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THE STUDY OF Na^+,K^+-ATPase IN THE ETIOLOGY OF OBESITY IN THE OBESE (Ob/Ob) MOUSE

By

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ABSTRACT

THE STUDY OF Na^+,K^+-ATPase IN THE ETIOLOGY OF OBESITY IN THE OBESE (Ob/Ob) MOUSE

By

Ming Huey Lin

The possible involvement of Na^+,K^+-ATPase in the etiology of obesity in the ob/ob mouse was explored. The number of Na^+,K^+-ATPase enzyme units in skeletal muscle, liver, and kidneys from lean and obese mice was estimated from saturable (^3H)-ouabain binding to particulate fractions. A 28% reduction in Na^+,K^+-ATPase enzyme units in skeletal muscle of genetically obese (ob/ob) mice is observed as early as 14 days of age. This represents one of the earliest reported developmental differences in an enzyme system between lean and obese mice. Later in development differences in number of Na^+,K^+-ATPase units (per mg skeletal muscle protein) between lean and obese mice are even greater. The enzyme units are 25% to 36% lower in 4 and 8 week old obese mice than in their lean littermates. The number of Na^+,K^+-ATPase enzyme units in livers of 14 day old obese mice is not reduced. But, four and eight week old obese mice have 19% and 54% fewer enzyme units per mg liver protein, respectively, than their lean littermates. Na^+,K^+-ATPase enzyme units in kidneys of obese mice are not lower than in lean littermates at any stage of

development.

Since ob/ob mice have been suggested to be hypothyroid, the response of Na^+,K^+-ATPase in these mice to thyroid treatment was also investigated. The number of Na^+,K^+-ATPase enzyme units in each of the three organs of obese mice increased to a much greater extent after thyroid hormone injection than did the enzyme in these tissues of lean mice. These results agree with the lower response of Na^+,K^+-ATPase to thyroid hormone administration in euthyroid rats than hypothyroid rats and suggest that obese mice are functionally hypothyroid.

Because the increased number of Na^+,K^+-ATPase enzyme units in obese mice can be increased by exogenous thyroid treatment, I determined whether obese mice would be able to alter Na^+,K^+-ATPase enzyme units under other conditions as well. Exposure to cold is known to increase Na^+,K^+-ATPase activity in lean rodents. Although obese mice cannot survive a severe cold stress, I demonstrated that young obese mice can survive a moderate, chronic cold exposure (14^oC) and that Na^+,K^+-ATPase in obese mice responds to chronic cold-exposure to approximately the same extent observed in lean mice. Further changes in Na^+,K^+-ATPase parallel the increased heat production in the cold-exposed mice. The increases in Na^+,K^+-ATPase units in the tissues of cold-exposed mice also parallel

those obtained in mice given thyroid hormones.

It has been demonstrated that concentrations of Na^+,K^+-ATPase in diaphragm and heart of rats are influenced by thyroid status; concomitant changes in intracellular sodium (Na^+) and potassium (K^+) concentrations are observed. Since the reductions in Na^+,K^+-ATPase in muscle and livers of obese mice are comparable to those observed in hypothyroid rats, I further examined the possibility that obese mice also have alterations in tissue Na^+ and K^+. Obese mice have lower K^+ content in muscle and liver and higher Na^+ content in muscle than lean counterparts. Calculated intracellular Na^+/K^+ ratios were 56% higher in livers of obese mice and 116% higher in muscle of obese mice than those observed in lean mice. Ouabain, a specific inhibitor of Na^+,K^+-ATPase, decreased oxygen consumption less in obese mice (12 to 25%) than in lean mice (19 to 38%). These results suggest that Na^+ pump activity might be reduced in obese mice and this, in turn, may contribute to reduced cellular thermogenesis in these animals.

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INTRODUCTION

Obesity is now recognized as a major health hazard in the United States (1,2). It can be considered as a condition in which there is an excessive amount of body fat. It is generally thought that a person maintains a constant body weight via some accurate control mechanism which ensures that intake and output of energy are equal. However, this control mechanism may not operate with equal efficiency in all individuals. Some individuals seem to have a propensity to change their body weight (3) while others appear to have a much more stable weight.

The concept that obesity usually results from eating more than normal is difficult to disprove. Nevertheless, several studies indicate that most obese subjects do not eat more than their slim counterparts (4,5). Most experiments studying control of energy balance in man have concentrated on the short-term regulation of energy intake (6). Any study in controlling energy intake must take account of variations both in the same individual from day to day and between individuals. Therefore, in order to achieve accurate results, the system must operate over a long period. In addition to studying the regulation of energy intake, studying the control of energy output as well could provide a much more comprehensive profile for energy balance.

Predisposition to obesity may have a genetic basis in man (2). But cultural differences may affect genetic expression of obesity. Continued examination of human obesity suggests that there are several types of obesity in which the etiologies may vary considerably (7-9). Since we don't have well-established criteria for distinguishing among the human obesities, it is useful to investigate the etiology of obesity by using more clearly defined animal models.

Animal models for obesity have been classified by Mayer (2) into regulatory and metabolic obesities. Regulatory obesities generally include goldthioglucose obese mice and ventromedial hypothalamus lesioned rats. Metabolic obesities include mice in which there are some primary metabolic defects, such as hyperglycemic obese mice, New Zealand obese mice, and obese yellow mice. Among these models, the genetically hyperglycemic obese (C57BL/6J-ob/ob) mouse has been most extensively studied (10).

APPETITE IN ANIMALS

There has been much study on the factors influencing appetite in animals, but little research has been undertaken in man. The hypothalamus is known to be involved in appetite control of animals. Destruction of ventromedial nuclei of the hypothalamus in rats, either by electrolytic lesions or treatment with goldthioglucose, leads to hyperphagia

(11). Conversely, aphagia can be induced by bilateral destruction of the lateral hypothalamic nuclei. The ventromedial nuclei have been designed as the "satiety center" whereas the nuclei in the lateral hypothalamus act as a primary "feeding center". This lateral "feeding Center" which can be inhibited by the ventromedial "satiety center" is very sensitive to glucose. Therefore, destruction of ventromedial nuclei would essentially represent a removal of inhibition on the "feeding center". The ventromedial hypothalamus nuclei of obese mice appear to be relatively insensitive to insulin and resistant to destruction by goldthioglucose (12). These observations might indicate a defective feedback control mechanism in hypothalamic appetite system of obese mice. It seems, however, in the great majority of cases that the cause of obesity is multifactorial. An insensitive hypothalamus in the obese mice is unlikely to be the only contributing factor to the obesity, or it may even be just a result, instead of a cause, for obesity in this model.

ENDOCRINE CHANGES IN OBESITY

Many endocrine and metabolic abnormalities have been reported in obesity (13). Considerations of the etiology have led to attempts to differentiate metabolic characteristics which are secondary to the development of obesity from those presumably causal factors that precede it. Recent studies have shown that many hormonal changes are secondary

to development of obesity and are not necessarily causative factors that precede the obesity (14).

A. INSULIN

Elevated serum insulin concentrations are a common feature of obesity observed in virtually all animal models of this syndrome as well as in man. The occurrence of hyperinsulinemia was first noted to coincide with the period of rapid weight gain (15, 16). In 1962, Rabinowitz and Zierler (17) first suggested that elevated blood insulin associated with the obese state reflects a diminished responsiveness of peripheral tissues to insulin, specifically muscle and adipose tissue. It is still unclear to what extent insulin resistance plays a deleterious role in the development of obesity. It seems likely that insulin resistance might be involved in the increased incidence of diabetes mellitus associated with obesity.

The most thoroughly studied animal model of genetic obesity and insulin resistance probably is the obese (ob/ob) mouse. Adult obese mice are insulin resistant, but tissue sensitivity to insulin changes during development of obesity (18). For example, adipose tissue of young obese mice is supersensitive to insulin, whereas later it becomes resistant (18). Insulin resistance in muscle may appear much earlier in development of obesity than in liver or adipose tissue (19). Thus, the early muscular lesion is

followed by compensatory increased circulating insulin levels with increased lipogenesis by adipose tissue. Eventually an increased cell size of adipocytes results, which in turn leads to a further increase in circulating insulin. Olefsky has reported that large adipocytes from older, fatter rats have decreased numbers of insulin receptors (20, 21) and that the dose response curve for insulins' antilipolytic action was shifted to the right (21). These results suggested that large adipocytes were more resistant to antilipolytic effects of insulin (22). Consequently, increased lipid causes an increase in cell size and renders the tissue relatively more resistant to insulin.

B. THYROID HORMONE

Despite earlier reports of euthyroidism (23) it has recently been reported that the genetically obese (ob/ob) mouse is functionally hypothyroid (24-26). Many of the characteristics of hypothyroidism have been identified, they included a reduced body temperature (24), lower protein-bound iodine (24) and a diminished uptake of ^{131}I by the thyroid (24). Furthermore, these metabolic abnormalities were improved by treatment with thyroid hormones. But a relatively higher dose of thyroid hormone was required to obtain comparable response of adipose tissue lipolysis to thyroid hormone in obese mice (27). The mechanism underlying the hypothyroidism of obese mice is not completely understood. Recent studies (28) have shown that the possible

relationship between thyroid function and hyperadrenocorticism of obese mice. It is unlikely, however, that hyperadrenocorticism is responsible for all of the changes in thyroid function of obese mice, since the appearance of thyroid abnormalities probably precedes that of the adrenal malfunction (28). Also the recent demonstration of imperfect thermoregulation at as early as 2 weeks of age (29) suggests that hypothyroidism may occur before hyperinsulinemia, insulin resistance, hyperphagia and obesity of obese mice. In obese patients, however, thyroid function is usually normal although there are some obese patients with hypothyroidism (30). The obese patients with euthyroidism are usually considered to be less sensitive than obese patients with hypothyroidism to the metabolic effects of thyroid hormone (30). Thus, the evidence suggests that genetically obese (ob/ob) mice may represent a good model for some types of obese individuals, but not for others.

C. ADRENAL FUNCTION

Adrenal cortical hyperactivity develops with obesity, but the mechanism remains unknown. The physiological importance of this hormonal change is not established. However, retention of water and electrolytes (13) and increased levels of vasopressin and aldosterone (13, 31) are frequently noted.

D. PITUITARY FUNCTION

Several studies reported that there are abnormalities in growth hormone and prolactin secretion in obese mice (32). It has also been suggested that the abnormalities in pituitary function may be related to the disorders of appetite control in obesity (32).

LIPID METABOLISM

Concomitant differences in carbohydrate and lipid metabolism exist, as evidenced by dissimilarity between obese and lean hepatic and adipose tissue enzyme profiles, incorporation of various substrates into lipid components and rates of lipogenesis and lipolysis.

A. LIPOGENESIS

Numerous investigations on lipogenesis in obese mice have shown that glucose, acetate and pyruvate oxidation in liver and adipose tissue are diminished, whereas incorporation of these substrates into total lipids is elevated (34-37). This elevation of lipid synthesis is especially evident in young animals during dynamic phase of obesity (38). Concomitant with increased rates of hepatic lipogenesis in obese mice, the activities of several hepatic enzymes are increased. These include α-glycerophosphate dehydrogenase (39), glucose-6-phosphate dehydrogenase (40), glucokinase (41), pyruvate kinase (41), citrate lyase (42), acetyl CoA carboxylase (43) and fatty acid

synthetase (43). In general, increased activities of these hepatic enzymes are consistent with a major role of liver for total lipogenesis. Hepatic lipogenesis accounts for 50% of total lipogenesis in obese mice and only for 20% in the controls (44).

Fatty acid synthesis in fat cells from obese (ob/ob) mice is dramatically increased and accounts for much of the excess glucose utilization compared to lean littermates (18). But obesity caused by goldthioglucose administration exhibited only slightly increased adipose tissue fatty acid synthesis. In addition, obesity caused by high fat diet did not increase lipogenic enzyme activities. Thus, it is still unclear whether this increased lipogenic activity plays a primary role in the etiology of the ob/ob syndrome; it may be entirely secondary to marked hyperinsulinemia in these animals and to consumption of a high-carbohydrate diet.

B. LIPOLYSIS

Alteration in lipolysis, such as reduced basal rates and decreased lipase activity in adipose tissue may be another contributing factor to obesity. Obesity associated with a diminished lipolytic response to several hormones has been described in several animal models (45-50). The "second messenger" concept has helped to demonstrate the key role of cyclic AMP in mediating the action of lipolytic hormones (51, 52). These hormones bind to specific receptors on the plasma membrane of fat cells and the resulting

adenylate cyclase activation provokes an accelerated conversion of ATP to cyclic AMP. Thus, it is likely that the coupling mechanism of hormone receptors to the catalytic subunit of the edenylate cyclase system and/or the catalytic subunit itself, are defective in obese (ob/ob) mice (53).

METABOLIC EFFICIENCY

The metabolic efficiency of the body can be altered in two ways; either by a change in efficiency of ATP, and other high energy intermediates, generation or by a change in the requirements of various metabolic processes for ATP.

A. CHANGES IN THE EFFICIENCY WITH WHICH ATP IS GENERATED

The efficiency of generation of ATP might vary due to changes in the proton conductance pathway.

PROTON CONDUCTANCE PATHWAY -- Himms-Hagen suggested that a relative lack of the specific proton conductance pathway in brown adipose tissue mitochondria and a defect in the control of the amount of this pathway could explain why the ob/ob mouse has an impaired response to cold stress (54). This might account for the obesity since a reduced amount of this pathway at temperatures below the thermoneutral zone for the mouse would result in less wasting of substrates for heat production and thus a greater metabolic efficiency (54).

B. CHANGES IN PROCESSES REQUIRING ENERGY AND THEIR EFFECT ON METABOLIC EFFICIENCY

Futile cycles, protein turnover and ion pumping have been thought to account for a significant fraction of the basal energy expenditure (55). But there is very little research on these aspects of energy metabolism in obesity.

FUTILE CYCLES --- Glucose and fat sources are utilized with about the same efficiency when assessed in terms of the potential yield of ATP (55) from isocaloric intakes of glucose and fat. Protein, however, is somewhat less efficient as a source of energy, because some energy is lost during urea biosynthesis (56). It also should be realized, that the estimation of the utilizable energy from carbohydrate, fat or protein sources, based on maximal ATP generation, may have limitations in relation to assessment of substrate utilization in vivo. For example, there are a number of futile cycles in metabolic pathway, such as between glucose and glucose-6-phosphate, fructose-6-phosphate and fructose-6,6-diphosphate, and between phosphoenolpyruvate and pyruvate. Significant energy cost are also associated with storage and later utilization of fuels, such as between adipose tissue fatty acid and liver fatty acid. Substrate cycling plays a role in metabolic regulation in the whole organism (57). Perhaps, a variation in the activity of these cycles may also provide a partial explanation for differences in the propensity of individuals

with generous energy intake to either become obese or to remain thin (58).

PROTEIN TURNOVER --- The energy requirement for protein synthesis is quite substantial when the energy needs for amino acid transport, synthesis of cell constituents such as RNA, and peptide formation are considered. Munro (59) and Brody (60) have demonstrated a close relationship between the intensity of body protein metabolism and rate of energy utilization in mammals. In mammalian cells it has been calculated that the energy required for protein turnover varies from 10% to 50% of basal metabolic rate (55). It has been suggested that decreased rate of whole protein turnover might be another contributing factor in obesity (61). Preliminary results suggest that many obese subjects have normal protein turnover, but that in a few subjects the rates are low (61). The latter results indicate that, at least, some obese individuals, may decrease protein turnover more efficiently than normal when energy supply is limited. Consequently, the efficiency of therapeutic diet for treatment of obesity would be reduced in these obese subjects.

ION PUMPING --- Ion pumping is another important component of metabolism requiring substantial amounts of energy. From currently available data, it is estimated that the sodium pump may contribute appreciably (15% to 50%) to total cellular energy utilization in mammalian tissues

(62, 63). Support for the concept that ion pumping is an important component of basal energy metabolism is suggested from the increased metabolic rate in response to thyroid hormone and cold exposure which appears to be attributed in part to changes in sodium pump activity and resulting increase in ATP turnover (64-68). Thus, under certain conditions at least, the energy demands of the Na^+/K^+ transport system may be of importance in the overall regulation of energy metabolism in an animal. Since ob/ob mice have been suggested to be hypothyroid (24-26) and since thyroidal state has been shown to affect tissue content of Na^+,K^+-ATPase (64-68), a difference in Na^+,K^+-ATPase enzyme units between tissues of lean and of obese mice might be expected.

THE OBJECTIVES OF MY RESEARCH

There seems little evidence as yet that any of the documented differences between obese and lean mice are key features which lead to the development of obesity. Very little work on the involvement of the sodium pump in energy metabolism has been done. Most in vitro assessments of sodium pump activity have been largely confined to studies on thyroid hormone treatment and cold exposure in the rats (64-68). An exploration of possible differences in Na^+,K^+-ATPase activity in lean and obese mice forms the basis of the present investigation. The specific focus is upon the tissue content of Na^+,K^+-ATPase in thermogenic organs, namely, skeletal muscle, kidney, and liver, of lean and genetically obese

(ob/ob) mice.

A. CHANGES IN Na^+,K^+-ATPase ENZYME UNITS DURING DEVELOPMENT OF OBESITY

The obese hyperglycemic syndrome in ob/ob mice first becomes visually evident at about 4 weeks of age; therefore, we used 4 week old mice to examine changes in Na^+,K^+-ATPase which might be present early in the development of obesity and 8 week old mice to evaluate changes after the obesity was readily apparent. A reduction in oxygen consumption and increased body fat content of the genetically obese (ob/ob) mice have been detected as early as 7 to 14 days of age (69). Therefore, the number of enzyme units in 14 day old obese and lean littermates was compared to determine whether the changes in Na^+,K^+-ATPase underlie the above phenomenon.

B. THE EFFECT OF THYROID HORMONE AND COLD EXPOSURE ON Na^+,K^+-ATPase ENZYME UNITS IN OBESITY

It has been suggested that a change in thyroid status or cold exposure produces a concomitant change in the activity of this enzyme in rats (64-68). Since many systems in obese mice appear to respond normally to endogenous thyroid hormones (24, 27, 70) additional studies are needed to focus on the relationship of thyroid hormones to Na^+,K^+-ATPase in obese mice. Therefore, I compared the response of Na^+,K^+-ATPase to thyroxine treatment in lean and obese (ob/ob) mice.

Obese mice, unlike lean mice, die of hypothermia when acutely exposed to 4^{o} because of a reduced capacity for cold-induced thermogenesis (71-73). Additionally, obese mice also display hypothermia when maintained at normal ambient temperatures (24, 74, 75). These results suggest that obese mice have an alteration in thermoregulatory thermogenesis (73, 75) which could partially explain the high efficiency of energy retention observed in these mice. Therefore, I also investigated the ability of young obese and lean mice to alter Na^{+},K^{+}-ATPase in response to warm (33^{o}) or cold (14^{o}) environments.

C. CHANGES IN TISSUE Na^{+} AND K^{+} CONCENTRATIONS IN RELATION TO CHANGES IN Na^{+},K^{+}-ATPase ACTIVITY

To provide further information on the correlates of Na^{+}, K^{+}-ATPase enzyme units in obese mice, I also measured the concentrations of Na^{+} and K^{+} in plasma, muscle, kidney and liver from lean and obese mice.

D. THE INFLUENCE OF OUABAIN ON OXYGEN CONSUMPTION OF INTACT LEAN AND OBESE MICE IN VIVO

Ouabain inhibits $Na,^{+},K^{+}$-ATPase. If Na^{+},K^{+}-ATPase activity contributes significantly to whole animal energy expenditure, injection of ouabain into mice should decrease their rate of oxygen consumption. Thus, I evaluated the influence of ouabain injections on oxygen consumption of intact lean and obese mice.

The data to be reported here indicate differences in Na^+,K^+-ATPase enzyme units in genetically obese mice as compared to their thin littermates and support the hypothesis of impaired cellular thermogenesis in these animals as a significant aspect of the obese condition.

Na^+,K^+-ATPase ENZYME UNITS IN FOUR AND EIGHT WEEK OLD, AND IN THYROXINE INJECTED OBESE (Ob/Ob) MICE

INTRODUCTION

Obese-hyperglycemic (ob/ob) mice retain a greater portion of ingested energy than lean mice (76, 77). This improved energy efficiency in obese mice is associated with a lower body temperature (24), reduced heat production per unit body weight (78), and a thermogenic impairment during cold stress (73) as compared to their lean counterparts. The gross obesity in this model might thus result from reduced cellular thermogenesis coupled with normal (79) or increased (76, 77) energy intake.

From currently available data, it is estimated that the sodium pump may contribute appreciably (15 to 50%) to total cellular energy utilization in mammalian tissues (62, 63). The stimulation in cellular thermogenesis observed in hyperthyroid and cold-exposed animals has been attributed in part to changes in sodium pump activity and the resulting increase in ATP turnover (64-68). Thus, under certain conditions at least, the energy demands of the Na^+/K^+ transport system may be of importance in the overall regulation of energy metabolism in the animal.

Na^+,K^+-ATPase (EC 3.6.1.3) (80) is a membrane bound enzyme considered to be the enzymatic expression of the sodium pump. This enzyme is inhibited by the cardiac glycoside-ouabain, the amount of ouabain bound to this enzyme correlates directly with the reduction of Na^+,K^+-ATPase activity (81). Previous work from this laboratory

demonstrated that the number of Na^+,K^+-ATPase enzyme units, using the (^3H)-ouabain binding method, was more than 50% lower in hindlimb skeletal muscle preparations from 8 week old obese (ob/ob) mice than in their lean littermates (82). The lower Na^+,K^+-ATPase activity in skeletal muscle might contribute to the lower energy expenditure and resultant obesity observed in these mice. York et al. (70) have also postulated that alterations in Na^+,K^+-ATPase may contribute to obesity in ob/ob mice.

The obese-hyperglycemic syndrome in ob/ob mice first becomes visually evident at about 4 weeks of age; therefore, in the present report we used 4 week old mice to examine changes in Na^+,K^+-ATPase which might be present early in the development of obesity and 8 week old mice to evaluate changes after the obesity was readily apparent. To further establish whether the reduction in Na^+,K^+-ATPase enzyme units in skeletal muscle of obese mice was specific for skeletal muscle or was associated with a decrease in enzyme units in other organs as well, liver and kidney tissues were also examined. Since ob/ob mice have been suggested to be hypothyroid (24, 26, 27) and since thyroidal state has been shown to affect tissue content of Na^+,K^+-ATPase (64-68), we compared the response of Na^+,K^+-ATPase to thyroxine treatment in lean and obese (ob/ob) mice.

METHODS

ANIMALS

The male obese (C57 BL/6J-ob/ob) and lean (ob/+ or +/+) mice used in these studies were obtained from our breeding colony or were purchased from Jackson Laboratories in Bar Harbor, Maine. They were weaned at 3 weeks of age and were housed in solid-bottom cages with wood shavings for bedding. A stock diet (Wayne Lab-Blox from Allied Mills, Inc., Chicago, Illinois) and water were available ad libitum. Ambient temperature was maintained at 25 ± 2^{o} throughout all experiments. Room lights were on 12 hours each day.

Mice were studied at 4 and 8 weeks of age in the first experiment. In the second experiment, 6-week old lean and obese mice were injected intraperitoneally with either L-thyroxine or the vehicle (physiological saline solution) once daily for 2 weeks. Lean mice received 200 ug thyroxine per kg body weight per day (approximately 5 ug per mouse). Obese mice were injected with the same amount of thyroxine as injected into lean mice. Obese mice, thus, received an average of 153 ug thyroxine per kg body weight per day. Direct measurements of the heat produced by the thyroxine-treated and control mice were made using a gradient-layer calorimeter (Thermonetics Corp., San Diego, California) (83) which had been calibrated using a small heating device. Heat production was measured before the treatments began and every third day throughout the two week experiment.

Pairs of mice from the same treatment group were placed in wire mesh cages within the calorimeter for each determination. After 45 to 60 minutes, heat production was recorded and averaged over a 10 minute time span. Soda lime was used to remove carbon dioxide from the chamber air and oxygen was supplied by a volume meter (Med Science Electronics Inc., St. Louis, Missouri).

Estimates of body energy in the mice were obtained at the end of the second experiment. After the liver, kidneys and hindlimb muscles were removed for enzyme assays, the remaining carcass was softened in an autoclave at 100^{o} for 30 minutes and then homogenized (Polytron, Brinkman Instruments, Westbury, NY) in distilled water. The total homogenate was dried to a constant weight in an oven at 50^{o}. The energy content of each dry carcass sample was measured in a bomb calorimeter (Parr Instrument Co., Inc., Moline, Illinois). Values were expressed as kcal per carcass; the liver, kidneys, and hindlimb muscles were excluded.

TISSUE Na^{+},K^{+}-ATPase PREPARATION

The mice were killed by cervical dislocation and the vena cava was immediately incised just anterior to the liver. The mouse was then perfused with 10 ml of cold saline solution via the left ventricle of the heart. Liver, both kidneys and hindlimbs were rapidly removed. The hindlimb muscle was removed from the bones and stripped of all visible fat.

These tissues were homogenized with 9 volumes of an ice-cold solution containing 0.25 M sucrose, 5 mM disodium EDTA, 5 mM histidine, 0.1% sodium deoxycholate and 10 uM dithiothreitol (pH adjusted to 7.0 with Tris base). Deoxycholate was included in the homogenization buffer to expose binding sites unavailable to ouabain in the absence of a detergent (84, 85). The minced hindlimb muscle was first homogenized with a Polytron homogenizer (Brinkman, Westbury, New York) fitted with a PT 10 generator and operated at a setting of 4 for two 5 second intervals. This suspension was further homogenized by ten passes in a motor driven glass homogenizer fitted with a tight Teflon pestle. The liver and kidney were homogenized with ten passes in a tight Teflon pestle homogenizer. The homogenates from hindlimb muscle, liver, and kidney were then centrifuged at 100,000xg for 60 minutes; the resulting pellets were resuspended in 10 mM Tris-HCl buffer (pH 7.4) using 5 passes in a tight Teflon pestle and centrifuged again at 100,000xg for 30 minutes. This step is necessary to remove deoxycholate from the final enzyme preparation. The final washed pellet was resuspended in 10 mM Tris-HCl buffer (pH 7.4) and frozen at -20^{o} for a maximum of three days. Storing the frozen preparations for three days did not diminish ouabain binding. All preparative procedures were performed at 0 to 4^{o}.

The particulate fraction, rather than a purified membrane preparation, was used in the subsequent ouabain binding assays because it was not known to what extent obesity might alter the sedimentation properties of the subcellular particles and thereby influence the recovery of the enzyme. Recovery of the enzyme could also be influenced by solubilization of the enzyme during homogenization with deoxycholate. This possibility was not investigated; however, others have reported minimal solubilization of Na^+,K^+-ATPase when low concentrations of deoxycholate are included in the homogenization buffer (85).

(^{3}H)-OUABAIN BINDING ASSAY

Tissue preparations were incubated at 37^o in a total volume of 2 ml. The incubation mixture contained 1.0 mM Tris-inorganic phosphate (orthophorphoric acid; titrated to pH 7.4 with Tris base), 1.0 mM $MgCl_2$, 10 mM Tris-HCl buffer (pH 7.4), 0.4 uM (^{3}H)-ouabain (1.9 Ci/mmole) and various concentrations of nonlabeled ouabain as indicated in the legends of tables. A preliminary study showed a linear relationship between protein concentration and (^{3}H)-ouabain binding over a 1.0 to 1.3 mg protein range.

Specific (^{3}H)-ouabain binding to liver, kidney, and skeletal muscle from rats is difficult to assay with the millipore filtration method, since bound ouabain is released rapidly during the filtration step (86). Similarly, the ouabain Na^+,K^+-ATPase complex from mouse preparations

dissociates during millipore filtration. Therefore, an equilibrium, rapid centrifugation method was utilized in the present studies. After the incubation, the tubes were rapidly cooled in ice cold water and then immediately centrifuged, at 100,000 x g for 30 minutes, to separate bound ouabain from unbound ouabain. The resulting pellet, which contained bound ouabain, was dissolved in 0.5 ml of 0.1 N NaOH with periodic shaking in a boiling water bath. The solution containing the dissolved pellet was neutralized with HCl, and the radioactivity was determined after adding scintillation cocktail (3a70 from Research Products International, Elk Grove Village, Illinois), The protein content of the pellet was estimated by the biuret method (87).

The time course of specific (^{3}H)-ouabain binding to hindlimb muscle, liver, and kidney preparations was determined. Binding of (^{3}H)-ouabain increased rapidly to reach a maximum at 3 to 5 minutes in both muscle and kidney preparations and at 8 minutes in liver preparations from both lean and obese mice. Since the binding capacity decreased slightly after reaching the maximum, 5, 5, and 8 minutes incubations were employed in the subsequent muscle, kidney, and liver ouabain binding assays, respectively.

ESTIMATION OF KINETIC PARAMETERS FOR OUABAIN BINDING

(^{3}H)-Ouabain binding to Na^+,K^+-ATPase follows classical Michaelis-Menten kinetics (88). The amount of

saturable (^{3}H)-ouabain bound to Na^+,K^+-ATPase in the presence of a fixed concentration of labeled ouabain, the dissociation constant for the ouabain, and the binding site concentration were determined as previously described (82, 86, 88). Binding of (^{3}H)-ouabain to low-affinity, nonspecific, nonsaturable sites was determined in the presence of 4 mM nonlabeled ouabain.

STATISTICAL CALCULATIONS

The data are presented as means and MS_E. Statistical comparisons were made with Bonferroni-t-test (89).

MATERIALS

All conventional reagents were reagent grade. (^{3}H)-Ouabain (specific activity; 1.9 Ci/mmole) was prepared by mixing nonradioactive ouabain with randomly labeled (^{3}H)-ouabain (Amersham/Searle Corp., Arlington Heights, Illinois). Ouabain and Na-L, 3,5,3',5'-thyroxine were purchased from Sigma Chemical Company, St. Louis, Missouri.

RESULTS

Body weights and hindlimb skeletal muscle weights of the mice at 4 and 8 weeks of age are summarized in Table 1. The obese mice weighed more than the lean mice. The difference in body weight was greater at 8 weeks than at 4 weeks. Skeletal muscle (both hindlimbs) weights were similar in both groups at 4 weeks of age. By 8 weeks, lean mice had doubled the weight of their hindlimb muscle; but

Table 1. Body weight, hindlimb muscle weight and (^{3}H)-ouabain binding to muscle preparations from lean and obese mice[A].

	Age				
	4 wk old		8 wk old		MS_E[B]
	Lean	Obese	Lean	Obese	
Body weight-g	16.7[C,a]	19.1[b]	24.6[c]	39.2[d]	2.7
Hindlimb muscle:					
Total weight-g	1.1[a]	0.9[a]	2.1[b]	1.3[c]	0.05
Particulate protein-mg/g muscle	73[a]	73[a]	74[a]	73[a]	57
Specific (^{3}H)-ouabain binding-[D] pmoles/mg protein	2.3[a]	1.7[b]	1.6[b]	1.0[c]	0.1
Nonspecific (^{3}H)-ouabain binding-[E] pmoles/mg protein	1.5[a]	1.4[a]	1.7[b]	1.4[a,c]	0.2
K_d value[F]-uM	0.045[a]	0.042[a]	0.036[a]	0.030[a]	0.142[a]
(^{3}H)-ouabain binding site concentration[F]-pmoles/mg protein	2.5[a]	1.9[b]	1.7[b]	1.1[c]	0.2
pmoles/total hindlimb muscle	192[a]	126[b]	269[c]	111[b]	2240

[A]Values represent mean for 11 pairs of 4 week old lean and obese mice or 10 pairs of 8 week old mice. The values for the 8 week old mice have been presented previously (82) and are presented again for comparative purposes.

[B]MS_E = mean square of error.

Table 1 (cont'd.).

[C]Means on the same line with different superscript lowercase letters are different ($p<0.05$).

[D](^{3}H)-Ouabain (0.4 uM) binding minus (^{3}H)-ouabain (0.4 uM) binding observed in the presence of excess nonlabelled ouabain (4 mM).

[E](^{3}H)-Ouabain (0.4 uM) binding observed in the presence of excess nonlabelled ouabain (4 mM).

[F]Calculated as described elsewhere (88).

muscle weight increased only 40% from 4 to 8 weeks of age in obese mice. These results are consistent with a previous report (79) which showed that obese mice accumulate less total protein, but 4 to 5 times more fat, than lean mice from 3 to 8 weeks of age. This occurs even though male obese mice consume only slightly more energy than lean counterparts during this period (79).

Results of the (^{3}H)-ouabain binding studies performed on hindlimb preparations from lean and obese mice at 4 and 8 weeks of age are summarized in Table 1. The content of particulate protein in hindlimb muscle, expressed as mg per g of muscle, was not significantly influenced by phenotype or age of the mice. The specific saturable binding of (^{3}H)-ouabain to skeletal muscle preparations from obese mice was lower at both ages than observed in lean mice (Table 1). In both lean and obese mice, specific saturable binding of (^{3}H)-ouabain decreased with age. Nonspecific, nonsaturable binding of ouabain to obese muscle preparations of 8 week old mice was less than observed for lean muscle preparations.

The apparent reduction in the saturable, specific (^{3}H)-ouabain binding to hindlimb muscle of obese mice could result from either a reduction in the affinity of the binding site for ouabain or from a reduction in binding site concentration. To evaluate these two possibilities, K_d values (dissociation constant, reciprocal index of the

affinity) and binding site concentration were estimated. The advantages of the method used herein over conventional methods have been discussed previously (88).

Neither phenotype nor age altered the K_d value for ouabain binding in the hindlimb muscle preparations. Hence, the affinity of Na^+,K^+-ATPase for ouabain in skeletal muscle remained unchanged, but the number of binding sites per mg protein was lower in obese mice. Since the hindlimb muscle of the obese mice weighed less than hindlimbs of the lean mice, total binding site number in the hindlimbs of obese mice was 35% lower at 4 weeks of age and more than 50% lower at 8 weeks of age than observed in hindlimbs of lean mice. Binding site number in the hindlimbs of lean mice increased with age; whereas, the number of binding sites in the hindlimbs of obese mice was slightly decreased with age (Table 1).

There was no difference in liver weights of lean and obese mice at 4 weeks of age (Table 2). From 4 to 8 weeks of age, obese mice increased their liver weight about 150%, whereas livers of lean mice increased in weight only 50%. The content of particulate protein in the liver (mg/g liver) was greater at 8 weeks than at 4 weeks of age. The saturable (^3H)-ouabain binding per mg protein in the livers from obese mice was less than that from lean mice at both 4 weeks and 8 weeks of age. This decrease in (^3H)-ouabain binding is specific for the saturable binding, since nonsaturable

Table 2. Liver weight and (^{3}H)-ouabain binding to liver preparations from lean and obese mice[A].

	Age				MS_E[B]
	4 wk old		8 wk old		
	Lean	Obese	Lean	Obese	
Total liver weight-g	1.0[C,a]	1.4[a]	1.5[a]	3.8[b]	0.1
Particulate protein-mg/g liver	44[a]	47[a]	58[b]	60[b]	42
Specific (^{3}H)-ouabain binding[D] pmoles/mg protein	0.24[a]	0.17[b]	0.18[b]	0.08[c]	0.003
Nonspecific (^{3}H)-ouabain binding[E] pmoles/mg protein	1.2[a]	1.1[a]	1.2[a]	1.0[a]	0.02
K_d value[F]-uM	10.4[a]	12.1[a]	12.4[a]	13.5[a]	3.7
(^{3}H)-ouabain binding site concentration[F] pmoles/mg protein	6.4[a]	5.2[a]	5.6[a]	2.6[b]	2.8
pmoles/total liver	289[a]	323[a]	495[b]	585[b]	26,300

[A-F] See Table 1.

binding was not influenced by phenotype or age (Table 2). As in skeletal muscle, the K_d values obtained in liver preparations from obese mice were similar to those obtained in preparations from lean mice. The binding site concentration per mg hepatic protein was not influenced by phenotype at 4 weeks of age, but was markedly reduced in the obese at 8 weeks of age. Since the livers from obese mice were heavier, the number of ouabain binding sites expressed on a total liver basis was not lower in the 8 week old obese mice than in lean counterparts. The number of ouabain binding sites per liver increased between 4 and 8 weeks of age in both phenotypes because liver weight increased.

Kidney weights of the mice were influenced by age of the mice, but not by phenotype (Table 3). No significant differences were observed in particulate protein content in kidneys of the mice. Unlike the observations in skeletal muscle and liver, specific (^{3}H)-ouabain binding to kidney preparations, expressed per mg protein, was not altered by phenotype or age of the mice. Neither K_d values nor the number of ouabain binding sites per mg protein was altered by phenotype or age. The number of ouabain binding sites per both kidneys, however, was significantly higher in 8 week old obese mice than in 4 week old obese mice (Table 3).

Since thyroid hormone administration has been reported to increase oxygen consumption in mice (78) and Na^+,K^+-ATPase activity in rats and mice (64-68, 70), we next evaluated the

Table 3. Kidney weight and (^{3}H)-ouabain binding to kidney preparations from lean and obese mice[A]

	Age				MS_E[B]
	4 wk old		8 wk old		
	Lean	Obese	Lean	Obese	
Weight of both kidneys-g	0.21[C,a]	0.20[a]	0.25[b]	0.26[b]	0.0003
Particulate protein-mg/g kidney	60[a]	64[a]	54[a]	56[a]	25
Specific (^{3}H)-ouabain binding[D]-pmoles/mg protein	2.5[a]	2.3[a]	2.2[a]	2.6[a]	0.2
Nonspecific (^{3}H)-ouabain binding[E]-pmoles/mg protein	1.4[a]	1.4[a]	1.7[a]	1.6[a]	0.06
K_d value[F]-uM	9.2[a]	9.5[a]	9.5[a]	9.6[a]	0.6
(^{3}H)-ouabain binding site concentration[F]-pmoles/mg protein	60[a]	56[a]	55[a]	65[a]	132
pmoles/both kidneys	691[a]	732[a]	886[a]	1140[b]	3950

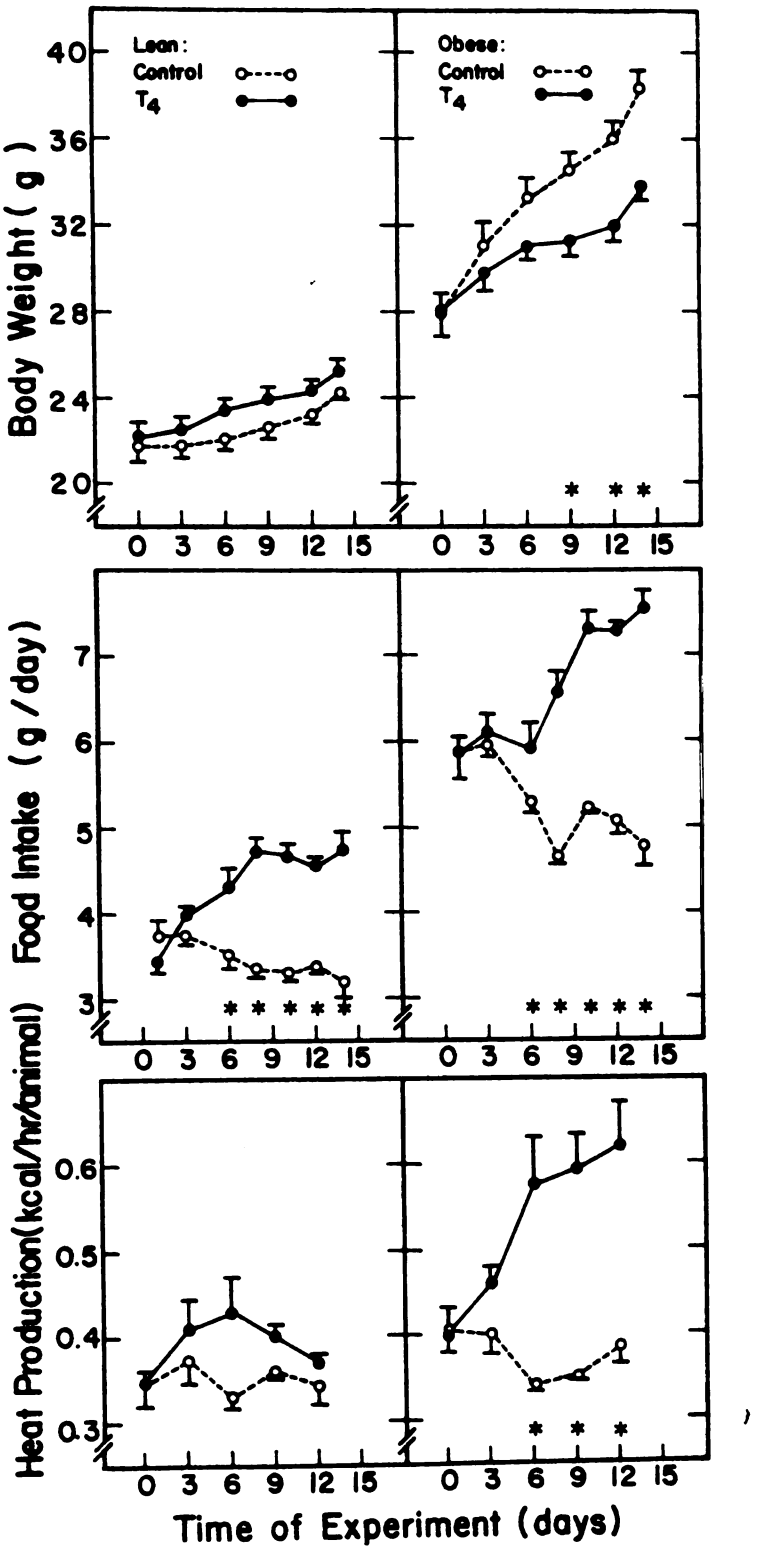
[A-F] See Table 1.

influence of thyroxine treatment on whole animal heat production and on ouabain binding to tissue preparations from lean and obese mice. The effects of thyroxine treatment on body weight, food intake, and heat production of lean and obese mice are presented in Figure 1. The control obese mice weighed more initially and gained body weight faster during the 2 week period than did control lean mice. The control obese mice consumed more food (79 ± 3g vs 52 ± 1g) than the control lean mice; however, heat production, expressed per animal, was similar in the control obese and lean mice. Since the obese mice weighed more than the lean mice, heat production per unit body weight, was less in the obese mice.

Thyroxine administration significantly depressed the rate of weight gain of obese mice, but had no effect on weight gain of lean mice (Figure 1). Food intake of thytoxine-treated lean and obese mice was significantly greater following the sixth day of treatment when compared with that of their respective controls. Total food intake over the 14-day period was increased 22% in both lean and obese thyroxine treated mice. Thyroxine treatment increased heat production by 14% (average increase on days 6, 9, and 12) in the lean mice and by 58% in the obese mice.

The final body weights of the lean mice were not altered by thyroxine treatment, but body weights of thyroxine-treated obese mice were lower than those of

Figure 1. Body weight, food intake and heat production of 6 week old lean and obese mice injected daily with either 5 ug L-thyroxine (T_4) or 0.15 M NaCl (control) for 2 weeks. Data obtained from lean and obese mice are presented in the left- and right-hand graphs, respectively. Each point represents the mean ± SEM obtained from 6 control mice (open circles) or 9 T_4-injected mice (closed circles). Asterisks indicate significant differences ($p<0.05$) between control and T_4-injected mice.


Lean:
Control
T4
Obese:
Control
T4
Body Weight (g)
Food Intake (g / day)
Heat Production(kcal/hr/animal)
Time of Experiment (days)

saline-treated obese mice (Table 4). As expected, carcasses of obese mice contained approximately three times as much energy as carcasses of lean mice. Thyroxine treatment did not alter carcass energy in lean mice, but depressed carcass energy by 24% in obese mice.

As in experiment 1, hindlimb muscle weight was lower in 8 week old obese mice than in lean mice (Table 4). Neither muscle weights nor particulate protein content in hindlimb muscle was influenced by thyroxine injection. Specific binding of ouabain, expressed per mg protein, in skeletal muscle from control obese mice was lower than in lean mice as observed in experiment 1. After thyroxine administration, the specific binding of ouabain to muscle preparations from obese mice increased 64%, whereas specific binding of ouabain to muscle preparations from lean mice increased only by 19%. Consequently, the specific ouabain binding in thyroxine-treated lean and obese mice was not different (Table 4). Nonspecific binding of ouabain to muscle preparations was lower in obese thyroxine-treated mice than in either lean thyroxine-treated mice or obese control mice. The reason for this is unclear. The K_d values of skeletal muscle Na^+,K^+-ATPase for ouabain were not influenced by phenotype or thyroxine treatment, but the number of binding sites increased in thyroxine treated mice due to the increase in specific ouabain binding. Because the hindlimbs of obese mice weighed less than those

Table 4. Body weight, body energy, hindlimb muscle weight and (^{3}H)-ouabain binding to muscle preparations from saline or thyroxine-treated lean and obese mice[A].

	Saline		Thyroxine		MS_E[B]
	Lean	Obese	Lean	Obese	
Body weight-g	24.3[C,a]	38.5[b]	25.3[a]	33.8[c]	2.6
Body energy-Kcal/carcass	50[a]	155[b]	47[a]	118[c]	67
Hindlimb muscle:					
Total weight-g	2.0[a]	1.3[b]	2.1[a]	1.1[b]	0.04
Particulate protein-mg/g muscle	66[a]	66[a]	65[a]	66[a]	51
Specific (^{3}H)-ouabain binding[D] pmoles/mg protein	1.6[a]	1.1[b]	1.9[c]	1.8[c]	0.03
Nonspecific (^{3}H)-ouabain binding[E] pmoles/mg protein	2.4[a]	2.1[b]	2.2[b]	1.6[c]	0.1
K_d value[F] uM	0.036[a]	0.033[a]	0.032[a]	0.028[a]	0.06
(^{3}H)-ouabain binding site concentration[E] pmoles/mg protein	1.7[a]	1.2[b]	2.0[c]	1.9[c]	0.04
pmoles/total lindlimb	217[a]	101[b]	274[c]	141[d]	995

[A]Values represent mean for 6 pairs of saline-treated lean and obese mice or 9 pairs of thyroxine-treated lean and obese mice.

Table 4. (cont'd.).

[B] MS_E = mean square of error.

[C] Means on the same line with different superscript lowercase letters are different ($p<0.05$).

[D] (^3H)-Ouabain (0.4 uM) binding minus (^3H)-ouabain (0.4 uM) binding observed in the presence of excess nonlabelled ouabain (4 mM).

[E] (^3H)-Ouabain (0.4 uM) binding observed in the presence of excess nonlabelled ouabain (4 mM).

[F] Calculated as described elsewhere (88).

of lean mice, the total number of binding sites in the hindlimbs of obese mice was still only about half the number observed in hindlimbs of lean mice, even after thyroxine treatment.

The liver weights were not changed in the lean mice after thyroxine treatment, but livers of thyroxine-injected obese mice weighed less than those of saline-injected obese mice (Table 5). The particulate protein content of liver (mg/g liver) was not changed by thyroxine treatment of phenotype. In agreement with experiment 1, specific (^{3}H)-ouabain binding in livers of obese saline-treated mice was lower than in livers from lean mice. Thyroxine treatment increased the specific (^{3}H)-ouabain binding in both lean and obese mice; however, the magnitude of the increase was greater in obese mice (130%) than in lean mice (28%). Nonspecific binding of ouabain was not altered by either phenotype or thyroxine treatment (Table 5). As in skeletal muscle, the K_d values were not changed by the treatment. Consequently, the concentration of ouabain binding sites, per mg particulate protein, reflected the specific binding values. The number of ouabain binding sites per liver, was higher in obese saline- or thyroxine-treated mice than in their lean counterparts.

Kidney weights of the thyroxine-treated mice were heavier than in saline-control mice and kidney weights of obese mice injected with thyroxine were heavier than kidneys

Table 5. Liver weight and (^{3}H)-ouabain binding[A] to liver preparations from saline or thyroxine treated lean and obese mice[A].

	Saline		Thyroxine		MS_E[B]
	Lean	Obese	Lean	Obese	
Total liver weight-g	1.5[C,a]	4.1[b]	1.5[a]	2.7[c]	0.05
Particulate protein-mg/g liver	50[a]	48[a]	51[a]	51[a]	16
Specific (^{3}H)-ouabain binding[D]- pmoles/mg protein	0.18[a]	0.10[b]	0.23[c]	0.23[c]	0.002
Nonspecific (^{3}H)-ouabain binding[E]- pmoles/mg protein	1.3[a]	1.3[a]	1.5[a]	1.3[a]	0.04
K_d value[F]-uM	12.4[a]	13.5[a]	12.8[a]	14.1[a]	3.1
(^{3}H)-ouabain binding site concentration[F]- pmole/mg protein	5.6[a]	3.4[b]	7.6[c]	8.2[c]	2.3
pmoles/total hindlimb muscle	404[a]	680[b]	587[a,b]	1150[c]	51,200

[A-F] See Table 4.

of lean counterparts (Table 6). Again, particulate protein concentration in the kidneys was not influenced by thyroxine treatment. Specific (^{3}H)-ouabain binding to kidneys was similar in saline-control lean and obese mice, but was higher in obese thyroxine-injected than in lean counterparts. Obese mice were thus more responsive to this relatively large dose of thyroxine than lean mice. This increase was specific for saturable binding since nonsaturable binding of ouabain was not influenced by thyroxine treatment. Finally, the concentration of ouabain binding sites and the total number of binding sites increased in kidneys of both lean and obese thyroxine-treated mice, but the percentage increase was greater in obese mice (108%) than in lean mice (71%).

DISCUSSION

Between 3 and 8 weeks of age male obese (ob/ob) mice consume only slightly more (9%) stock diet, but gain nearly 5 times as much fat as lean mice (79); thus, the obese mice are much more efficient in retaining ingested energy. Hyperglycemia (15), hyperinsulinemia (15), and reduced growth hormone and prolactin secretion (32) are among the metabolic parameters which become progressively more divergent in the obese mice during this period. However, the site of the primary defect which allows the obese mice to store larger amounts of energy as fat remains to be

Table 6. Kidney weight and (^{3}H)-ouabain binding to kidney preparations from saline or thyroxine treated lean and obese mice[A]

	Saline		Thyroxine		MS_E[B]
	Lean	Obese	Lean	Obese	
Weight of both kidneys-g	0.28[C,a]	0.30[a]	0.34[b]	0.39[c]	0.0004
Particulate protein-mg/g kidney	46[a]	53[a]	49[a]	49[a]	28
Specific (^{3}H)-ouabain binding[D]- pmoles/mg protein	2.7[a]	2.9[a]	3.7[b]	5.0[c]	0.1
Nonspecific (^{3}H)-ouabain binding[E]- pmoles/mg protein	2.3[a]	2.3[a]	2.2[a]	2.2[a]	0.1
K_d value[F]-uM	9.5[a]	9.6[a]	9.2[a]	9.4[a]	0.1
(^{3}H)-ouabain binding site concentration[F]- pmoles/mg protein	67[a]	72[a,b]	89[b,c]	122[d]	69
pmoles/both kidneys	872[a]	1130[a,b]	1492[b,c]	2348[d]	13,500

A-F See Table 4.

established. A number of studies indicate that the obese mice may have reduced cellular thermogenesis (70, 73, 82); consequently, in this report we focused on Na^+,K^+-ATPase as a possible contributor to altered heat production (78) in the obese mice.

We used (^3H)-ouabain binding as an index of the number of Na^+,K^+-ATPase enzyme units present, since the amount of ouabain bound to receptor sites correlates with the amount of this enzyme (81). Measuring ouabain binding to the particulate fractions, as opposed to purified membrane preparations, should minimize the possibility that any observed differences between obese and lean mice might be due to differences in the fraction of Na^+,K^+-ATPase recovered in enzyme preparations.

Marked species and tissue-dependent differences in the sensitivity of Na^+,K^+-ATPase to ouabain have been reported (90). In the mice used in the present study the affinity of binding sites for ouabain was highest in the skeletal muscle, intermediate in the kidney, and relatively low in the liver. The K_d values for ouabain binding in these three tissues of the mice were comparable with values reported in rats (86). Despite these tissue dependent differences in affinity of the binding sites for ouabain, the affinity of the binding sites for the drug was not influenced by phenotype or by thyroxine treatment. Thus, the major factor accounting for the observed changes in ouabain binding in

the tissues was an alteration in concentration of ouabain receptors, rather than a change in apparent affinity for ouabain.

Skeletal muscle is a major source of heat production in mammals (91). Earlier studies indicated that obese mice accumulate less hindlimb muscle than their lean counterparts (92), and that the number of ouabain binding sites was lower in 8 week old obese mice than in lean mice (82). A comparison of ouabain binding sites per mg hindlimb protein in 4 and 8 week old mice indicated that the difference in number of binding sites between lean and obese mice was slightly greater at 8 weeks of age than at 4 weeks of age. A much greater difference was observed when the number of ouabain binding sites was expressed per total hindlimb muscle, because the differences in muscle weight were more pronounced at 8 weeks of age than at 4 weeks of age. This greater divergence between the lean and obese mice at 8 weeks of age is consistent with the progressive hormonal and energy storage changes in the obese mice during this stage of development.

At both 4 and 8 weeks of age the hindlimbs represent approximately 25% of the total skeletal muscle mass in lean and obese mice (unpublished observation). If it is assumed that the concentration of ouabain binding sites in the hindlimbs reflects the concentration in the skeletal muscle as a whole, then it can be estimated that skeletal muscle

of 8 week old lean mice binds approximately 970 pmoles of ouabain (average of 8 week old mice in experiment 1 and saline-injected control mice in experiment 2). Comparable binding occurs in the kidneys of the lean mice (880 pmoles of ouabain bound), but less ouabain binding occurs in the liver (450 pmoles ouabain bound). Similar calculations for 8 week old obese mice demonstrates that the skeletal muscle contains fewer ouabain binding sites (425 pmoles ouabain bound) than observed in either the kidneys (1135 pmoles ouabain bound) or liver (630 pmoles ouabain bound). On a whole organ basis, alterations in ouabain binding in the skeletal muscle of the obese mice are more pronounced than for the other tissues examined.

Pooled hindlimb muscles were utilized in the present study to estimate the number of Na^+,K^+-ATPase enzyme units. The hindlimbs, however, contain a mixture of red and white muscle fibers and red fibers have been reported to have a higher concentration of Na^+,K^+-ATPase enzyme units than white fibers (93). Thus, further studies will be required to determine to what extent the number of Na^+,K^+-ATPase units are altered in specific muscle fibers of the obese mice or if fiber type is different between phenotypes.

While the concentration of ouabain binding sites per mg protein was lower in livers of 4 week old obese mice and even lower yet in livers of 8 week old obese mice than in livers of their respective lean counterparts, the number

of ouabain binding sites in the total liver tended to be greater in obese mice than in lean mice. York et al. (70) recently reported that Na^+,K^+-ATPase activity in liver homogenates of 12 week old obese mice, estimated by measuring phosphate release from ATP and expressed per mg protein, was lower than observed in preparations from lean mice. Our results from ouabain binding studies are in agreement with their measure of Na^+,K^+-ATPase activity in these mice. Despite the alterations in Na^+,K^+-ATPase specific activity in livers from obese mice, the increased liver size may negate the direct involvement of the hepatic sodium pump in the reduced heat production in obese mice. Studies in perfused livers of normal rats have suggested that the energy demand for active Na/K transport in the liver may be too small to significantly contribute a cellular thermogenesis (94).

In contrast to our findings in skeletal muscle and liver, we found no change in (^3H)-ouabain binding, per mg protein, in kidneys of obese mice. York et al (70) reported a reduced Na^+,K^+-ATPase activity in a single kidney membrane preparation from obese mice. They utilized older mice, a different tissue preparation technique, and a different assay; these factors may have contributed to the conflicting results.

Ismail-Beigi and Edelman (66-68) have presented evidence that a significant portion of the thermogenic response

to thyroid hormones arises from the increased energy expenditure for transmembrane active sodium transport. The response of Na^+,K^+-ATPase in hypothyroid rats injected with thyroid hormones is greater than observed in euthyroid rats (67). Injection of thyroid hormones into hypothyroid rats increased Na^+,K^+-ATPase activity by 75% in skeletal muscle, 55% in liver, and 69% in kidneys (64, 67). Comparable increases in the number of ouabain binding sites per mg protein were observed in the present study when the obese mice were injected with thyroxine; the increases averaged 58, 141, and 69% in skeletal muscle, liver, and kidneys, respectively. A smaller increase, however, was observed when the lean mice were injected with thyroxine; values were increased 18, 36, and 33% in skeletal muscle, liver, and kidneys, respectively. Since many systems in obese mice appear to respond normally to endogenous thyroid hormones (24, 27, 70), additional studies are needed to focus on the relationship of thyroid hormones to Na^+,K^+-ATPase in obese mice.

York et al. (70) recently reported that treatment with triiodothyronine did not increase the activity of Na^+,K^+-ATPase in the livers of obese (ob/ob) mice. It is not possible from available data to resolve the apparent discrepancy between this report and our results, but differences in the dose and type of thyroid hormone utilized, the number of days the animals were injected, the age of the

animals, and the enzyme assay procedures may have contributed to the conflicting results. Nevertheless, our results suggest that obese (ob/ob) mice possess a thyroid-dependent Na^+,K^+-ATPase which is more responsive to the large dose of thyroxine we administered than that in lean mice. Hepatic glycerophosphate dehydrogenase activity in obese mice is also more responsive to exogenous thyroxine than that in lean mice (24).

Sodium-transport-dependent respiration has been suggested to account for a significant portion of the increase in oxygen consumption associated with thyroid hormones (64-68). In the present study thyroid hormone administration to obese mice increased heat production by 58% and the total number of ouabain binding sites in skeletal muscle, liver, and kidneys by 85% (assuming that hindlimb muscle represents 25% of total muscle). In lean mice, heat production increased by 14% and the number of ouabain binding sites in the three tissues examined increased by 38% after the thyroxine injections. While these results cannot be used to imply a causal relationship between changes in Na^+,K^+-ATPase and heat production in the mice, they do suggest that the parameters change in concert. Further studies are needed to determine the mechanism(s) responsible for the altered number of ouabain binding sites in the obese (ob/ob) mice and to determine the extent to which these changes influence energy balance.

Na^+, K^+-ATPase ENZYME UNITS IN SKELETAL MUSCLE AND LIVER OF 14 DAY OLD LEAN AND OBESE (Ob/Ob) MICE

INTRODUCTION

A reduction in oxygen consumption of genetically obese (ob/ob) mice has been detected as early as 5 days after birth (69). While preweaned obese mice consume less oxygen than lean littermates (74, 95, 96), their energy intakes are similar (79). Consequently, the obese mice are able to retain more energy than lean littermates and this is reflected in an increased body fat content of the obese mice as early as 7 days of age (69). Reduced energy expenditure is thus an important contributing factor to the development of obesity in these mice. The underlying biochemical mechanism responsible for the improved energy efficiency in obese mice, however, has not been identified.

Energy required for Na^+,K^+-ATPase, the enzymatic equivalent of the sodium pump, is thought to represent a significant component of maintenance energy expenditure of mammals (62, 63). Observations that 3 to 6 week old obese mice have a 40% lower maintenance energy requirement than lean littermates (97) and that 4 to 8 week old obese mice have 35 to 50% fewer Na^+,K^+-ATPase enzyme units in skeletal muscle than lean littermates (82, 98) are consistent with the hypothesis that Na^+,K^+-ATPase is involved in the etiology of obesity in these obese mice. However, it would be important to establish if changes in Na^+,K^+-ATPase occur early and therefore possibly contribute to the development of the obesity, or are merely a secondary consequence of

the obesity.

In this report we identified 14 day old obese and lean littermates by their reduced oxygen consumption and then measured the number of Na^+,K^+-ATPase enzyme units in skeletal muscle and liver preparations. Body fat content of the mice was also determined.

METHODS

Fourteen day old male obese (ob/ob) and lean (ob/+ or +/+) littermate mice were obtained from litters of heterozygote breeding pairs (C67BL/6J-ob/+ from Jackson Laboratory, Bar Harbor, Maine). Breeding mice were fed a stock diet (Wayne Lab-Blox, Allied Mills, Inc., Chicago, Illinois) ad libitum. Ambient temperature was 25 ± 2°. On days 12, 13, and 14 oxygen consumption of individual pups was measured at 30° as previously described (69) and expressed as an average rate for the three days. At 14 days of age, lean and obese male littermates were selected, on the basis of differences in oxygen consumption, and were killed by cervical dislocation. The vena cava was immediately incised. After perfusing each mouse with 10 ml of cold saline solution via the left ventricle of the heart to remove blood, the forelimbs and hindlimbs and liver were quickly removed. The skeletal muscle, stripped of all visible fat, was removed from the bone. Food residue was removed from the stomach and the carcasses (except liver,

and forelimb and hindlimb skeletal muscle) were homogenized in an equal weight of water. Carcass fat was extracted with chloroform/methanol (3:2 v/v) and quantitated gravimetrically.

Particulate fractions from combined forelimb and hindlimb muscle preparations and from liver were prepared as described (82, 98) and were assayed for the number of Na^+,K^+-ATPase enzyme units (E.C. 3.6.1.3), using the (^3H)-ouabain binding method. (^3H)-Ouabain binding to Na^+, K^+-ATPase follows classical Michaelis-Menten kinetics (88). The amount of specific (^3H)-ouabain bound to Na^+,K^+-ATPase in the presence of a fixed concentration of labeled ouabain, the dissociation constant for (^3H)-ouabain binding to Na^+,K^+-ATPase, and the high-affinity (^3H)-ouabain binding site concentration in skeletal muscle and liver were determined as previously described (82, 98). Binding of (^3H)-ouabain to low-affinity, nonspecific, nonsaturable sites was determined in the presence of the 4 mM nonlabeled ouabain.

The data were analyzed by the paired t-test (89) and were presented as means ± SEM.

RESULTS

Body weight, body fat content, and oxygen consumption of the lean and obese mice are presented in Table 7. Obese

Table 7. Body weight, body fat content, and oxygen consumption of 14 day old lean and obese mice[a]

Parameter	Lean	Obese
Body weight, g	7.0 ± 0.3	7.8 ± 0.3*
Body fat, %	8.3 ± 0.7	14.2 ± 1.1*
Oxygen consumption, ml/g/hr	4.06 ± 0.07	2.90 ± 0.08*

[a]Mean ± SEM for six pairs of lean and obese mice. The asterisk indicates a significant different ($p<0.05$) between lean and obese mice.

mice weighed more and contained more fat than their lean littermates. The lower oxygen consumption of obese mice is readily apparent at this age. These results are in agreement with a previous report (69) and indicate that alterations in energy metabolism occur early in the life of the obese mouse.

Results of the (^{3}H)-ouabain binding studies performed on skeletal muscle from 14 day old lean and obese mice are summarized in Table 8. Total forelimb and hindlimb muscle weights and particulate protein content were not influenced by phenotype. Nonspecific binding of (^{3}H)-ouabain to muscle preparations was not altered by phenotype. [The specific (^{3}H)-ouabain binding to muscle from obese mice was 26% lower than that from lean mice] (Table 8). Obese and lean mice exhibited similar K_d values of skeletal muscle Na^+,K^+-ATPase for (^{3}H)-ouabain binding, as we also noted previously with older mice (82, 98). Thus, the lower (^{3}H)-ouabain binding capacity in muscle of obese mice, coupled with no change in binding affinity, indicates that obese mice have a lower number of (^{3}H)-ouabain binding sites than lean mice. The total (^{3}H)-ouabain binding site number in the mixture of forelimb and hindlimb muscles from obese mice was 28% lower than in lean littermates.

Liver weights and particulate protein content and nonspecific binding of (^{3}H)-ouabain to liver preparations of 14 day old obese mice were similar to respective values in

Table 8. Combined forelimb and hindlimb muscle weights, liver weights and (^{3}H)-Ouabain binding to muscle and liver preparations of 14 day old lean and obese mice[a]

	Forelimb & Hindlimb Muscles		Liver	
	Lean	Obese	Lean	Obese
Total tissue weight-g	0.63 ± 0.07	0.66 ± 0.04	0.28 ± 0.02	0.32 ± 0.01
Particulate protein-mg/g tissue	56 ± 2	55 ± 1	44 ± 3	45 ± 2
Nonspecific (^{3}H)-ouabain binding[b] pmoles/mg protein	2.4 ± 0.1	2.4 ± 0.1	1.76 ± 0.10	1.68 ± 0.09
Specific (^{3}H)ouabain binding[c] pmoles/mg protein	2.7 ± 0.08	2.0 ± 0.10*	0.26 ± 0.03	0.25 ± 0.05
K_d value[d] uM	0.039 ± 0.003	0.038 ± 0.003	14.1 ± 1.3	13.4 ± 2.0
(^{3}H)-Ouabain binding site concentration[d] pmoles/mg protein	3.0 ± 0.09	2.2 ± 0.1*	9.8 ± 1.1	8.9 ± 1.5
pmoles/total tissue	109 ± 12	79 ± 7*	115 ± 7	119 ± 13

[a]Mean ± SEM for six pairs of mice. The asterisk indicates a significant difference ($p<0.05$) between lean and obese mice.

[b](^{3}H)-Ouabain (0.4 uM) binding observed in the presence of excess nonlabeled ouabain (4 mM).

[c](^{3}H)-Ouabain (0.4 uM) binding minus (^{3}H)-ouabain (0.4 uM) binding observed in the presence of excess nonlabeled ouabain (4 mM).

[d]Calculated as described elsewhere (82).

lean littermates (Table 8). In contrast to the lower specific (^{3}H)-ouabain binding in skeletal muscle of obese mice, phenotype did not influence specific (^{3}H)-ouabain binding in liver preparations. As in skeletal muscle, K_d values for specific (^{3}H)-ouabain binding to Na^+,K^+-ATPase in the livers were not altered by phenotype. The number of (^{3}H)-ouabain binding sites per total liver in the 14 day old obese mice was similar to the values obtained in their lean counterparts.

DISCUSSION

We used (^{3}H)-ouabain binding as an index of the number of Na^+,K^+-ATPase enzyme units present in skeletal muscle and liver of the mice. Previous reports (81, 99) have shown that the amount of ouabain bound to receptor sites correlates with the number of Na^+,K^+-ATPase enzyme units. The 14 day old obese mice had 28% fewer Na^+,K^+-ATPase units in skeletal muscle than did lean mice. This represents one of the earliest reported developmental differences in an enzyme system between obese (ob/ob) and lean mice. Later in development the differences in number of Na^+,K^+-ATPase enzyme units (per mg skeletal muscle protein) between lean and obese mice are even greater (82, 98). Further, obese mice accumulate less skeletal muscle than lean mice between 3 and 8 weeks of age (92). Consequently, 8 week old obese mice have less than 50% as many hindlimb skeletal muscle

Na^+,K^+-ATPase enzyme units as observed in their lean counterparts (82, 98).

In contrast to observations in skeletal muscle, the number of Na^+,K^+-ATPase enzyme units in livers of the 14 day old obese mice was not reduced. But, later in development differences are observed. Four and eight week old obese mice have 19% and 54% fewer Na^+,K^+-ATPase enzyme units per mg liver protein, respectively, than their lean counterparts (98). Thus, the onset of observable alterations in Na^+,K^+-ATPase enzyme units in livers of obese mice occurs well after the initial appearance of obesity. Increases in liver weight of obese mice during development, however, offset the lower number of Na^+,K^+-ATPase enzyme units per mg protein. Consequently, adult obese mice do not have fewer Na^+,K^+-ATPase enzyme units per total liver than their lean counterparts (98). The number of Na^+, K^+-ATPase enzyme units in kidneys of the 14 day old mice was not evaluated, but we previously noted that the number of Na^+,K^+-ATPase enzyme units in kidneys of older obese mice was similar to the numbers observed in their lean counterparts (98).

Obese (ob/ob) mice have been suggested to be hypothyroid (24, 27), but the levels of Na^+,K^+-ATPase in three thyroid hormone responsive tissues (skeletal muscle, liver, and kidney) of the obese mice suggest that additional factors are also involved in the regulation of Na^+,K^+-ATPase

in obese mice. For example, the number of Na^+,K^+-ATPase units in skeletal muscle of obese mice is lower than in their lean counterparts before weaning, but the enzyme is not changed in the livers of the obese mice until later in development (98). The kidney enzyme appears less responsive to phenotype than does the enzyme in the other two organs (98). The number of Na^+,K^+-ATPase enzyme units in each of the three organs of obese mice, however, increased to a greater extent after thyroid hormone injection than did the enzyme in these tissues of lean mice (66). Thus, the hyperresponsiveness of Na^+,K^+-ATPase to thyroid hormone administration in obese mice is similar to the effects of thyroid hormone administration on the enzyme in hypothyroid rats (18). Additional studies are needed to clarify the role of thyroid hormones in the regulation of Na^+,K^+-ATPase enzyme units in tissues of obese mice.

Adult obese mice are insulin resistant, but tissue sensitivity to insulin changes during development of obesity (18). For example, adipose tissue of young obese (ob/ob) mice is supersensitive to insulin, whereas later it becomes resistant (19). Insulin resistance in muscle may appear much earlier in development of obesity than in liver or adipose tissue (19). Insulin has also been shown to increase K^+ uptake and Na^+,K^+-ATPase in muscle preparations (100). Thus, the possibility that the development of insulin resistance and the lower number of Na^+,K^+-ATPase enzyme units in skeletal muscle of obese mice are interrelated warrants

further study.

In summary, the lower number of Na^+,K^+-ATPase enzyme units in skeletal muscle of 14 day old obese mice represents one of the earliest reported enzymatic alterations in these mice. The extent to which this alteration contributes to the lower energy expenditure and subsequent increased energy storage in these mice remains to be established.

HEAT PRODUCTION AND Na^+, K^+-ATPase ENZYME UNITS IN LEAN AND OBESE (Ob/Ob) MICE HOUSED AT 33^o OR 14^o

INTRODUCTION

Obese (ob/ob) mice, unlike lean mice, die of hypothermia when acutely exposed to 4^{o} because of a reduced capacity for cold-induced thermogenesis (71, 72, 73). Obese mice also display hypothermia when maintained at normal ambient temperatures (24, 74, 75). Thus, the high efficiency of energy retention observed in obese mice (40, 76, 77, 101) may be related to their low energy expenditure for thermoregulatory thermogenesis (73, 75).

It has been suggested that active sodium transport contributes significantly to cold-induced (65, 102, 103) and thyroid hormone-induced (66, 67) thermogenesis. In this regard, obese mice exhibit a lower number of Na^{+},K^{+}-ATPase enzyme units in skeletal muscle as early as 2 weeks of age concomitant with a low capacity for thermogenesis (104). Later in development alterations are also evident in hepatic Na^{+},K^{+}-ATPase (70, 98). The extent to which changes in Na^{+},K^{+}-ATPase influence energy balance in obese mice has not been completely elucidated, nor is it clear to what extent Na^{+},K^{+}-ATPase in obese mice can be altered. York et al. (70) reported that injections of triiodothyronine did not increase the activity of Na^{+},K^{+}-ATPase in livers of obese mice, but increased it in lean counterparts. In contrast, we observed that thyroxine injections increased the number of Na^{+},K^{+}-ATPase enzyme units in liver, skeletal muscle, and kidneys as well as heat production to a much greater extent

in obese mice than in lean mice (98). These latter results indicate that Na^+,K^+-ATPase and heat production in obese mice change in concert, at least under certain conditions.

Because of reports of reduced capacity for cold-induced thermogenesis in obese mice, the present study was undertaken to evaluate the ability of young obese and lean mice to alter Na^+,K^+-ATPase in response to warm (33^o) or cold (14^o) environments. Na^+,K^+-ATPase enzyme units in skeletal muscle, liver, and kidneys, were estimated from (^3H)ouabain binding to tissue preparations (82, 98). These values were compared with whole animal heat production, and food intake and body weight changes in the same animals.

METHODS

Five week old male obese (ob/ob) and lean (ob/+ or +/+) mice (C57BL/6J), which had been housed at 23^o, were purchased from Jackson Laboratories, Bar Harbor, Maine. For 3 weeks mice were placed in chambers maintained at either 14 ± 1^o or 33 ± 1^o. Mice were housed in separate plastic cages and wood shavings were provided for bedding. Paper toweling (approximately 600 cm^2) was also provided as nesting material for mice housed at 14 ± 1^o, but this was removed after 10 days. Lights were on within each chamber from 0700 to 1900 hours. Mice were fed a stock diet (Wayne Lab-Blox, Allied Mills, Inc., Chicago, IL) and both food and water were available ad libitum. Individual food

intake and body weight were measured three times per week.

Body temperature and heat production of the mice were measured after 10 and 20 days of exposure to the warm or cold temperature. Between 0900 and 1200 hours a lubricated, rapid-response, telethermometer (Yellow Springs Instrument Co., Yellow Springs, OH) was inserted 2 cm into the rectum, after 30 seconds body temperature was recorded. Heat production was measured directly with a gradient layer calorimeter (Thermonetics Corporation, San Diego, CA). The calorimeter was placed in a chamber maintained at either 14 ± 1^{o} or 33 ± 1^{o} for heat production measurements of mice exposed to those temperatures, respectively. Mice were placed in the calorimeter for 60 to 90 minutes and heat production was recorded and averaged over the final 20 minutes. Heat production was calculated as total kcal produced per animal and as kcal produced per $kg^{0.75}$ body weight.

At the end of the 3 week experimental period, mice were killed by cervical dislocation and liver, kidneys, and hindlimb muscles were removed for Na^{+},K^{+}-ATPase determinations. The remaining carcass was softened in an autoclave at 100^{o} for 30 minutes and homogenized (Polytron, Brinkman Instruments, Westbury, NY) in an equal weight of water. This total homogenate was dried to a constant weight in an oven at 50^{o} and duplicate samples of each homogenate were ignited in a bomb calorimeter (Parr Adiabatic Calorimeter

Parr Instrument Co., Moline, IL) to determine carcass energy.

Liver, kidney, and hindlimb muscle were homogenized and centrifuged as previously described (82, 98). The number of Na^+,K^+-ATPase enzyme units in the particulate fraction of each tissue was estimated using the ouabain binding method (82, 88, 98). Nonspecific and specific (^3H)-ouabain binding (82, 88, 98) were assayed. Dissociation constants (K_d) for (^3H)-ouabain binding to Na^+,K^+-ATPase and (^3H)-ouabain binding site concentrations were calculated (88). Protein content was estimated by the biuret method (87).

The data were subjected to analysis of variance and were presented as means ± mean square error (MS_E). Statistical comparisons were made with the Bonferroni-t-test (89).

RESULTS

Table 9 lists body weight, food intake, and body energy data obtained from 5 week old male obese and lean mice housed at either 33^o or 14^o for 3 weeks. Initially, 5 week old obese mice weighed significantly more than did their lean counterparts. Obese mice housed at 33^o gained three times more body weight than lean mice at the same temperatures. At 14^o body weight gain of obese mice was slightly more than that of lean mice, but this difference was not statistically significant. Environmental temperature failed to significantly affect weight gain of lean mice, but obese

Table 9. Initial body weight, body weight gain, food intake, and body energy of lean and obese mice housed at either 33° or 14° for 3 weeks[A]

Parameter	Room Temperature				MS_E[B]
	33°		14°		
	Lean	Obese	Lean	Obese	
Initial body weight (g)	19.1[C,a]	22.8[b]	19.1[a]	23.8[b]	11.1
Body weight gain (g)	3.3[a]	10.4[b]	2.7[a]	5.7[a]	8.5
Food intake (g)[D]	52[a]	82[b]	119[c]	126[c]	114
Body energy (kcal)[E]	43[a]	143[b]	42[a]	109[c]	128

[A]Values represent mean for 9 or 10 mice.

[B]Mean square error.

[C]Means on the same line with different superscripts lowercase letters are different ($p \leq 0.05$).

[D]Measured over the first 20 days of the experiment.

[E]Total carcass energy after liver, kidneys, and hindlimb muscle were removed.

mice at 33° gained more weight than did obese mice at 14°.

Obese mice consumed more food than lean mice at the warmer temperature, but lean and obese mice ate similar amounts of food in the cold (Table 9). The colder temperature significantly increased food intake of both obese and lean mice (54% and 128%, respectively) above that of obese and lean mice at 33°. During the first week of cold exposure, food intake of obese and lean mice increased rapidly when compared with food consumption of obese and lean mice at 33°. No further cold-induced increase in food intake occurred during the final 2 weeks of the experiment (data not presented).

At the conclusion of the experiment obese mice had significantly more carcass energy than lean mice at both temperatures (Table 9). Cold exposure did not affect carcass energy of lean mice, but obese mice housed at 14° had 24% less carcass energy than obese mice kept at 33° for 3 weeks.

Body temperature and heat production were measured on days 10 and 20 of the study. These data and body weights recorded at those times are presented in Table 10. Obese mice weighed significantly more than lean mice. Cold exposure did not affect the body weight of lean mice, but after 20 days, obese mice at 14° weighed significantly less than obese mice at 33°. Body temperatures of obese and lean mice were similar at 33°, but at 14° body temperature of lean mice was significantly higher than that of obese mice.

Table 10. Body weight, body temperature, and heat production of lean and obese mice housed at either 33° or 14° for 3 weeks[A]

Day	Room Temperature				MS_E[B]
	33°		14°		
	Lean	Obese	Lean	Obese	
	Body weight (g)				
10	20.0[C,a]	27.9[b]	19.8[a]	25.3[b]	6.75
20	21.8[a]	33.1[b]	21.1[a]	28.6[c]	6.71
	Body temperature (°C)				
10	35.9[a]	35.7[a,b]	35.8[a]	35.0[b]	0.52
20	36.2[a]	36.3[a]	37.0[a]	34.1[b]	0.63
	Heat production (kcal/hr/animal)				
10	0.265[a]	0.306[a]	0.651[b]	0.758[c]	0.003
20	0.248[a]	0.334[a]	0.715[b]	0.744[b]	0.007
	Heat production (kcal/hr/$kg^{0.75}$)				
10	5.05[a]	4.53[a]	12.36[b]	12.34[b]	1.22
20	4.39[a]	4.34[a]	12.83[b]	10.64[c]	1.33

[A]Values represent means for 9 or 10 mice

[B]Mean square error

[C]See Table 9.

After 20 days of cold exposure, body temperature of obese mice was almost 3^{o} less than that of lean mice.

Total heat production (kcal/hr/animal) of both lean and obese mice was more than two times greater at 14^{o} than at 33^{o} (Table 10). Heat production of obese mice tended to be slightly greater than that of lean mice at both temperatures, but this difference was statistically significant only at 14^{o} on day 10. Because obese mice were heavier than lean mice, heat production was also expressed per unit of metabolic body weight (kcal/hr/$kg^{0.75}$) (105). Lean and obese mice produced similar amounts of heat per unit of metabolic body weight at 33^{o}. This was also true on day 10 at the cooler temperature. By the end of the experimental period (day 20), however, heat production per unit of metabolic body weight of cold-exposed obese mice was lower than that of lean mice at 14^{o}. Since shivering was not observed in either obese or lean mice after the first few days at 14^{o}, we assume that the increased heat production at 14^{o} resulted from nonshivering mechanisms.

Heat production of cold-acclimated mice was measured at 25^{o} to determine if these mice would reduce heat production when the cold stimulus was removed. Obese and lean mice housed at 14^{o} for two weeks produced 0.79 ± 0.08 (mean ± SEM) and 0.66 ± 0.07 kcal heat per hour per mouse, respectively, at that temperature. Within one hour after these mice were transferred to a chamber maintained at 25^{o},

their heat production decreased to 0.41 ± 0.02 and 0.50 ± 0.03 kcal per hour per mouse for obese and lean mice, respectively. These values were similar to those of obese and lean mice continuously maintained at 25° (0.44 ± 0.05 and 0.49 ± 0.01 kcal heat per hour per mouse). Thus, both obese and lean mice are able to rapidly switch off thermogenesis when the cold stimulus is removed.

Hindlimb muscle weights and (^{3}H)-ouabain binding to muscle preparations of lean and obese mice housed at either 33° or 14° for 3 weeks are summarized in Table 11. In agreement with earlier reports (92, 106) obese mice had less hindlimb muscle than did lean mice. This appears to be related to a faster fractional breakdown rate of muscle in obese mice than in lean mice (106). Environmental temperature altered muscle mass in obese mice, but not in lean mice. Obese mice housed at 14° had only 57% as much hindlimb muscle at the end of the experiment as did obese mice housed at 33°. Additional studies are needed to determine the cause for the low accumulation of muscle in cold-exposed obese mice.

The concentration of particulate protein in hindlimb muscles was not influenced by either phenotype or environmental temperature. Likewise, neither phenotype nor environmental temperature affected nonspecific, nonsaturable binding of (^{3}H)-ouabain to the hindlimb muscle preparations. Specific binding of (^{3}H)-ouabain to muscle preparations of

Table 11. Hindlimb muscle weight and (^{3}H)-ouabain binding to muscle preparations of lean and obese mice housed at either 33° or 14° for 3 weeks[A]

	Room Temperature				MS_E[B]
	33°		14°		
	Lean	Obese	Lean	Obese	
Hindlimb muscle:					
Total weight-g	1.9[C,a]	1.4[b]	1.7[a]	0.8[c]	0.04
Particulate protein-mg/g muscle	71[a]	73[a]	68[a]	69[a]	4.3
Nonspecific (^{3}H)-ouabain binding[D] pmoles/mg protein	2.3[a]	1.9[a]	2.1[a]	1.8[a]	0.09
Specific (^{3}H)-ouabain binding[E] pmoles/mg protein	1.2[a]	1.0[a]	2.0[b]	2.0[b]	0.09
K_d value[F]-uM	0.031[a]	0.035[a]	0.033[a]	0.036[a]	0.033
(^{3}H)-ouabain binding site concentration[F] pmoles/mg protein	1.2[a]	1.0[a]	2.2[b]	2.1[b]	0.1
pmoles/total hindlimb muscle	164[a]	103[b]	252[c]	122[b]	1040

[A] Values represent mean for 9 or 10 mice.

[B] Mean square error.

[C] See Table 9.

[D] (^{3}H)-Ouabain (0.4 uM) binding observed in the presence of excess nonlabeled ouabain (4 uM).

[E] (^{3}H)-Ouabain (0.4 uM) binding minus (^{3}H)-ouabain (0.4 uM) binding observed in the presence of excess nonlabeled ouabain (4 mM).

[F] Calculated as described elsewhere (88).

both obese and lean mice exposed to 14° was nearly twice as high as was the specific binding of (^{3}H)-ouabain to muscle preparations of mice exposed to 33°. Phenotype did not alter specific ouabain binding to muscle preparation. Since the affinity of the binding sites for ouabain was not changed by either phenotype or environmental temperature, the concentration of (^{3}H)-ouabain binding sites in the muscle paralleled the specific (^{3}H)-ouabain binding results. The total number of ouabain binding sites in the hindlimbs of obese mice exposed to 33° and 14°, however, was 37% and 52% lower, respectively, than was the number of binding sites in hindlimbs of lean counterparts. This occurred because the hindlimb muscle weights of the obese mice were significantly lower than that of their lean counterparts. Cold exposure elevated the number of ouabain binding sites in hindlimb skeletal muscle of lean mice by 54%, but no significant elevation was observed in obese mice because of the low amount of muscle in the cold-exposed obese mice.

Livers of obese mice weighed more than did livers of lean mice (Table 12). Obese mice exposed to 14° had lighter livers than did obese mice maintained at 33°, but environmental temperature did not influence liver weights of lean mice. Particulate protein content, expressed as mg protein per g liver, was not significantly altered by phenotype or environmental temperature nor was nonspecific binding of (^{3}H)-ouabain to liver preparations influenced by phenotype

Table 12. Liver weight and (^{3}H)-ouabain binding to liver preparations of lean and obese mice housed at either 33° or 14° for 3 weeks[A]

	Room Temperature				MS_E[B]
	33°		14°		
	Lean	Obese	Lean	Obese	
Total liver weight-g	1.4[C,a]	2.5[b]	1.5[a]	2.1[c]	0.07
Particulate protein-mg/g liver	51[a]	47[a]	51[a]	51[a]	22
Nonspecific (^{3}H)-ouabain binding[D] pmoles/mg protein	1.5[a]	1.4[a]	1.5[a]	1.4[a]	0.04
Specific (^{3}H)-ouabain binding[E] pmoles/mg protein	0.13[a]	0.09[a]	0.23[b]	0.22[b]	0.001
K_d value-uM[F]	10.3[a]	11.7[a]	12.9[a]	11.5[a]	2.1
(^{3}H)-ouabain binding site concentration[F] pmoles/mg protein	3.7[a]	2.7[a]	7.5[b]	6.4[b]	1.1
pmoles/total liver	268[a]	323[a]	586[b]	697[b]	17300

A-F See Table 11.

or temperature. The specific (^{3}H)-ouabain binding to livers of mice housed at 14^o was approximately double the values observed in mice housed at 33^o, but phenotype did not alter specific (^{3}H)-ouabain binding to liver preparations. The affinity of Na^+,K^+-ATPase for ouabain was unchanged by phenotype and environmental temperature, as observed in muscle preparations. The concentration of (^{3}H)-ouabain binding sites in liver was higher at the lower temperature, but phenotype did not influence the results. Even though obese mice had heavier livers than lean mice, the total number of ouabain binding sites in livers of obese mice was not significantly greater than levels observed in their lean counterparts.

Kidney weights were not altered by phenotype, but kidneys were substantially larger in mice housed at 14^{o}C than in mice housed at 33^o (Table 13). Presumably, this cold-induced enlargement of kidneys represents, in part, a compensatory response to an added functional load. As with the other tissues, neither phenotype nor temperature influenced particulate protein concentration in kidneys or nonsaturable, nonspecific binding of ouabain to kidney preparations. Mice housed at 14^o had higher specific binding of (^{3}H)-ouabain to kidney preparations than did preparations from mice housed at 33^o, but the temperature effect was somewhat less than observed for skeletal muscle and liver preparations. Specific binding of (^{3}H)-ouabain to

Table 13. Kidney weight and (^{3}H)-ouabain binding to kidney preparations of lean and obese mice housed at either 33° or 14° for 3 weeks[A]

	Room Temperature				MS_E[B]
	33°		14°		
	Lean	Obese	Lean	Obese	
Weight of both kidneys-g	0.24[C,a]	0.23[a]	0.33[b]	0.33[b]	0.0001
Particulate protein-mg/g kidney	61[a]	57[a]	58[a]	60[a]	83
Nonspecific (^{3}H)-ouabain binding[D] pmoles/mg protein	1.6[a]	1.9[a]	2.1[a]	1.9[a]	0.10
Specific (^{3}H)-ouabain binding[E] pmoles/mg protein	1.5[a]	2.2[b]	2.7[c]	3.0[c]	0.16
K_d value-[F]uM	9.8[a]	9.7[a]	9.3[a]	9.0[a]	0.2
(^{3}H)-ouabain binding site concentration[F] pmoles/mg protein	39[a]	55[b]	65[c]	70[c]	98
pmoles/both kidneys	564[a]	703[a]	1240[b]	1350[b]	24900

[A-F] See Table 11.

Na^+,K^+-ATPase in kidneys of obese mice housed at 33^o was 47% higher than was specific binding of (^3H)-ouabain in kidneys of lean counterparts; however, this difference was not apparent at 14^o. As observed in skeletal muscle and liver, affinity of Na^+,K^+-ATPase for ouabain in kidney preparations was not changed by phenotype or environmental temperature. Since the K_d value for ouabain binding was not altered, the concentration of (^3H)-ouabain binding sites in kidney paralleled specific binding of (^3H)-ouabain. The higher specific binding of (^3H)-ouabain in cold-exposed mice coupled with the heavier kidneys of these mice resulted in a two-fold greater number of ouabain binding sites in kidneys of cold-exposed mice than in their warm-exposed counterparts.

DISCUSSION

When obese mice are acutely exposed to severe cold (3^o to 4^o), they fail to increase their metabolic rate, develop hypothermia, and die (71, 72, 73); but, it is now clear that young obese mice are capable of increased thermogenesis when chronically exposed to less severe cold (14^o). Heat production of obese mice maintained at 14^o for 10 to 20 days was approximately equal to that of their lean counterparts and was 25% higher than heat production of obese mice maintained at 25^o and injected with a large dose of thyroxine (5 ug daily for 14 days) (98). A larger dose of thyroxine did not increase heat production further (78). Lean mice exposed to

cold also produced more heat (60% more) than lean mice injected with thyroxine (98). Thus, the cold-induced thermogenesis produced a greater heat production in both obese and lean mice than did exogenous thyroid hormone-induced thermogenesis.

Cold-exposed obese mice failed to increase their food intake sufficiently to fully compensate for the greater heat loss and consequently, gained less body energy than warm-exposed obese mice. Likewise, obese mice injected with thyroxine increased their heat production more than their food intake (98). Lean mice, however, precisely regulated energy intake under these conditions to offset changes in energy loss and to maintain body energy (78, 98). While heat output of cold-exposed obese mice equaled that of lean mice, obese mice exhibited moderate hypothermia which is also observed in obese mice maintained at 20^{o} to 25^{o} (24, 75). At 33^{o}, however, rectal temperature of obese mice equaled that of lean mice. The obese mouse, thus, appears to regulate its body temperature at a level slightly lower than that of lean mice, except when exposed to a warm environment or exogenous thyroxine (24, 75).

(^{3}H)-Ouabain binding was used as an index of the number of Na^{+},K^{+}-ATPase enzyme units present in the tissue preparations, since the amount of (^{3}H)-ouabain bound to receptor sites correlates with the amount of this enzyme (81, 99). Two kinetic parameters, the dissociation constant for

(^{3}H)-ouabain binding to Na^+,K^+-ATPase and the (^{3}H)-ouabain binding site concentration in the tissues, were measured. Affinity of binding sites for (^{3}H)-ouabain was not influenced by either phenotype or environmental temperature. Thus, the observed changes in specific ouabain binding in the tissues were due to alterations in number of Na^+,K^+-ATPase enzyme units.

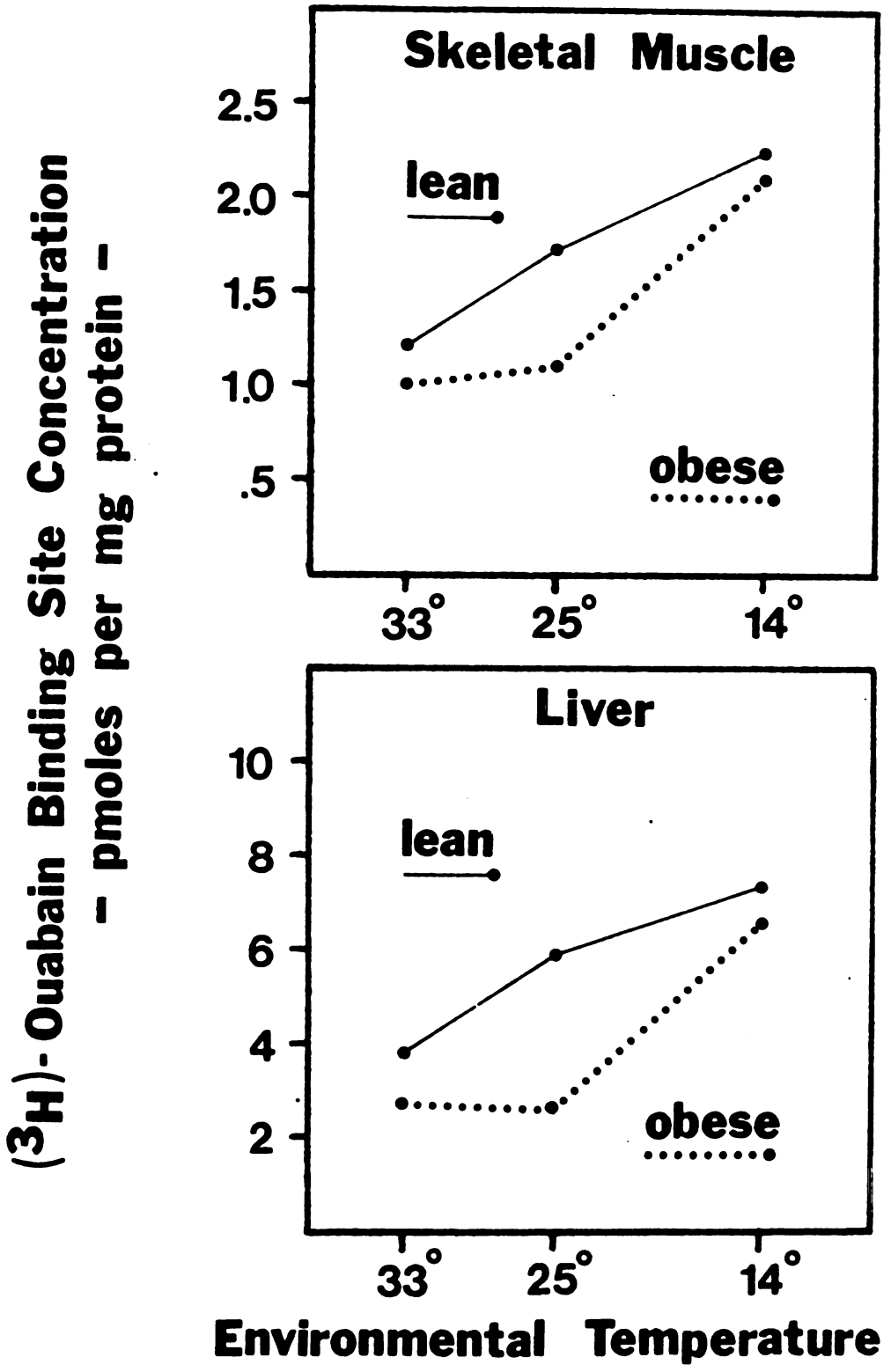
The concentration of Na^+,K^+-ATPase enzyme units, estimated from specific (^{3}H)-ouabain binding, in skeletal muscle and liver of cold-exposed mice was approximately double the number observed in warm-exposed mice, responses in kidneys were somewhat less. A comparison of heat production and Na^+,K^+-ATPase enzyme units indicated that lean mice exposed to 14^o produced 188% more heat and had 90% more Na^+,K^+-ATPase enzyme units in the three tissues examined (assuming that hindlimbs represent 25% of total muscle mass) than lean mice exposed to 33^o. Likewise, obese mice maintained at 14^o produced 123% more heat and had 76% more Na^+,K^+-ATPase enzyme units in muscle, liver, and kidneys than obese mice maintained at 33^o. Others have measured in vitro respiration rates in muscle, liver and kidneys of rats (65, 103), mice (102), and hamsters (107) and noted that approximately 20 to 100% of the increase in respiration in these tissues of cold-exposed animals is Na^+ dependent and ouabain-sensitive. Both Na^+-dependent and ouabain-sensitive (65, 66-67) respiration are associated with active Na^+

transport. Together these results suggest that the elevated heat production observed in animals during chronic cold exposure may be mediated in part through an increase in number of Na^+,K^+-ATPase units and a concomitant increase in Na^+ pump activity.

Obese mice maintained at 33^o had the same concentration of Na^+,K^+-ATPase enzyme units in skeletal muscle and liver as did lean mice. Likewise at 14^o, obese and lean mice had similar numbers of Na^+,K^+-ATPase enzyme units per mg protein even though the number of enzyme units was higher in the cold-exposed mice than in the warm-exposed mice. These results are in contrast to our earlier report (98) where differences were observed between obese and lean mice, but these mice had been maintained at 25^o. At this intermediate temperature the concentration of Na^+,K^+-ATPase enzyme units in muscle and liver of 8 week old obese mice is 35 and 54% lower, respectively, than in tissues of lean counterparts (98).

We plotted the concentration of (^3H)-ouabain binding sites in muscle and liver versus environmental temperature and found that lean and obese mice respond differently (Figure 2). The number of binding sites in lean mice decreased linearly as environmental temperature was raised from 14^o to 25^o to 33^o. In skeletal muscle, the decrease averaged 0.05 pmoles (^3H)-ouabain bound per mg protein for each degree increase in environmental temperature

Figure 2. ^{3}H-Ouabain binding site concentration in skeletal muscle and liver of 5 week old lean and obese mice housed at 33°, 25°, or 14° for 3 weeks. Data points represent mean values for 9 or 10 mice. Values for mice housed at 25° have been published (98) and are presented again for comparative purposes.


Skeletal Muscle
lean
obese
2.5
2.0
1.5
1.0
.5
33°
25°
14°
Liver
lean
obese
10
8
6
4
2
33°
25°
14°
(3H)-Ouabain Binding Site Concentration
- pmoles per mg protein -
Environmental Temperature

(correlation coefficient = 0.99) and in liver binding decreased 0.20 pmoles per mg protein for each degree increase in temperature (correlation coefficient = 0.99). The response in obese mice, however, was not linear. The decrease in (^{3}H)-ouabain binding in skeletal muscle and liver of obese mice as environmental temperature increased fitted a single-term exponential curve; the correlation coefficients for skeletal muscle and liver were 0.95 and 0.89, respectively. There was a marked reduction in (^{3}H)-ouabain binding site correlation in both muscle and liver of obese mice as environmental temperature was raised from 14^o to 25^o, but between 25^o and 33^o the binding of (^{3}H)-ouabain to muscle and liver preparations failed to decrease further. Thus, while Na^+,K^+-ATPase in the mice examined responds in the same direction to changes in environmental temperature, the response curves of the obese and lean mice differ. As a result, the greatest differences in Na^+,K^+-ATPase enzyme units between obese and lean mice is observed not at 14^o or 33^o but at an intermediate temperature (98).

Thyroid hormones (66, 67) and catecholamines (108) contribute to the induction and mediation of nonshivering thermogenesis during cold exposure, in part at least, through their involvement in the regulation of Na^+,K^+-ATPase. Few studies have focused on these hormones in cold-exposed obese mice, however, recent evidence indicates that obese mice housed at 20^o have higher circulating levels of

thyrotropin than when housed at 28^{o}, whereas lean mice housed at either 20^{o} or 28^{o} had the same concentration of thyrotropin (109). Likewise, the capacity for nonshivering thermogenesis in response to an injection of catecholamines was greater in lean mice than in obese mice when the animals were housed at 31^{o}, but at 10^{o} both groups of mice responded similarily (75). These subtle differences between lean and obese mice in response to alterations in environmental temperature may contribute to the temperature related changes in Na^{+},K^{+}-ATPase in these young mice.

The reduction in heat production observed when mice were transferred from 14^{o} to 25^{o} indicates that these mice possess mechanisms to rapidly regulate cold-induced thermogenesis. It is, however, unlikely that the number of Na^{+}, K^{+}-ATPase enzyme units in the mice changed significantly during this time (approximately one hour). Thus, mechanisms either exist to rapidly alter in vivo function of Na^{+},K^{+}-ATPase or alternatively, Na^{+},K^{+}-ATPase may not be involved in cold-induced thermogenesis. In brown adipose tissue, at least, it appears that catecholamines may regulate in vivo functioning of Na^{+},K^{+}-ATPase (108, 110). Our data indicate that the capacity of the Na^{+},K^{+}-ATPase system in skeletal muscle, liver, and kidneys is increased in cold-acclimated mice, but the in vivo activity of the system was not determined. Further data are required before it will be possible to establish the contribution of Na^{+},K^{+}-ATPase to cold-induced

thermogenesis in intact animals.

Acute cold exposure increases the activities of glycerokinase, α-glycerophosphate dehydrogenase, lipase (111), and mitochondrial atractyloside-insensitive purine nucleotide binding (54) in brown adipose tissue of lean mice, but not in brown adipose tissue of obese mice. It will be important to determine if brown adipose tissue of obese mice is incapable of responding to cold, or whether obese mice merely require a longer period of cold exposure than employed in these acute experiments. Since Na^+,K^+-ATPase is probably involved in brown adipose tissue thermogenesis (108, 110), and since this organ may contribute significantly to heat production in cold-exposed animals (112, 113), additional studies are needed to evaluate Na^+,K^+-ATPase in brown adipose tissue of obese mice.

In summary, it is clear that young obese mice can survive a moderate, chronic cold exposure and that Na^+,K^+-ATPase is increased in several tissues of these cold-exposed mice. Further studies are required to determine the extent these enzyme changes contribute to the total heat production of the mice and to determine if this system is involved in the failure of obese mice to survive severe cold stress.

FUNCTIONAL CORRELATES OF Na^+,K^+-ATPase IN OBESE (Ob/Ob) MICE

INTRODUCTION

Evidence from several sources suggest that Na^+,K^+-ATPase may be associated, either directly (64, 66, 67) or indirectly (114), with significant energy expenditure in animals. Adult obese (ob/ob) mice have lower concentrations of Na^+,K^+-ATPase enzyme units in skeletal muscle and liver than lean counterparts (98). Na^+,K^+-ATPase activity, estimated from ouabain inhibitable ATP hydrolysis, is also lower in liver homogenates obtained from obese mice (70). Additionally, obese mice exhibit less ouabain-suppressible respiration in intact hemidiaphragms and liver slices than lean mice (115). These results suggest that Na^+,K^+-ATPase may be of importance in the etiology of obesity in ob/ob mice.

Ismail-Beigi and Edelman have shown that tissue Na^+,K^+-ATPase concentrations are influenced by thyroid status in rats (66, 67) with a concomitant change in intracellular sodium (Na^+) and potassium (K^+) concentrations in diaphragm and heart (116). Although obese mice appear to have normal circulating levels of thyroid hormones (117, 109) and a normal thyroid-pituitary axis (109), they have reduced nuclear binding of triiodothyronine (115), thus suggesting a reduced effectiveness of circulating thyroid hormones. Reduction in Na^+,K^+-ATPase in obese mice (70, 98) are comparable to those observed in

hypothyroid rats (64, 66, 67). Thus, we have examined the possibility that obese (ob/ob) mice also have alterations in tissue Na^+ and K^+. Additionally, ouabain was injected into intact lean and obese mice to obtain data on ouabain-suppressible oxygen consumption in these animals.

METHODS

Animals: The male obese (ob/ob) and lean (ob/+ or +/+) mice used in these studies were from our breeding colony (C57BL/6J - ob/+ from Jackson Laboratory, Bar Harbor, Maine). They were weaned at 3 weeks of age and were housed in solid-bottom cages with wood shavings for bedding. A stock diet (Wayne, Lab-Blox from Allied Mills, Inc., Chicago, Illinois) and water were available ad libitum. Lights were on from 0700 hr to 1900 hr daily. Ambient temperature was maintained at 23^o to 25^oC, unless otherwise indicated.

Experiment 1: To provide information on the in vivo functioning of Na^+,K^+-ATPase in lean and obese mice, we measured the concentrations of sodium (Na^+) and potassium (K^+) in selected tissues. Eight-week-old lean and obese mice were utilized. Na^+ and K^+ content of plasma, liver, kidney, and gastrocnemius muscle were estimated. Extracellular water content of tissues from another group of eight-week-old lean and obese mice was estimated from the determination of inulin space. Mice, deprived of food and water for an hour, were injected intraperitoneally with

0.1 ml of saline containing 1.66 uCi of inulin-^{14}C (Amersham/Searle Corp.). After one hour, a blood plasma sample and liver and gastrocnemius muscle samples (10-30 mg) were taken for determination of inulin-^{14}C. The inulin space of kidneys was not determined because this organ accumulates inulin.

Experiment 2: This experiment was designed to evaluate fraction of oxygen consumption which is inhibited by ouabain. In order to eliminate the difference in body weight between lean and obese, four-week-old mice were used. Oxygen consumption of lean and obese mice was measured with a volume meter (Med Science Electronics, Inc., St. Louis, Missouri). Food was available to the mice until oxygen consumption measurements commenced. Mice were placed in flasks immersed in water baths maintained at either 14 ± 1^{o}, 25 ± 1^{o} or 33 ± 1^{o}C. After 20 minutes, oxygen consumption was recorded and averaged over a span of 20 minutes. Immediately, after this 40 minute control period, mice received an intraperitoneal injection of 0.3 or 0.9 ug ouabain (Sigma Chemical Company, St. Louis, Missouri) per g body weight. A preliminary study indicated that oxygen consumption of lean and obese mice was not significantly altered by intraperitoneal injection of physiological saline. Rates of oxygen consumption decreased during the first 20 minutes after ouabain injection and then plateaued at this lower rate for at least 2

hours. Rates of oxygen consumption were continuously recorded between 20 and 40 minutes after ouabain injection. Immediately after measurement of oxygen consumption, mice were anesthetized and samples of plasma, liver, kidney and gestrocnemium muscle were obtained for Na^+ and K^+ estimation.

Experiment 3: To correlate effects of ouabain injections on oxygen consumption of intact lean and obese mice with uptake of ouabain by selected tissues, groups of four-week-old lean and obese mice were injected intraperitoneally with ^{3}H-ouabain (0.3 ug ouabain/g body weight; 2.8 uCi per ug ouabain). ^{3}H-Ouabain was from Amersham/Searle Corporation. Mice were maintained at 14 ± 1°, 25 ± 1° or 33 ± 1°C for 40 minutes before injection with ^{3}H-ouabain. Forty minutes after administration of ^{3}H-ouabain mice were anesthetized, a blood sample and duplicate samples (10-30 mg) of brain, heart, kidney, liver and gastrocnemium muscle were rapidly removed for determination of ^{3}H-ouabain content.

Na^+ and K^+ measurements: Mice were anesthetized with ether, and blood was collected in a heparinized syringe by cardiac puncture. Blood was centrifuged to obtain plasma. Duplicate samples of liver, kidney and gastrocnemium muscle were rapidly removed. After recording wet weight, tissues were dried overnight at 90°C, reweighed, and the difference equaled water content of the tissue.

Tissues were then extracted in nitric:perchloric acid (4:1) for one hour at 100°C. Tissue extracts and plasma were analyzed for Na^+ and K^+ content (IL 453 atomic absorption/ emission spectrophotometer).

Plasma and tissue radioactivity measurements: Tissue samples and plasma containing ^{14}C-inulin or ^{3}H-ouabain were placed in 1.0 ml of tissue solubilizer (BTS 450, Beckman Instruments, Inc., Fullerton, California) at 55°C for 5 hours. Samples were decolorized with 50 ul of 30% H_2O_2 solution. Ascorbic acid (50 ul of a 15% solution in water) was also added to reduce chemiluminescence. Ten ml of scintillation cocktail containing 4.5 g of 2,5-diphenyl-oxazole and 0.09 g of 1,4-bis-2-(4-methyl-5-phenyloxazolyl)-benzene and toluene to 1 liter was added to each sample. Radioactivity was measured in a Packard Tri Carb Scintillation Spectrophotometer.

Statistical calculations: Data were analyzed by analysis of variance and presented as means ± standard error (Tables 14 and 15, and Figures 3 and 4) or means ± mean square error (Table 16). Statistical comparisons (89) were made with paired-t-test (Tables 14 and 15) or Tukey test (Table 16 and Figures 3 and 4).

RESULTS

Table 14 shows concentrations of water, Na^+ and K^+ in plasma, liver, kidney and gastrocnemius muscle, and kidneys obtained from 8-week-old lean and obese mice housed

Table 14. Tissue water content, and Na^+ and K^+ content of tissues from lean and obese mice[A].

	Lean	Obese
Plasma		
Na^+, meq/l plasma	151 ± 13	137 ± 10
K^+, meq/l plasma	5.3 ± 0.4	5.5 ± 0.4
Liver:		
Total water content, ml/100 g	69.9 ± 1.0	59.2 ± 1.1*
Na^+, meq/l tissue water	40 ± 2	44± 2
K^+, meq/l tissue water	136 ± 5	120 ± 5*
Muscle:		
Total water content, ml/100 g	78.5 ± 0.5	74.4 ± 0.8*
Na^+, meq/l tissue water	42 ± 2	58 ± 5*
K^+, meq/l tissue water	146 ± 6	132 ± 5*
Kidney:		
Total water content, ml/100 g	74.8 ± 0.4	74.5 ± 0.5
Na^+, meq/l tissue water	85 ± 7	84 ± 6
K^+, meq/l tissue water	122 ± 6	126 ± 7

[A]Means ± SEM for 18 pairs of 8-week-old mice. Lean and obese mice weighed 26 ± 1 and 37 ± 1 g, respectively. Asterisks indicate significant differences ($p<0.05$) between lean and obese mice.

at 23° to 25°C. Water content of liver and gastrocnemius muscle from obese mice was lower than from lean mice, but no differences were observed in kidneys. Neither plasma Na^+ nor plasma K^+ were altered by phenotype. Liver Na^+ concentration, per liter tissue water, was not influenced by phenotype, but muscle Na^+ concentration was elevated in obese mice. K^+ concentrations in both liver and muscle were lower in obese mice than in lean mice. Neither Na^+ nor K^+ concentrations in kidneys were altered by phenotype.

To investigate whether the observed differences in Na^+ and K^+ content, expressed per liter tissue water, resulted from an alteration in the ratio of extracellular to intracellular water, or from a shift in intracellular ion content, the extracellular inulin space was measured in 8 pairs of lean and obese mice. Results indicated that the ratio of extracellular water to intracellular water in liver and muscle was not influenced by phenotype. Based on inulin space measurements, percentage of water in the intracellular space averaged 75 ± 3% and 70 ± 3% in liver of lean and obese mice, respectively; values averaged 82 ± 1% and 82 ± 2% in muscle of lean and obese mice, respectively. Livers of rats accumulate radioactive inulin continuously and this causes an underestimate of cell water (118). Since the calculated value for intracellular water

in livers of our mice averaged only 70 to 75% of tissue water, it appears that inulin-^{14}C also underestimates intracellular water space in livers of mice. Nevertheless, phenotype did not appear to alter distribution of inulin-^{14}C within livers. In subsequent calculations inulin-^{14}C space data obtained in muscle are applied to livers to enable us to estimate intracellular Na^+ and K^+ in livers.

Calculated intracellular Na^+ and K^+ concentrations in liver and muscle of lean and obese mice are presented in Table 15. Intracellular Na^+ concentrations in both liver and muscle were higher in obese mice than in lean mice. Conversely, calculated intracellular K^+ concentrations in both organs were lower in obese mice than in lean mice. The intracellular ratio of Na^+ to K^+ was, thus, 56% higher in livers of obese mice and 116% higher in muscle of obese mice than observed in their lean counterparts.

Ouabain inhibits Na^+,K^+-ATPase (80, 119, 120). Ouabain does not appear to inhibit other systems at least directly (121), and therefore a decrease in oxygen consumption induced by ouabain is likely to indicate that Na^+,K^+-ATPase activity supports a substantial fraction of respiration under normal conditions. Mice were first exposed to three environmental temperatures to vary their metabolic rates. At 14^o both lean and obese mice consumed more than twice as much oxygen as at 33^o (Table 16). Obese mice, however, consumed less oxygen than did lean mice at both 14^o and

Table 15. Intracellular Na^+ and K^+ concentration of liver and gastrocnemius muscle from lean and obese mice[A].

Tissue	Phenotype	Na^+	K^+	Na^+/K^+
		meq/l cell water[B]		
Liver	Lean	17.8 ± 1.6	165 ± 7	0.109 ± 0.009
	Obese	24.4 ± 2.0*	145 ± 6*	0.170 ± 0.008*
Muscle	Lean	19.3 ± 2.2	177 ± 7	0.109 ± 0.009
	Obese	58.0 ± 5.0*	160 ± 5*	0.235 ± 0.021*

[A]Means ± SEM for 18 pairs of 8-week-old mice.

[B]Values were calculated from several measured values (i.e. total tissue water, Na^+ and K^+ contents; plasma and tissue insulin-^{14}C assays; and plasma Na^+ and K^+ concentrations). In these calculations, estimates of muscle inulin space were substituted for those obtained in the liver to calculate hepatic Na^+ and K^+. Asterisks indicate a significant difference ($P<0.05$) between lean and obese mice.

Table 16. Body weight and oxygen consumption of mice injected with ouabain[A]

14°		25°		33°		MS_E[B]
Lean	Obese	Lean	Obese	Lean	Obese	
Body weight, g						
15.9[C,a]	16.3[a]	15.7[a]	16.3[a]	16.0[a]	16.7[a]	13.7
Oxygen Consumption[D] Prior to Ouabain Injection						
7.73[a]	6.41[b]	4.20[c]	3.76[d]	3.02[e]	2.93[e]	0.399
Oxygen Consumption[D] After Injection of 0.3 ug Ouabain/g Body Weight						
6.31[a]	5.62[b]	3.03[c]	3.24[c]	2.25[d]	2.13[d]	0.106
Percent of Control						
79[a]	87[b]	73[c]	88[b]	75[c]	76[ac]	10.1
Change in Oxygen Consumption[E]						
1.66[a]	0.86[bc]	1.10[c]	0.44[d]	0.74[be]	0.64[de]	0.065
Oxygen Consumption[D] After Injection of 0.9 ug Ouabain/g Body Weight						
5.40[a]	5.45[a]	2.65[bc]	2.99[b]	2.37[c]	2.35[c]	0.315
Percent of Control						
72[a]	87[b]	62[c]	77[a]	77[a]	75[a]	27.9
Change in Oxygen Consumption[E]						
2.10[a]	0.88[b]	1.63[c]	0.85[b]	0.69[b]	0.73[b]	0.114

[A]Means for ten pairs of 4-week-old mice, half were injected with 0.3 and the other half with 0.9 ug ouabain/g body weight.

[B]Mean Square Error.

[C]Means on the same line with different superscript lowercase letters are different ($P<0.05$).

[D]Expressed as ml/hr/g body weight.

[E]Oxygen consumption prior to ouabain injection minus consumption after ouabain injection (ml/hr/g body weight).

25°, but no differences were observed at 33°. These observations are in close agreement with other reports which demonstrate that obese mice have less ability to increase metabolic rate when exposed to an acute cold stress than lean mice (71, 72, 73). While short-term measurement of metabolic rates at thermoneutrality (33°) failed to detect significant differences between lean and obese mice, long-term, paired-feeding experiments at 33° clearly demonstrate that obese mice do have lower metabolic rates at this temperature than lean mice (122).

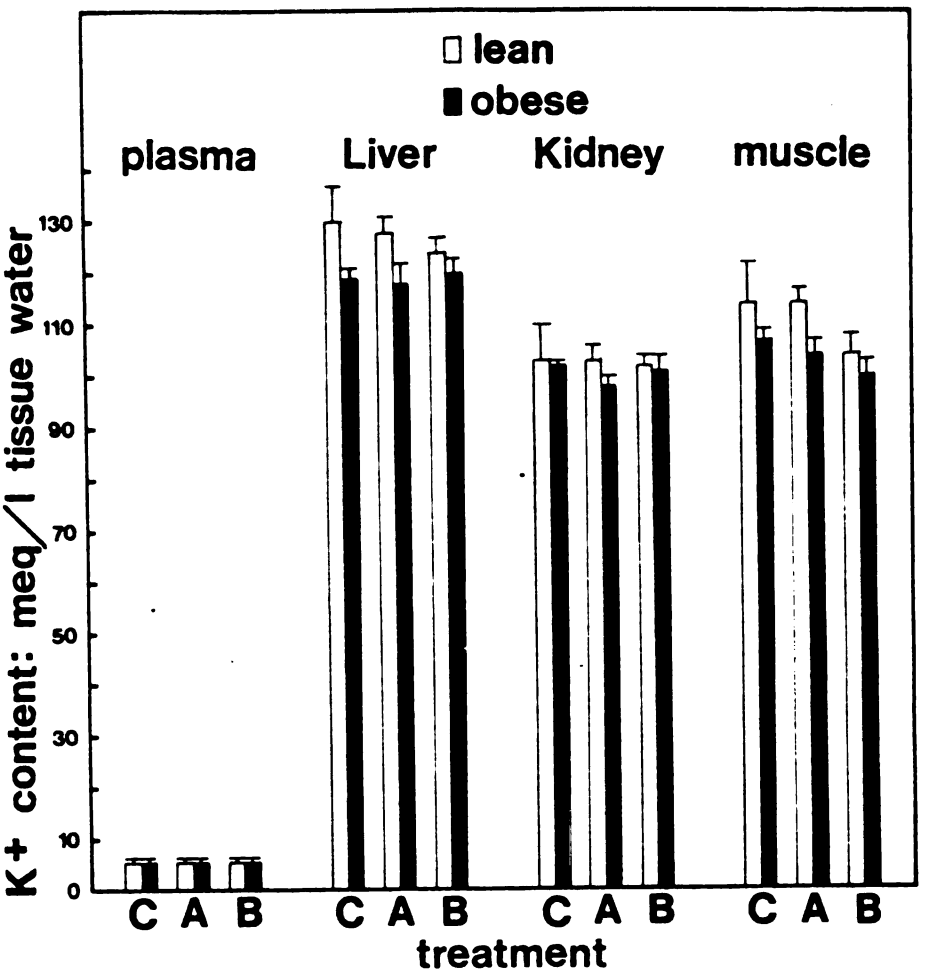
Ouabain (0.3 ug/g body weight) injection resulted in a 21 to 27% depression in oxygen consumption in lean mice at all three temperatures (Table 16). At 33°, obese mice also decreased their oxygen consumption to a similar extent. But less of a response to ouabain was observed in obese mice maintained at 14° or 25°; oxygen consumption decreased only 13 to 14%. Consequently, the depression in oxygen consumption at 14° or 25°, expressed ml/hr/g body weight, was significantly less in obese mice injected with ouabain than in lean mice. A three-fold increase in dose of ouabain (0.9 ug/g body weight) failed to further depress oxygen consumption in mice maintained at 33°. But oxygen consumption of mice maintained at 14° or 25° and injected with the larger dose of ouabain was decreased to a greater extent than observed when the smaller dose was injected. As with the lower dose of ouabain, obese mice at 14° or 25° responded

with less of a decrease in oxygen consumption than did their lean counterparts.

Effects of ouabain injection on distribution of Na^+ and K^+ in plasma, liver, kidney and skeletal muscle are presented in Figure 3. K^+ and Na^+ concentrations in plasma, liver, kidney and skeletal muscle of control mice were not significantly influenced by phenotype, although K^+ concentrations in liver and skeletal muscle of 4-week-old lean mice tended to be higher than those of their obese counterparts. Greater divergence in tissue K^+ at 8 weeks of age than at 4 weeks of age (Table 14 versus Figure 3) parallels differences in Na^+,K^+-ATPase at these two ages in lean and obese mice (98). Neither plasma K^+ nor kidney K^+ were altered by ouabain injection. Ouabain injection, however, tended to decrease K^+ concentration in liver and skeletal muscle of lean mice, but these changes were not significant. K^+ concentrations in liver and skeletal muscle of obese mice were not significantly influenced by ouabain injection. Injection of ouabain had no measurable effect on plasma or tissues Na^+ concentration of either lean or obese mice.

Ouabain content in selected tissues of mice exposed to various environmental temperatures is presented in Figure 4. Mice were killed 40 minutes after injection of ouabain; at this time oxygen consumption of the mice had plateaued. Ouabain content in heart, muscle, and liver increased

Figure 3. Na^+ and K^+ content of plasma, liver, kidney, and gastrocnemius muscle from 4-week-old lean and obese mice injected with saline (C), 0.3 ug ouabain/g body weight (A) or 0.9 ug ouabain/g body weight (B). Lean and obese mice weighed 15.8 ± 1.0 and 16.3 ± 0.7 g, respectively. Water content of liver, kidney and gastrocnemius muscle from lean and obese mice was not influenced by ouabain injection. Tissue water averaged 71.5 ± 1.0 in liver, 77.1 ± 0.7 in kidney, and 79.6 ± 1.5 ml/100 g tissue in muscle of lean mice; values averaged 69.3 ± 1.5 in liver, 76.0 ± 0.5 in kidney, and 77.6 ± 1.4 ml/100 g tissue in muscle of obese mice. Bars represent means ± SEM for 5 control mice maintained at 25° and 15 ouabain-treated mice. Five ouabain-treated mice were maintained at 33°, 25°, or 14°; since temperature did not alter tissue Na^+ or K^+, results were pooled.


lean
obese
plasma
Liver
Kidney
muscle
K+ content: meq/l tissue water
130
110
90
70
50
30
10
0
C A B C A B C A B C A B
treatment

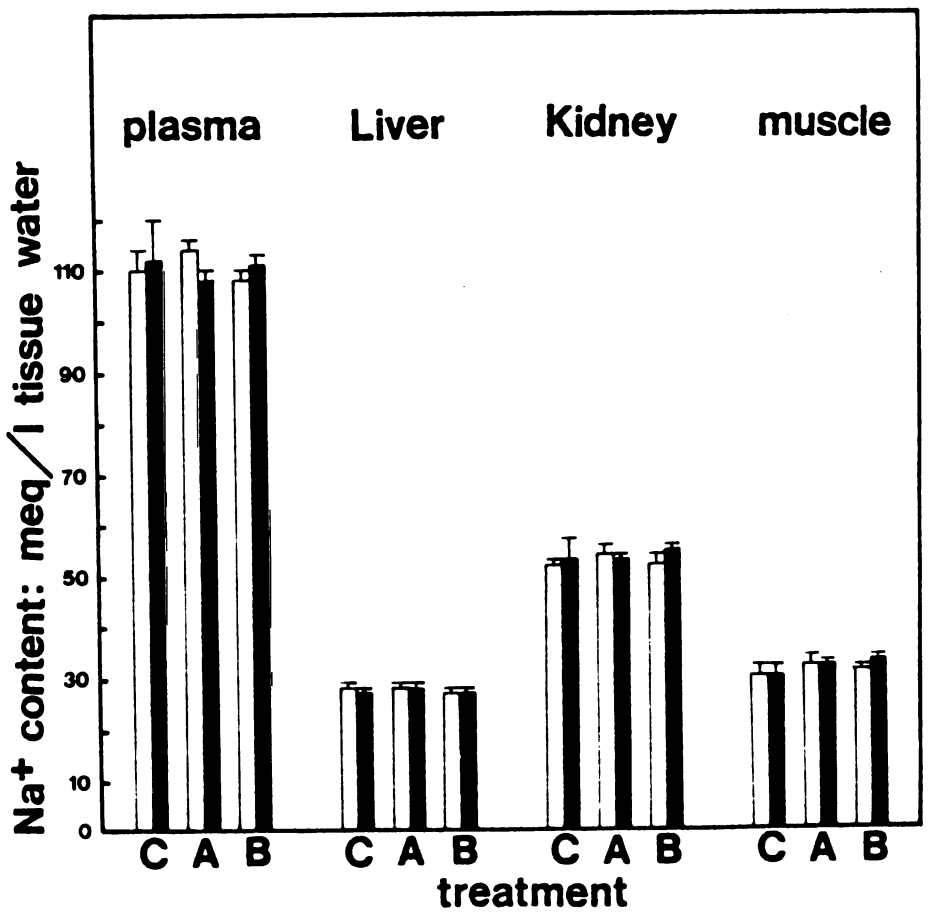
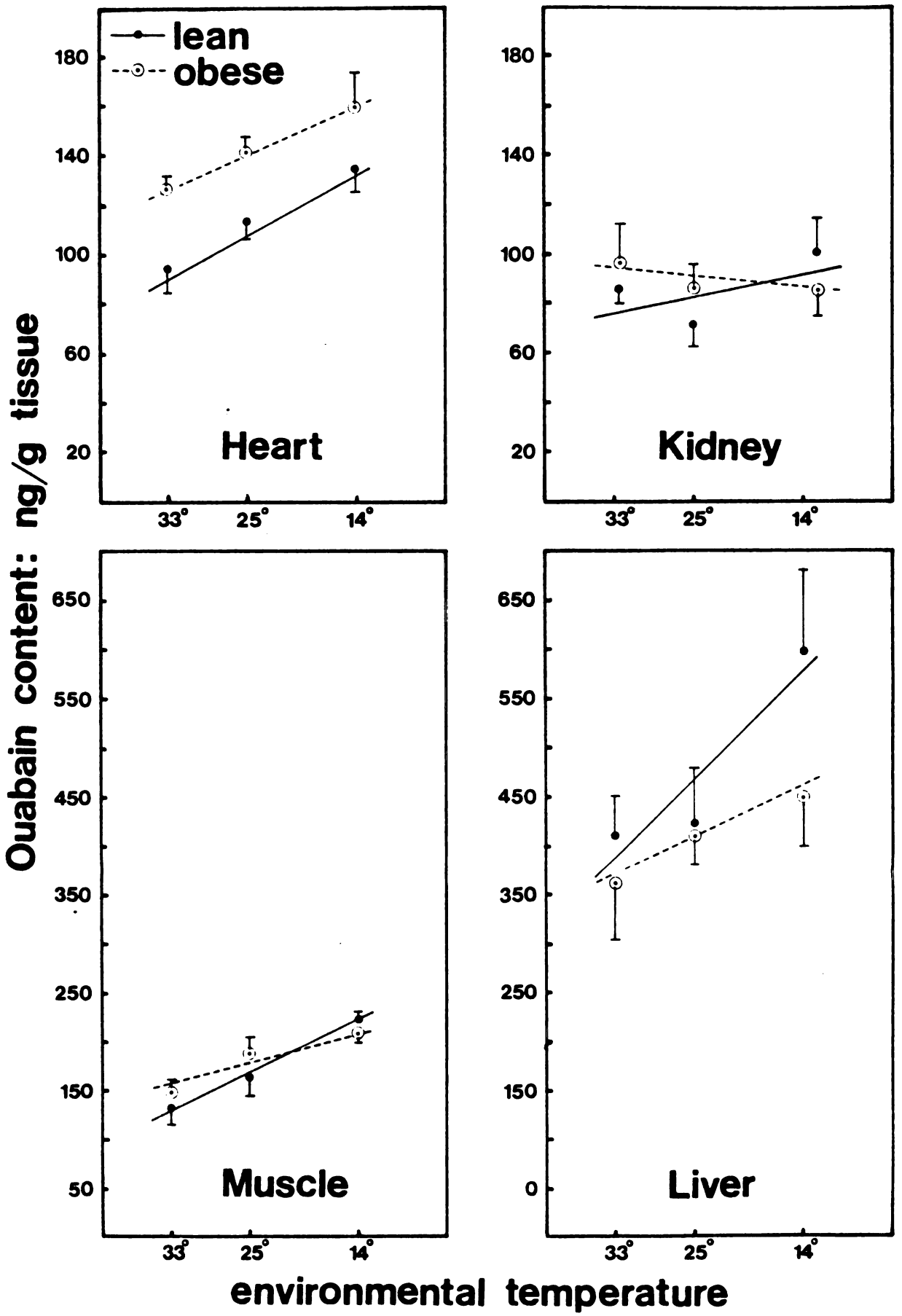

plasma
Liver
Kidney
muscle
Na+ content: meq/l tissue water
110
90
70
50
30
10
0
C A B C A B C A B C A B
treatment

Figure 4. Ouabain content of heart, kidney, muscle, and liver from 4-week-old lean and obese mice injected with (0.3 ug ouabain/g body weight; 2.8 uCi ^{14}C per ug ouabain). Data points represent mean values ± SEM for 6 mice. Lean and obese mice weighed 17.0 0.4 and 17.2 ± 0.7 g, respectively. Ouabain content of brain and plasma from lean and obese mice was not influenced by environmental temperature. Ouabain content averaged 4.5 ± 0.3 and 4.4 ± 0.4 ng/g brain of lean and obese mice, respectively; values averaged 27 ± 2 and 29 ± 2 ng/ml plasma of lean and obese mice, respectively.


lean
obese
Heart
Kidney
Muscle
Liver
Ouabain content: ng/g tissue
environmental temperature
180
140
100
60
20
650
550
450
350
250
150
50
0
33°
25°
14°

linearly ($P<0.05$) in both lean and obese mice as environmental temperature decreased. Rate of increase in ouabain content in muscle and liver tended to be greater in lean mice than in obese counterparts, but the differences were not significant. Ouabain content of kidneys was not altered by environmental temperature. Hearts of obese mice accumulated more ouabain than hearts of lean mice; similar responses were not apparent in other organs examined. The reason for greater accumulation of ouabain by hearts of obese mice is unclear; data on affinity of Na^+,K^+-ATPase from hearts of lean and obese mice for ouabain are not available. Ouabain content of brain was only one-seventh that observed in plamsa and was not altered by environmental temperature or phenotype (Figure 4 legend). Low ouabain uptake by brain was expected because of the low lipid solubility of ouabain.

DISCUSSION

Eight-week old obese mice have lower K^+ content in muscle and liver and higher Na^+ content in muscle than lean counterparts. Based on our calculations it appears that the intracellular ratio of Na^+ to K^+ in both livers and muscle is higher in obese mice than in their lean counterparts. These results parallel those of Ismail-Beigi and Edelman (116). They studied effects of thyroid status on electrolyte distribution in rats and observed that the

intracellular Na^+ to K^+ ratio in diaphragms and hearts of thyroidectomized rats was 24 to 51% higher than in rats administered triiodothyronine (116). While it has been argued that obese mice are not hypothyroid, a recent report suggests that obese mice exhibit reduced nuclear binding of triiodothyronine in liver and lungs (115). Consequently, circulating levels of thyroid hormones in obese mice, although generally thought to be normal (109, 117), may not be high enough to adequately maintain all thyroid-dependent systems.

An alternative explanation for the observed differences in tissue Na^+ and K^+ in obese mice is that intracellular ion concentration is not altered, but rather the ratio of intracellular to extracellular water is shifted in obese mice. To explain the observed alterations in tissue K^+ on this basis, it would be necessary for the ratio of intracellular to extracellular water to be 10% greater in obese mice than in lean mice. Livers and muscles of obese mice contain more fat than those of lean mice. This condition would not be expected to be associated with increased intracellular water. Additionally, total extracellular water in obese mice is slightly increased, but total body water is similar to values observed in lean mice (123). Thus, total body intracellular water should not be increased in obese mice. While limitations of methodology used to estimate intracellular water restrict us from negating

the possibility of a shift in intracellular to extracellular water in obese mice, it appears likely that an increase in the intracellular ratio of Na^+ to K^+ in liver and muscle explains a significant portion of the observed alterations in tissue Na^+ and K^+ in obese mice.

Regulation of intracellular Na^+ and K^+ results from a balance between active transport via the Na^+ pump and passive transport (120); K^+ is actively transported into cells to maintain high intracellular K^+ while Na^+ is transported to the outside to maintain low intracellular Na^+. We do not have direct data on either active or passive transport in liver or muscle of obese mice, but Na^+,K^+-ATPase enzyme units are reduced in these tissues of obese mice (98). Reductions are comparable to those observed in thyroidectomized rats (64). A reduction in number of enzyme units, however, does not necessarily imply less ion transport in vivo because the sodium pump likely possesses reserve capacity. An increase in intracellular Na^+ would normally be expected to increase activity of existing pump sites (124). Obese mice are insulin resistant. Since insulin affects the $K_{0.5}$ of Na^+,K^+-ATPase for Na^+ (125), it is possible that Na^+,K^+-ATPase in liver and muscle of obese mice is less responsive to elevated intracellular Na^+ than is the enzyme in lean mice. Supporting the hypothesis that lower Na^+,K^+-ATPase enzyme units may contribute to shifts in tissue ion concentration in obese mice is the

observation that neither Na^+ nor K^+ concentrations are altered in kidney of obese mice and Na^+,K^+-ATPase is not lowered in kidneys of obese mice either (98).

At room temperature (25^o) obese mice exhibited less ouabain-suppressible oxygen consumption than lean mice. Although other actions of ouabain cannot be excluded with certainty (126), the observed effects of ouabain on oxygen consumption may reasonably be attributed to inhibition of Na^+,K^+-ATPase activity (80, 119, 120). In agreement with this hypothesis are earlier findings that obese mice have lower concentrations of Na^+,K^+-ATPase in muscle and liver (98) and lower Na^+ transport-dependent respiration in diaphragm and liver slices (115) than lean mice.

Catecholamines turnover is accelerated during cold exposure (127). One effect of catecholamines is to stimulate Na^+,K^+-ATPase (108, 110). We, thus, examined ouabain-suppressible oxygen consumption in cold-exposed mice. As expected, lean mice exhibited more ouabain-suppressible oxygen consumption at 14^o than at 33^o. Obese mice, however, failed to alter their ouabain-suppressible oxygen consumption as environmental temperature decreased, although they were able to increase their total oxygen consumption somewhat. As a result, 50% of the cold-induced increase in oxygen consumption of lean mice (14^o versus 33^o and injected with 0.9 ug ouabain/g body weight) was ouabain-suppressed, whereas only 30% of the increase was

ouabain-suppressed in obese mice. Reasons for the blunted responsiveness of obese mice to acute cold have not been completely elucidated. Both thyroid hormones and catecholamines contribute to the induction and mediation of nonshivering thermogenesis, in part at least, through their involvement in the regulation of Na^+,K^+-ATPase. Injection of massive amounts of catecholamines elicits less of a thermogenic response in obese mice than in lean mice (75); however, obese mice injected with thyroid hormones exhibit increased survival during cold exposure (72). These results suggest that thyroid hormone status of obese mice may be less than optimal (24, 27), which would in turn reduce their responsiveness to B-adrenergic stimulation (128). The extent that B-adrenergic stimulation influences Na^+,K^+-APTase and other mechanisms of heat production (129) in obese mice remains to be established.

Ouabain uptake by hearts of the mice and total oxygen consumption increased linearly as environmental temperature decreased. These results agree with observations that ouabain uptake by the heart increases as myocardial blood flow and workload increases (130). A close correlation has also been related between the rate of Na^+,K^+ pumping and the rate of ouabain binding in isolated skeletal muscle and adipocyte preparations (100). Thus, it is possible that the increased ouabain content noted in skeletal muscle and liver of cold-exposed mice may be associated with

increased Na^+,K^+-ATPase activity prior to ouabain injection. In obese mice, however, increased ouabain content in muscle and liver was not associated with increased ouabain-suppressed oxygen consumption. Further studies are needed to examine the association of ouabain uptake by various tissues of lean and obese mice and ouabain-suppressed oxygen consumption. Since brown adipose tissue is an important site for thermogenesis in rodents (112, 113) and since blockage of Na^+,K^+-ATPase in catecholamine-stimulated brown adipocytes markedly depressed their rate of respiration (108, 110, 131), it will be important to evaluate ouabain uptake by brown adipose tissue of lean and obese mice.

In summary, results presented support and extend earlier observations (70, 98, 115), that obese mice have alterations in Na^+,K^+-ATPase. Present data demonstrated that the intracellular ratio of Na^+ and K^+ may be increased in liver and muscle, but not in kidneys of obese mice. Ouabain decreased oxygen consumption in both lean and obese mice, but the decreases were significantly less in obese mice at 25^o or 14^o than in lean counterparts. The extent that alterations in Na^+,K^+-ATPase contributes, directly or indirectly (114), to increased energy retention in obese mice is not yet known.

GENERAL CONCLUSIONS

In the present study, a 28% reduction in Na^+,K^+-ATPase enzyme units in skeletal muscle of genetically obese (ob/ob) mice is observed as early as 14 days of age. This represents one of the earliest reported developmental differences in an enzyme system between lean and obese mice. Later in development the difference in number of Na^+,K^+-ATPase units (per mg skeletal muscle protein) between lean and obese mice is even greater. The enzyme units are 25% to 36% lower in 4 and 8 week old obese mice than in their lean littermates. But the enzyme is not changed in the livers of 2 week old obese mice until later in development. The kidney enzyme, however, appears less responsive to phenotype than does the enzyme in the other two organs.

During the past 15 years many reports documenting tissue resistance to the full expression of insulin action on carbohydrate metabolism in obesity have appeared. In obese mice all three major insulin-sensitive target organs - liver, muscle and adipose tissue (19) have been found to exhibit this phenomenon. Recently, it has been reported that insulin resistance in muscle appears much earlier in development of obesity than in liver or adipose tissue (19). Insulin has also been shown to increase K^+ uptake and Na^+,K^+-ATPase in muscle preparations (100). While it is strictly speculative, it will be interesting to study the possibility that the development of insulin resistance and the lower number of Na^+,K^+-ATPase enzyme

units in skeletal muscle of very young obese mice are interrelated.

The reason for the inconsistent finding in obese kidney is not known; that is Na^+,K^+-ATPase was lower in liver and muscle of obese mice, but not in kidney. Glucocorticoids have been shown to increase Na^+,K^+-ATPase activity in the kidney (132). Obese mice have elevated circulating levels of corticosterone which may have contributed to the maintenance of the Na^+,K^+-ATPase in the kidneys of the obese mice. Na^+,K^+-ATPase in the kidney appears to be correlated with sodium excretion. The higher food intake and consequently higher sodium intake in the obese mice, particularly at 8 weeks of age, may also have helped maintain the enzyme at a level comparable to that observed in lean mice. Based on data from the present study, phenotype did not appear to alter the number of Na^+,K^+-ATPase enzyme units in the kidneys. However, it is not possible from these data to determine the in vivo catalytic activity of the enzyme. Whether the kidneys contribute to reduced heat production in obese mice via alterations in Na^+,K^+-ATPase is yet to be further investigated.

Since ob/ob mice have been suggested to be hypothyroid, the response of Na^+,K^+-ATPase in these mice to thyroid treatment was also investigated. The number of Na^+,K^+-ATPase enzyme units in each of the three organs of obese mice

increased to a much greater extent after thyroid hormone injection than did the enzyme in these tissues of lean mice. These results agree with the lower response of Na^+,K^+-ATPase to thyroid hormone administration in euthyroid rats than in hypothyroid rats (67) and suggest that obese mice are functionally hypothyroid.

In light of our surprising finding that tissue Na^+,K^+-ATPase activity from young ob/ob mice exhibit greater responsiveness to thyroxine, it is tempting to speculate how this might relate to the etiology of the obese, hyperglycemic syndrome. While the lower Na^+,K^+-ATPase enzyme units in obese mice can be overcome by thyroid treatment, it appeared of interest to see whether in other conditions in which obese mice would be able to alter Na^+,K^+-ATPase enzyme units. Exposure to cold may provide such condition since the thyroid gland activity may be increased during the development of the cold-acclimated state (133). In fact, it has been reported that active sodium transport contributes significantly to the increased calorigenesis manifested by tissues from cold-acclimated mice (102) and rats (65, 103). From present study, it is clear that young obese mice can survive a moderate, chronic cold exposure and that Na^+,K^+-ATPase in obese mice responds to chronic cold-exposure to approximately the same extent observed in lean mice and that changes in Na^+,K^+-ATPase parallel the increased heat production in the cold-exposed

mice. The increases in Na^+,K^+-ATPase units in the tissues of cold-exposed mice parallel those obtained in mice given thyroid hormones (98). It is therefore most interesting that Reichlin and coworkers (1973) have reported that in cold-acclimated animals, plasma levels of triiodothyronine increase up to 80% (134). Although it is known that hypothyroid animals are not able to acclimate to cold (135), there has been much controversy as to whether thyroid hormones exert more than a permissive role in nonshivering thermogenesis in acclimation to cold (133). Another hormone-catacholamones has also been shown to increase in cold-acclimated animals (126). Furthermore, the calorigenic effects of catacholamine on brown adipose tissue appear to be mediated by an increased activity of the sodium pump. Thus, the increased Na^+,K^+-ATPase units in cold-exposed mice may be produced by the interdependent effects of thyroid hormones and of catacholamines.

The last studies were designed to provide information on functional correlates of Na^+,K^+-ATPase in ob/ob mice. Obese mice have lower K^+ content in muscle and liver and higher Na^+ content in muscle than lean counterparts. Calculated intracellular Na^+/K^+ ratios were 56% higher in livers of obese mice and 116% higher in muscle of obese mice than those observed in lean mice. These results parallel those of Ismail-Beigi and Edelman (115). They studied effects of thyroid status on electrolyte

distribution in rats and observed that the intracellular Na^+ to K^+ ratio in diaphragms and hearts of thyroidectomized rats was 24 to 51% higher than in rats administered triiodothyronine (115).

Ouabain inhibits Na^+,K^+-ATPase (80, 118, 119). Ouabain does not appear to inhibit other systems at least directly (120), and therefore a decrease in oxygen consumption induced by ouabain is likely to indicate that Na^+,K^+-ATPase activity support a substantial fraction of the respiration under normal conditions. In the present study, oxygen consumption was measured in mice maintained at 14^o, 25^o or 33^o for 40 minutes and injected with 0.3 ug or 0.9 ug ouabain per g body weight. Ouabain decreased oxygen consumption less in obese mice (12 to 25%) than in lean mice (19 to 38%). These results suggest that Na^+ pump activity might be reduced in obese mice and this, in turn, may contribute to reduced cellular thermogenesis in these animals.

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