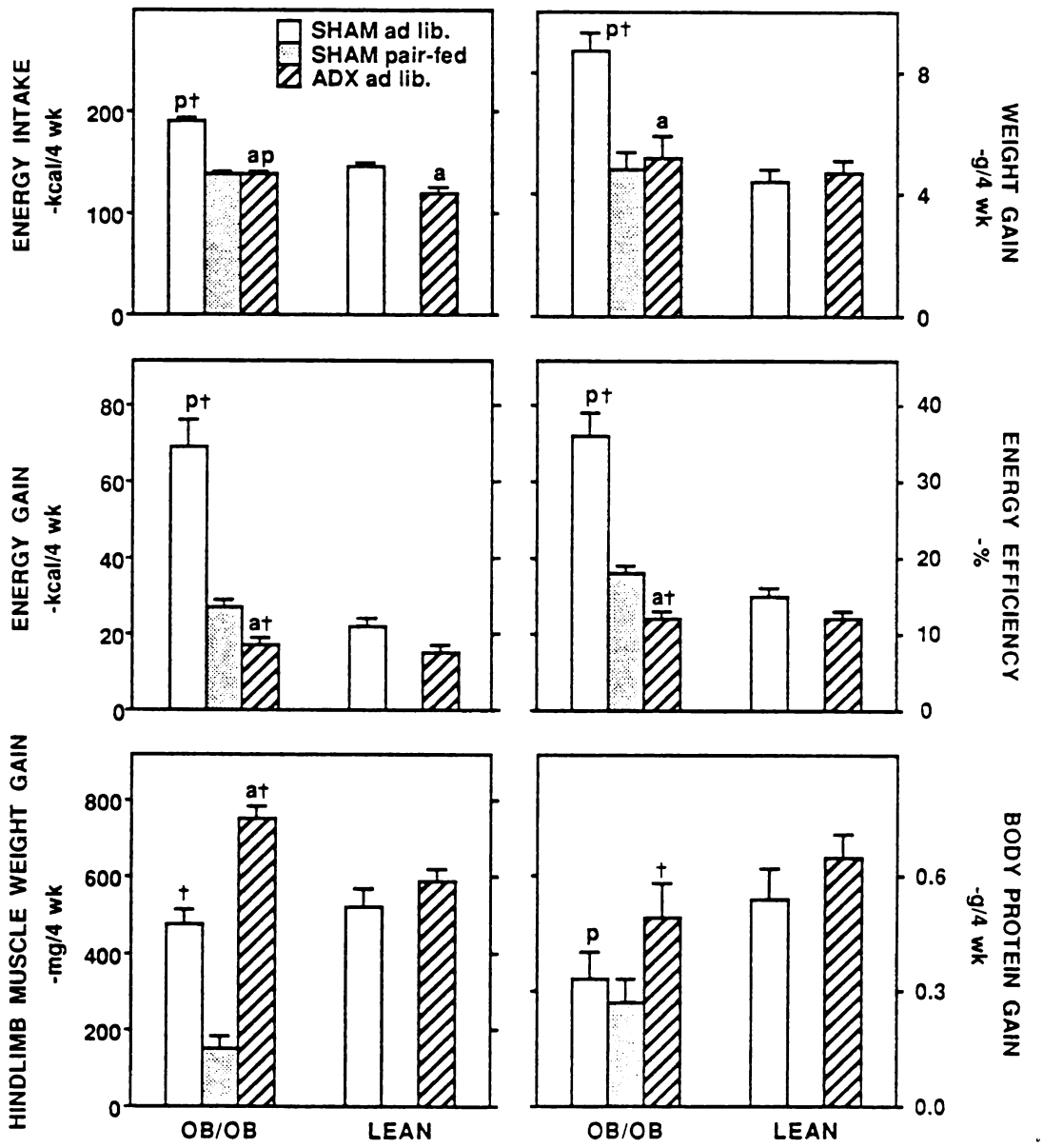


Fig. 4.1 Energy intake, body weight gain, energy gain, efficiency of energy retention, hindlimb muscle weight gain, and body protein gain in mice fed a high-starch diet and housed at 35°C for 4 wk. Each bar represents mean±SE for 10-12 mice. Initial body energy values were 41.7±3.0 and 20.7±1.3 kcal for ob/ob and lean mice, respectively. Initial hindlimb muscle weights averaged 776±33 and 898±47 mg for ob/ob and lean mice, respectively. Initial body protein content averaged 1.54±0.05 and 1.35±0.08 g for ob/ob and lean mice, respectively. a, Significant ($P<0.05$) effect of adrenalectomy within phenotype; p, Significant ($P<0.05$) effect of phenotype within same surgical group; +, Significant differences between pair-fed, sham-operated ob/ob and ad libitum-fed, sham-operated or adrenalectomized ob/ob mice.

ob/ob mice retained energy with a lower efficiency than ad libitum fed, sham-operated ob/ob mice, but with a higher efficiency than adrenalectomized ob/ob mice. Reduced energy intake per se played no role in the adrenalectomy-induced increase in hindlimb muscle mass and total body protein gain in ob/ob mice (Figure 4.1) as pair-fed, sham-operated ob/ob mice actually gained much less hindlimb muscle and body protein than either sham-operated, ad libitum fed ob/ob mice or adrenalectomized ob/ob mice. Adrenalectomy had minimal influences in lean mice.

Plasma glucose and hormone concentrations in mice fed for 4 weeks are presented in Figure 4.2. Plasma glucose concentrations were unaffected by the treatments. Plasma insulin concentrations of ad libitum fed, sham-operated ob/ob mice housed at 35'C were only three times higher than that of lean mice, contrary to the extreme hyperinsulinemia (more than 20 fold elevations often observed) of 8 week old ob/ob mice housed at 23-25'C (chapter 3). Adrenalectomy reduced plasma insulin concentrations in ob/ob mice largely as a result of lowered energy intake. Plasma thyroxine concentrations were lower in ad libitum fed, sham-operated ob/ob mice than their lean littermates, and restriction of energy intake had no effect on plasma thyroxine concentration in sham-operated ob/ob mice. Adrenalectomy increased plasma thyroxine concentrations in ob/ob mice to the same level as in lean mice. Plasma triiodothyronine concentrations were relatively unaffected by adrenalectomy or food restriction in ob/ob mice.

The combined interscapular and subscapular BAT depots of ad libitum-fed, sham-operated ob/ob mice housed at 35'C for 4 weeks were

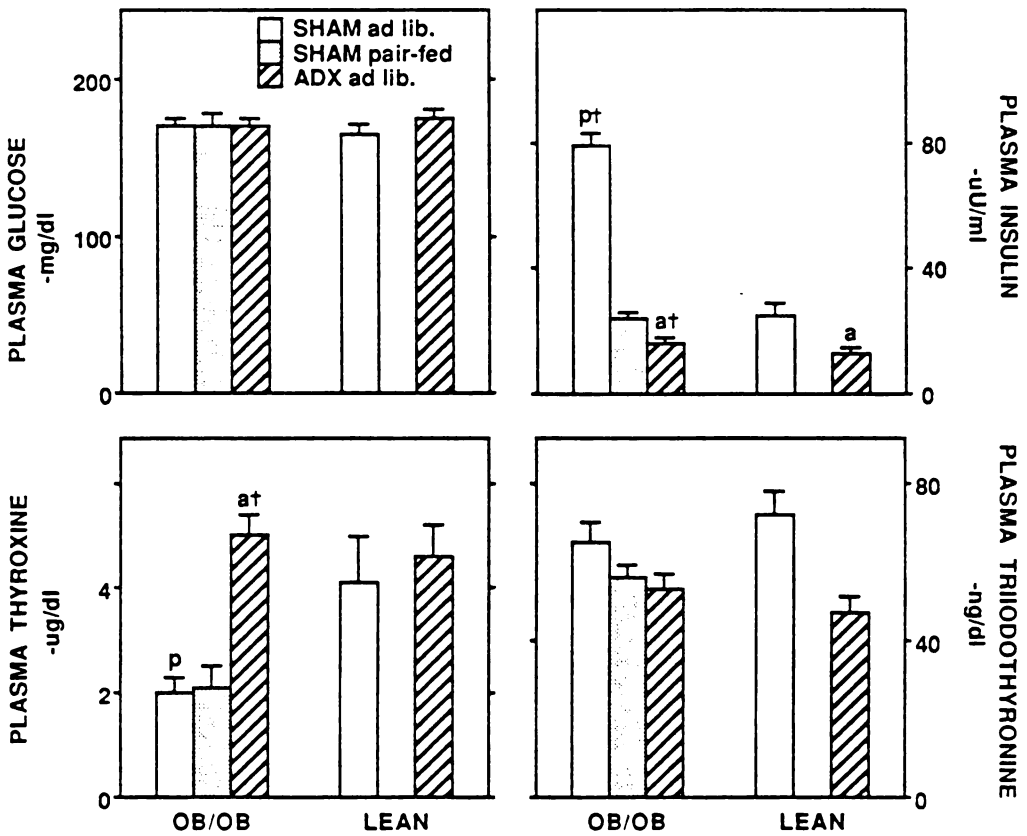


Fig. 4.2 Plasma glucose, insulin, thyroxine, and triiodothyronine concentrations of mice fed a high-starch diet and housed at 35°C for 4 wk. Each bar represents mean±SE for 20-24 mice. a, Significant ($P<0.05$) effect of adrenalectomy within phenotype; p, Significant ($P<0.05$) effect of phenotype within same surgical group; +, Significant differences between pair-fed, sham-operated ob/ob and ad libitum-fed, sham-operated or adrenalectomized ob/ob mice.

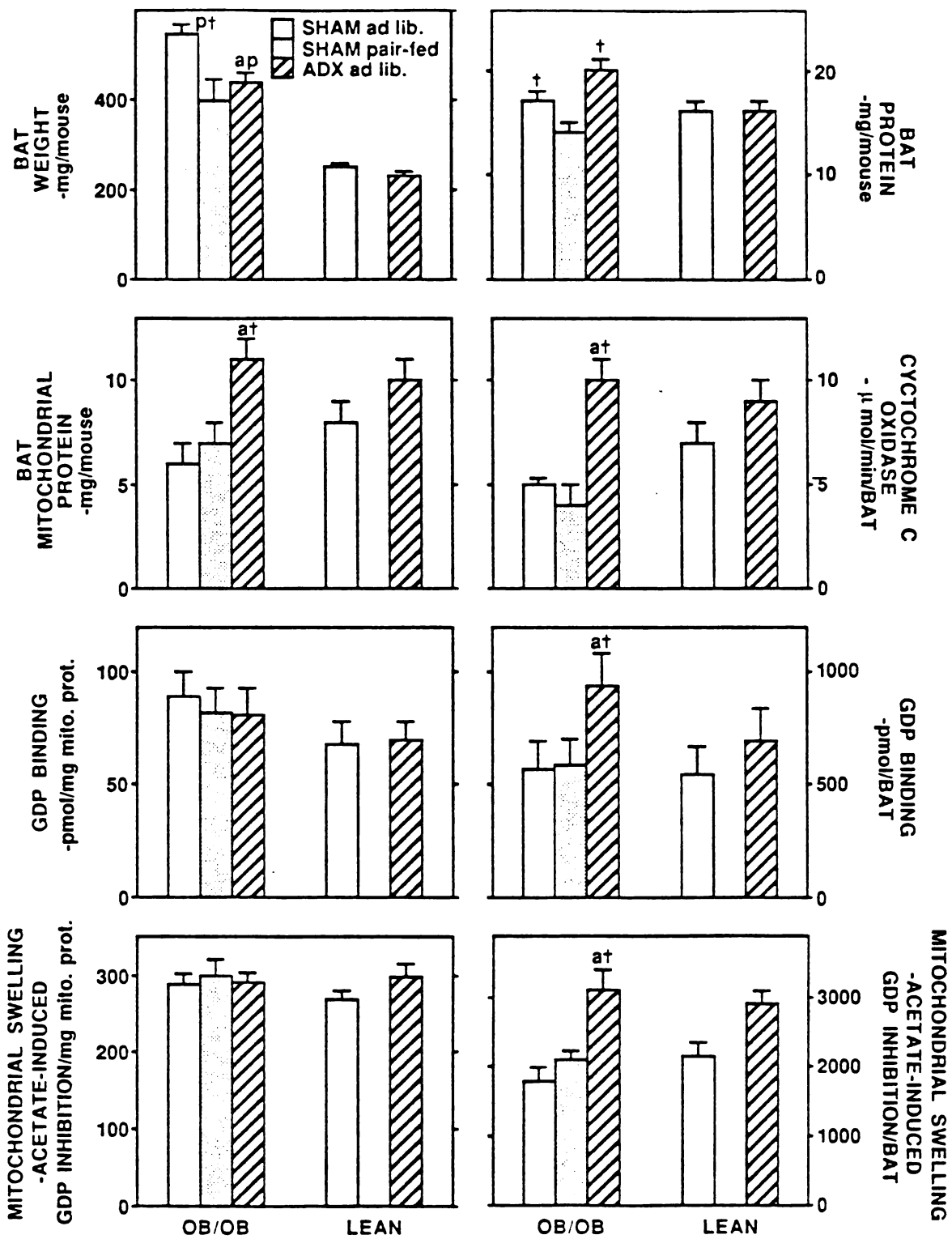
heavier than those from lean mice, but the protein content and (Figure 4.3). Adrenalectomy decreased the weight, and increased mitochondrial protein content and cytochrome c oxidase activity of BAT in ob/ob mice. The adrenalectomy-induced changes in BAT weight were probably a secondary consequence of lowered food intake in the adrenalectomized ob/ob mice since food restriction of sham-operated ob/ob mice also lowered BAT weight. However, the increases in mitochondrial mass as reflected by increased mitochondrial protein and cytochrome-c oxidase activity were not linked to lowered food intake per se.

Thermogenic activity of BAT was determined by measuring GDP binding to BAT mitochondria, and by acetate-induced, GDP-inhibitable mitochondrial swelling (Figure 4.3). GDP binding and mitochondrial swelling expressed per mg of mitochondrial protein were unaffected by phenotype, adrenalectomy or restriction of energy intake. However, total GDP binding and mitochondrial swelling expressed per combined interscapular and subscapular BAT depots were increased by adrenalectomy in ob/ob mice, but not in lean mice, because of increased mitochondrial mass.

To examine effects of adrenalectomy and warm exposure on ob/ob mice at an earlier time, mice were killed after 10 days of treatment. Plasma glucose concentrations were unaffected by adrenalectomy or phenotype (Figure 4.4). Plasma insulin concentrations in sham-operated ob/ob mice were higher than in leans, and adrenalectomy lowered plasma insulin concentrations of ob/ob mice to similar level as leans (Figure 4.4).

BAT protein, mitochondrial protein and cytochrome c oxidase activity from mice housed at 35'C for 10 days were unaffected by

Fig. 4.3 Interscapular plus subscapular brown adipose tissue (BAT) depot weight, protein, mitochondrial protein, cytochrome c oxidase activity, GDP binding to BAT mitochondria, and acetate-induced GDP-inhibitable mitochondrial swelling in BAT of mice fed a high-starch diet and housed at 35'C for 4 wk. Each bar represents mean±SE for 6 analyses, each obtained from pooled BAT depots from 2 mice. Cytochrome c oxidase activity was expressed as umol cytochrome c oxidized/min at 25'C. Total GDP binding/BAT was calculated from the recovery of cytochrome c oxidase and GDP binding/mg mitochondrial protein. Recovery of cytochrome c oxidase activity was calculated from the % of total homogenate cytochrome c oxidase recovered in mitochondrial preparations. Mitochondrial swelling was expressed in arbitary units as change in absorbance at 520 nm/min. GDP-inhibitable BAT mitochondrial swelling was calculated from the difference of changes in absorbance in the presence or absence of 0.1 mM GDP. a, Significant ($P<0.05$) effect of adrenalectomy within phenotype; p, Significant ($P<0.05$) effect of phenotype within same surgical group; +, Significant differences between pair-fed, sham-operated ob/ob and ad libitum-fed, sham-operated or adrenalectomized ob/ob mice.


SHAM ad lib.
SHAM pair-fed
ADX ad lib.
BAT WEIGHT -mg/mouse
BAT PROTEIN -mg/mouse
BAT MITOCHONDRIAL PROTEIN -mg/mouse
CYCTOCHROME C OXIDASE - μ mol/min/BAT
GDP BINDING -pmol/mg mito. prot.
GDP BINDING -pmol/BAT
MITOCHONDRIAL SWELLING -ACETATE-INDUCED GDP INHIBITION/mg mito. prot.
MITOCHONDRIAL SWELLING -ACETATE-INDUCED GDP INHIBITION/BAT
OB/OB
LEAN
OB/OB
LEAN

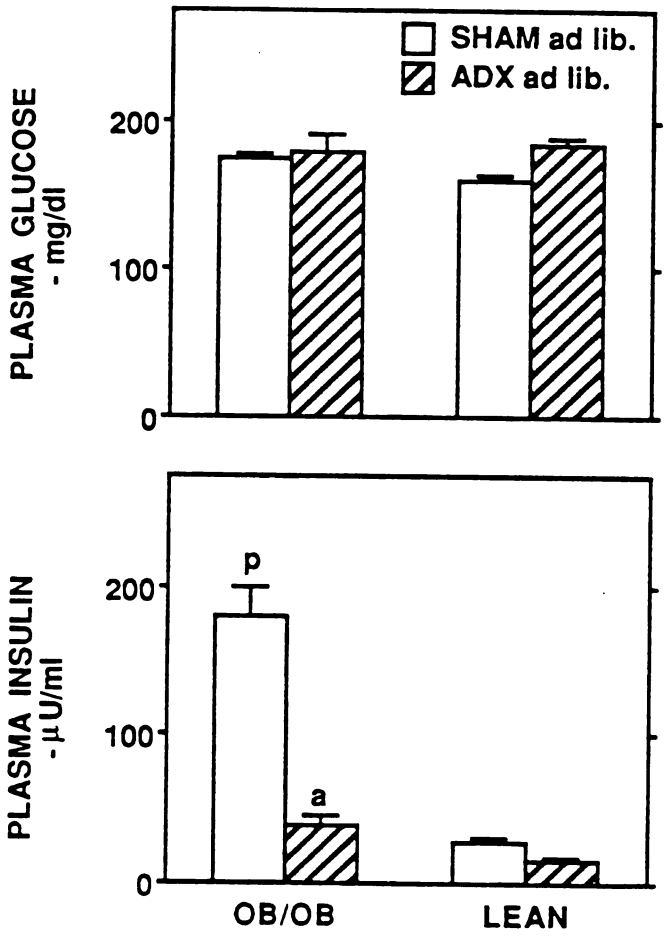


Fig. 4.4 Plasma glucose and insulin concentrations of mice fed a high-starch diet and housed at 35'C for 10 days. Each bar represents mean±SE for 20-25 mice. a, Significant ($P<0.05$) effect of adrenalectomy within phenotype; p, Significant ($P<0.05$) effect of phenotype within same surgical group.

phenotype or adrenalectomy (Figure 4.5). Thermogenic activity, assessed by GDP binding to BAT mitochondria, was similar in sham-operated ob/ob and lean mice. Adrenalectomy increased specific GDP binding slightly ($P<0.1$) and total GDP binding ($P<0.05$) in ob/ob mice without affecting lean mice. Activation of GDP binding sites by divalent cations (Mg++) was unaffected by phenotype or adrenalectomy. Activation of GDP binding by Mg++ was approximately 2 fold (180-217 %) for all groups.

Sympathetic nervous system stimulation of BAT thermogenesis was determined by measuring norepinephrine turnover. Combined inter- and subscapular BAT depots of sham-operated and adrenalectomized ob/ob and lean mice contained comparable amounts of norepinephrine (Table 4.1). Fractional rates (k) of norepinephrine turnover and calculated rates of norepinephrine turnover (NE content x fractional rate of NE turnover) in BAT of sham-operated ob/ob mice were similar to those of lean mice, and were elevated by adrenalectomy only in ob/ob mice (Table 4.1 and Figure 4.6). Heart weight, norepinephrine content, fractional rates of norepinephrine turnover, and calculated rates of norepinephrine turnover were generally comparable in ob/ob and lean mice, and adrenalectomy did not affect these parameters.

4.4. Discussion

Results of the present study confirm earlier findings (60,99) that ob/ob mice retain energy with a higher efficiency than lean mice even when thermoregulatory thermogenesis is suppressed by housing mice in a warm environment and hyperphagia is prevented. Circulating

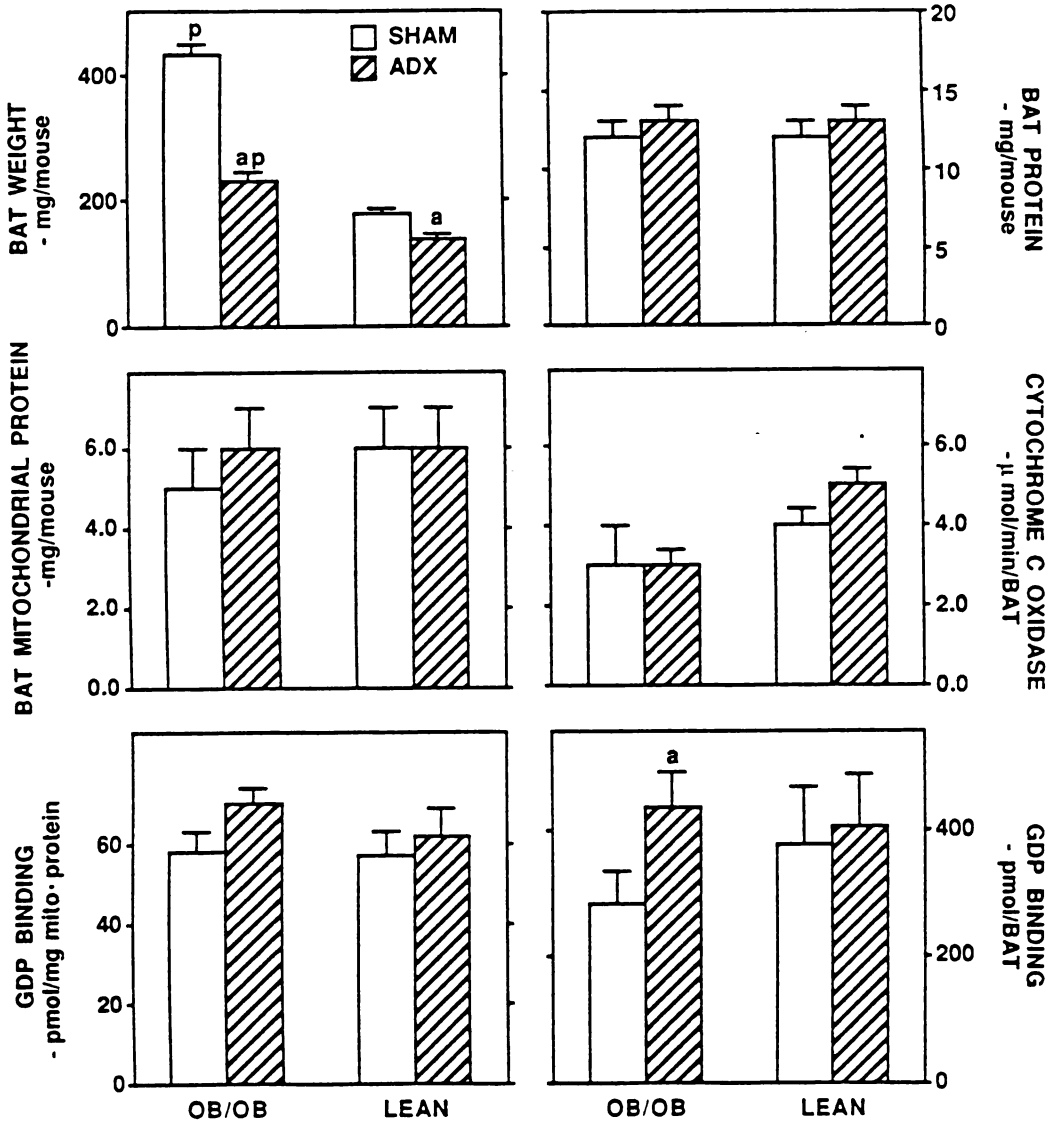


Fig. 4.5 Combined interscapular and subscapular brown adipose tissue (BAT) depot protein, BAT mitochondrial protein, cytochrome c oxidase activity, and GDP binding to BAT mitochondria of mice fed a high-starch diet and housed at 35'C for 10 days. Each bar represents means±SE for 8 analyses, each obtained from pooled BAT depots from 2 mice. a, Significant ($P<0.05$) effect of adrenalectomy within phenotype; p, Significant ($P<0.05$) effect of phenotype within same surgical group.

Table 4.1 Organ weights, norepinephrine content, and norepinephrine turnover in BAT and heart of mice fed a high-starch diet and housed at 35'C for 10 days

	ob/ob		Lean	
	Sham	ADX	Sham	ADX
BAT				
weight - mg	423±18[p]	205±24[ap]	173±9	136±11
NE content - ng	234±12	249±11	254±11	258±11
NETO - ng/h/BAT	28±2	42±4[ap]	34±1	29±2
Heart				
weight - mg	58±2	54±2	57±2	58±2
NE content - ng	69±4	79±4	83±4	88±6
NETO - ng/h/heart	5.2±.3	5.9±.6	5.4±.2	6.1±.7

Values are means±SE for 22-32 mice. BAT is pooled interscapular and subscapular pads. Rates of norepinephrine turnover (NETO) were calculated as the product of k (Figure 4.6) and norepinephrine content. a, Significant effect ($P<0.05$) of adrenalectomy within phenotype. p, Significant effect ($P<0.05$) of phenotype within same surgical group.

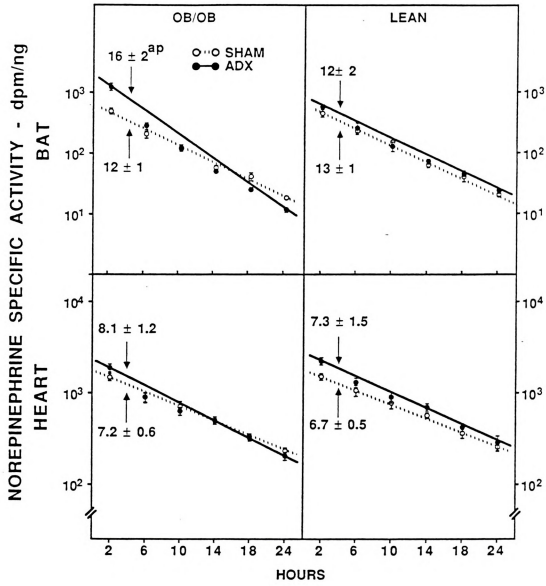


Fig. 4.6 Norepinephrine (NE) specific radioactivity after injection of ^{3}H-norepinephrine in mice fed a high-starch diet and housed at 35°C for 10 days. Each point represents means±SE for 6-8 mice killed 2.6.10.14.18. or 24 h after ^{3}H-NE injection. Numbers in each panel are fractional rates of NE turnover (k)±SE calculated from slopes (b) of each regression line (k = b/0.434). Brown adipose tissue (BAT) represents pooled interscapular and subscapular pads. a, Significant (P<0.05) effect of adrenalectomy within phenotype; p, Significant (P<0.05) effect of phenotype within same surgical group.

corticosterone concentrations were comparable in sham-operated ob/ob and lean mice housed at 35'C, unlike at 23-25'C where plasma corticosterone concentrations are higher in ob/ob mice than lean mice, but skeletal muscle growth was still depressed in ob/ob mice housed at 35'C. Thus, hyperphagia, high metabolic efficiency and stunted skeletal muscle growth in ob/ob mice are not exclusively dependent on higher plasma corticosterone concentrations than in lean counterparts.

Adrenalectomy prevented hyperphagia, stimulated skeletal muscle growth, lowered the efficiency of energy retention, lowered plasma insulin concentrations, and elevated plasma thyroxine concentrations in ob/ob mice housed at 35'C. The adrenalectomy-induced reduction in food intake contributed to the lowered energy retention and plasma insulin concentrations in ob/ob mice, but not to the increase in skeletal muscle mass or plasma thyroxine concentrations. These results parallel what is observed in adrenalectomized ob/ob mice housed at 23-25'C (chapter 3) and demonstrate that housing mice in a warm environment does not block adrenalectomy-induced alterations in body composition and plasma hormones in ob/ob mice.

My expectation was that adrenalectomy would not stimulate brown adipose tissue thermogenesis in the ob/ob mice because of the warm environment (35'C). However, adrenalectomy increased sympathetic nervous system stimulation of brown adipose tissue in ob/ob mice as assessed by norepinephrine turnover in the tissue, brown adipose tissue mitochondrial mass as measured by cytochrome c oxidase activity, and total thermogenic activity of the tissue as determined by GDP binding and swelling of isolated mitochondrial preparations. The percentage increases in norepinephrine turnover (approx. 50 %) and

total GDP binding (also approx. 50 %) in brown adipose tissue of adrenalectomized ob/ob mice housed at 35'C were comparable to those observed in mice housed at 23-25'C (chapter 3). GDP binding and GDP-inhibitable mitochondrial swelling per mg mitochondrial protein were not statistically increased in adrenalectomized ob/ob mice housed at 35'C, unlike at 23-25'C where thermogenic activity per mg mitochondrial protein is increased. Rather, the elevation in thermogenic activity resulted from brown adipose tissue mitochondriogenesis. Norepinephrine has been shown to trigger brown adipose tissue mitochondriogenesis both in vivo and in vitro (32,80).

Adrenalectomy failed to stimulate norepinephrine turnover in hearts of ob/ob mice, or in either brown adipose tissue or heart of lean mice. The mechanism of the tissue and phenotype specific response of the sympathetic nervous system to adrenalectomy is unknown, but has been observed before (chapter 2,3). Corticotropic releasing factor (CRF) synthesis and release would be expected to be increased in the adrenalectomized animals (26,112). CRF activates the sympathetic nervous system (16) and has recently been shown to selectively decrease body weight and plasma insulin concentrations in fa/fa rats without influencing lean counterparts (98). Possibly adrenalectomized ob/ob mice have an altered sensitivity or responsiveness to CRF which contributes to the selective stimulation of the sympathetic nervous system in their brown adipose tissue.

Although adrenalectomy stimulated the thermogenic activity of brown adipose tissue in ob/ob mice housed at 35'C, the quantitative contribution of this increase to the lowered efficiency of energy retention in adrenalectomized ob/ob mice is unknown. Adrenalectomy

also reduced food intake and increased skeletal muscle mass in ob/ob mice, and both factors would contribute to a lowered efficiency of energy retention. The adrenalectomy-induced lowering of plasma insulin concentrations in ob/ob mice would be another potential contributor to the lowered efficiency of energy retention. The overall changes in ob/ob mice induced by adrenalectomy involve several diverse metabolic pathways which are independent of a need for thermoregulatory heat production.

CHAPTER 5. CONCLUSIONS AND DIRECTIONS FOR FUTURE RESEARCH

Adrenalectomy prevents the development of obesity in ob/ob mice partly by stimulating the low thermogenic activity of brown adipose tissue (BAT) when they were fed high-carbohydrate stock diets. However, recently it was reported that adrenalectomized ob/ob mice fed a high-fat diet continued to develop obesity. This suggests that the outcome of adrenalectomy is diet dependent (120). Also eventhough BAT thermogenesis has been claimed to be an important factor for development of obesity in genetically obese mice, there is evidence that BAT plays only a minor role in development of obesity in ob/ob mice housed at a thermoneutral temperature (99). Therefore in the present studies, the outcome of adrenalectomy was examined with several different diet compositions and in a warm environment, where BAT is not needed for thermoregulatory thermogenesis, in an effort to elucidate the mechanism(s) of obesity in these adrenalectomized mice.

Consumption of a high-fat diet normalized the low sympathetic nervous system (SNS) activity and thermogenic activity of BAT in ob/ob mice without preventing their obesity (chapter 2). Selective stimulation of BAT SNS activity and thermogenesis of ob/ob mice fed a high-fat diet may be a consequence of interactions among fat metabolism, carbohydrate metabolism, central nervous system mechanism(s), and environmental temperature. Energy expenditure associated with deposition of body fat is less when a high-fat diet is fed than when a high-carbohydrate diet is fed. This lower energy expenditure related to nutrient assimilation would contribute to an increased demand for thermoregulatory thermogenesis in mice fed a high-fat diet and housed below their thermoneutral temperature.

Another possible mechanism is that ob/ob mice may have defect(s)

in afferent signal and(or) in the mechanism(s) which sense the signal from the diet to trigger an activation of the sympathetic nervous system (SNS). The nutrient-SNS signals generated by fat are not known. However, Bukowiecki et al. (11) suggested that products of lipolysis, especially palmitic acid, may mediate the cellular response to norepinephrine. Palmitic acid was able to mimic 80-85 % of the effect of norepinephrine in isolated brown adipocytes. Hormones stimulated by fatty acids could be another possibility that exert the stimulation of SNS by fat diet. The nutrient-SNS signals for carbohydrate have been suggested the insulin or insulin-mediated glucose metabolism (101). Therefore, it is possible that the sensing mechanism(s) for a high-fat diet, which is probably different from that of carbohydrate, functions normally in ob/ob mice and but that these mice have defects in their carbohydrate sensing mechanism(s). However, evidence that dietary fat per se is not the only factor contributing to the elevation of SNS activity and BAT thermogenesis is the finding that pre-obese ob/ob pups consuming high-fat milk have lower norepinephrine turnover rates and GDP binding of BAT than lean counterparts (36,61,128). Because these pups are able to huddle together in a nest, the temperature in the near environment of individual pups is unknown and may have influenced the results. Determination of energy balance and BAT thermogenesis in ob/ob mice fed a high-fat diet and housed at a thermoneutral temperature would provide more complete evidence.

Regardless of the mechanisms involved in the activation of the SNS in BAT of ob/ob mice fed a high-fat diet, these results show that there is not primary functional defect in BAT of ob/ob mice, or in

their ability to activate the SNS under appropriate environmental condition(s).

If effective BAT function of ob/ob mice is induced by high-fat diet and if defective BAT function is thought to underlie the high metabolic efficiency and development of obesity of these animals, then why do the ob/ob mice fed high-fat diet still develop obesity? Furthermore, ob/ob mice housed at 35'C also deposit energy with a higher efficiency than leans, but both ob/ob and lean mice housed at 35'C have equally low SNS activity and thermogenic activity of BAT. These results indicate factor(s) other than BAT thermogenesis are involved in the development of obesity in ob/ob mice under these conditions. Other tissues such as muscle, liver, and white adipose tissue, and(or) unknown mechanism(s) in BAT other than SNS regulated proton conductance pathway may be involved in this mechanism. The eventual explanation for high metabolic efficiency in ob/ob mice requires more exploration and understanding of the interactions between diet composition, environmental temperature and neuroendocrine regulation.

Adrenalectomy was ineffective in blocking development of obesity in ob/ob mice fed high-fat or high-glucose diets whereas it was effective in ob/ob mice fed a high-starch diet (chapter 3). Energy intake was the only parameter that was normalized to the level of lean counterparts independent of diet composition. The absence of any major effect of adrenalectomy on energy balance and BAT metabolism in ob/ob mice fed high-fat or high-glucose diets indicates that factor(s) other than adrenal secretions per se that can be influenced by diet is(are) responsible for development of obesity in these mice.

Selective interactions of glucocorticoids and diet composition to stimulate BAT metabolism of ob/ob mice may be a consequence of interactions among glucocorticoids, fat, carbohydrate, and central control.

One possibility for this mediator is insulin. Adrenalectomized ob/ob mice fed high-fat or high-glucose diets continue to have an impaired glucose tolerance (based on data generated independently this dissertation) and have higher plasma insulin concentrations than adrenalectomized ob/ob mice fed the starch diet. These results parallel the changes in metabolic efficiency in adrenalectomized ob/ob mice fed fat or diets varying in type of carbohydrate (chapter 2 and 3), and suggest that the diet consumed by ob/ob mice may influence the outcome of adrenalectomy by affecting plasma insulin concentrations. It would be interesting to see if injection of insulin to adrenalectomized ob/ob mice fed the high-starch diet in doses sufficient to elevate plasma insulin concentrations to the level of adrenalectomized ob/ob mice fed the high-fat or high-glucose diets would affect the metabolic efficiency.

There is some evidence that high parasympathetic nervous activity (PNS) in obese rodents is responsible for the defective BAT thermogenesis and high metabolic efficiency (102). Then, it is possible that adrenalectomy not only increases SNS activity but also decreases PNS activity in ob/ob mice. Since diet composition selectively stimulates SNS activity it could also be speculated that diet composition might selectively inhibit PSN activity. Selective inhibition of PSN activity with different diets in adrenalectomized ob/ob mice may explain different plasma insulin concentrations in

these mice. Additional studies are needed to examine the involvement of PSN activity in adrenalectomized ob/ob mice fed different diet compositions to provide more insights into the mechanism(s).

Another possibility to explain the different responses of the adrenalectomized ob/ob mice to the diets fed is that there might be another organ involved in providing small amounts of glucocorticoids, and different diet compositions may stimulate this organ differently. Indeed, I saw small amounts of corticosterone in the adrenalectomized mice, and slightly higher plasma corticosterone concentrations in adrenalectomized ob/ob mice fed the high-fat or high-glucose diets than in starch-fed mice. Since, ob/ob mice are more sensitive to glucocorticoids than lean counterparts (47), small differences in plasma corticosterone level in adrenalectomized ob/ob (0.26 ug/dl in starch fed group and 0.47-0.51 ug/dl in glucose or fat fed group) may exert a difference in metabolic efficiency and BAT metabolism observed. The possible involvement of the ovary as an organ for glucocorticoid secretion, and the interaction of glucocorticoid with different diet compositions are currently being investigated with combined adrenalectomy and ovariectomy of ob/ob mice.

Removal of negative feedback action of glucocorticoids by adrenalectomy stimulates hypothalamic corticotropin releasing factor (CRF) synthesis and release. CRF activates SNS activity and has recently been shown to selectively decrease body weight and plasma insulin level in fa/fa rats without influencing lean rats (8). Also, there are some suggestions that vasopressin (AVP) may be involved in the response of adrenalectomized animals. After adrenalectomy, the hypothalamic and hypophysial portal plasma concentration of AVP

increased more than that of CRF, and AVP potentiated the CRF action on ACTH secretion (26). However, the involvement of these hypothalamic neurohormones in adrenalectomized ob/ob mice awaits further investigation.

In summary, there is no primary functional defect in BAT of ob/ob mice or in their ability to activate the SNS under appropriate environmental conditions. Effective functioning of BAT could be induced by high-fat content of the diet, but high metabolic efficiency was maintained even with effective BAT function in ob/ob mice. In addition, the effects of adrenalectomy are diet dependent; fat and glucose diet abolished the effects of adrenalectomy whereas starch diet did not. Also, warm environment (35°C), where BAT thermoregulatory thermogenesis is not needed, failed to prevent the increase in BAT metabolism in adrenalectomized ob/ob mice which suggests that other mechanisms rather than BAT thermoregulatory heat production are involved in these mice.

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