MEASUREMENT OF THE EFFECT OF TRIIODOTHYRONINE ON Na^+, K^+-ATPASE IN GENETICALLY OBESE ZUCKER RATS USING THE (^3H)-OUABAIN BINDING METHOD

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MEASUREMENT OF THE EFFECT OF TRIIODOTHYRONINE ON Na^+,K^+-ATPase IN GENETICALLY OBESE ZUCKER RATS USING THE [^{3}H]-OUABAIN BINDING METHOD

An Abstract

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by

Teresa J. Webb

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ABSTRACT

The Zucker "fatty" rat is genetically obese. One of the proposed mechanisms for the obesity is a deficit or defect in Na^+,K^+-ATPase (sodium, potassium pump) function. This study was designed to compare basal levels of Na^+,K^+-ATPase in lean and obese Zucker rats and to determine whether the amount of enzyme changes in response to L-3,5,3'-triiodothyronine (T_3) in the same manner in the obese rat as in the lean Zucker rat. Two methods were developed and tested. One method quantitates Na^+,K^+-ATPase activity by measuring inorganic phosphorus released during the hydrolysis of ATP by the enzyme; the other method measures the amount of [^{3}H]-ouabain specifically bound to Na^+,K^+-ATPase in the presence of vanadate. The [^{3}H]-ouabain method was found to be more sensitive, and was used to study the Zucker rats.

Soleus and extensor digitorum longus (edl) muscle samples (5-10 mg) from lean and obese T_3-treated and untreated Zucker rats were incubated for 2 X 60 min at 37°C in vanadate buffer containing 1 μCi/ml [^{3}H]-ouabain. The samples were washed in unlabeled vanadate buffer for 4 X 30 min and then soaked in 0.3 M trichloroacetic acid overnight. Tritium activity was measured in a liquid scintillation counter and calculations were performed to determine pmol ouabain bound/g wet weight. Serum samples from the rats were assayed to determine T_3 and thyroxine (T_4) concentrations. Serum T_3 levels were elevated and T_4 levels depressed in both the lean and obese T_3-treated rats. Both the obese and lean treated rats showed significant increases over basal for [^{3}H]-ouabain binding to soleus but not to edl. These results are in agreement with a previous study on the sodium, potassium pump in liver tissue of obese Zucker rats and do not support a relationship between the enzyme and the obesity of the Zucker rat.

MEASUREMENT OF THE EFFECT OF TRIIODOTHYRONINE ON Na^+,K^+-ATPase OF GENETICALLY OBESE ZUCKER RATS USING THE [^{3}H]-OUABAIN BINDING METHOD

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September 1993

To the Graduate and Research Council:

I am submitting herewith a Thesis written by Teresa J. Webb entitled "Measurement of the Effect of Triiodothyronine on Na^+,K^+-ATPase in Genetically Obese Zucker Rats Using the [^{3}H]-Ouabain Binding Method." I have examined the final copy of this paper for form and content, and I recommend that it be accepted in partial fulfillment of the requirements for the degree Master of Science with a major in Biology.

Ruth A Young
Major Professor

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CHAPTER 1

INTRODUCTION

The Zucker "fatty" Rat

The Zucker "fatty" rat is genetically obese. The obesity of the animal is due to a single recessive gene (*fa*) (Zucker and Zucker 1961) located on chromosome five (Truett et al. 1991). The Zucker "fatty" rat was discovered in a laboratory in Stow, Massachusetts in 1961 by Lois and Theodore Zucker (Zucker and Zucker 1961). The mutation appeared spontaneously in a cross between the Merck Stock M and Sherman rats (Bray 1977).

The obese Zucker rat has many aberrant physiological processes. Some characteristics of these animals are hypothyroidism (Bray and York 1971), hyperinsulinemia (Martin et al. 1978), and hyperphagia (Bray and York 1972). Additionally, Zucker "fatty" rats have impaired reproductive function with males almost always being sterile (Zucker and Zucker 1961, Young et al. 1982b).

The obese animals are easily distinguishable from their lean littermates by five weeks of age (Zucker and Zucker 1961). The Zucker "fatty" rat has a greater body fat content than its lean littermate and an altered body shape. The obese rats have an increased number of adipocytes that are larger than normal (Bray 1977). Zucker "fatty" rats also have elevated plasma triglycerides that cannot be attributed to overeating alone (Barry and Bray 1969).

Young obese Zucker rats eat 50% more and drink more than their lean littermates or hypothalamic obese rats (Bray and York 1972) and their body composition is not normalized by food restriction (Bray et al. 1973, Young et al. 1985). The extra energy

taken in is stored as fat (Bray 1977). The rats have an increased efficiency of food utilization (Zucker 1975, Deb et al. 1976, Young et al. 1980) and continually accumulate fat throughout life (Bray 1977). This efficiency is partially due to the fact that the Zucker "fatty" rat directs nutrients toward adipocytes and away from muscle tissue (Young et al. 1980, Argiles 1989).

Early studies showed that Zucker "fatty" rats have lower oxygen consumption than their lean littermates (Milam et al. 1982) as well as impaired uptake and release of ^{131}I (Bray and York 1971). The early studies also showed that the Zucker "fatty" rat may have an impaired ability to form or distribute thyrotropin-releasing hormone (TRH) or the thyroid of the animal may have an impaired response to circulating thyrotropin (TSH) (York et al. 1971, Martin et al. 1978, Goldberg et al. 1988). When fed a low protein, high carbohydrate diet, obese Zucker rats, unlike their lean littermates, lack the ability to eliminate excess energy through increased thermogenesis (Young et al. 1980). The obese rats in the 1980 study by Young et al. showed no increase in oxygen consumption or circulating T_3 levels when fed the low protein diet. In 1982, Young et al. found that the change in T_3 levels in the lean rats was not related to changes in thyroid function but to an increase in serum thyronine-binding proteins. Young et al. (1982b) suggested that the increased oxygen consumption in the lean rats may be due to an increased tissue sensitivity to catecholamines or a stimulation of the sympathetic nervous system.

Although much is known about the obesity of the Zucker "fatty" rat, the underlying cause of the obesity has yet to be determined (Argiles 1989, Johnson et al. 1991). The hypothyroidism of the animal, however, coupled with its low metabolic rate, has led to

the question of decreased T_3-stimulated Na^+,K^+-ATPase activity as one cause.

Na^+,K^+-ATPase

The sodium, potassium pump (Na^+,K^+-ATPase) is a plasma membrane-bound protein complex that transports K^+ into and Na^+ out of animal cells. This process is coupled to the hydrolysis of ATP. The sodium, potassium pump is composed of the two subunits, α and β. The α-subunit is the larger of the two and contains the sites for ATP binding and phosphorylation. The function of the smaller β-subunit is still unknown (Gick et al. 1988b), but probably has some regulatory function (Horowitz et al. 1990). The Na^+ and K^+ gradients formed by the activity of the pump are used to perform a variety of functions, including the transport of amino acids, glucose and Cl^- as well as the generation and conduction of nerve and muscle action potentials (Gick et al. 1988a).

Thyroid hormones increase oxygen consumption in many mammalian tissues (McDonough et al. 1988). Previous studies have shown that L-3, 5, 3'-triiodothyronine (T_3) increases Na^+,K^+-ATPase activity by increasing synthesis of the enzyme in certain tissues such as kidney, skeletal muscle, and liver (Lin and Akera 1978). McDonough et al. (1988) measured mRNA concentrations in kidney tissue and found that T_3 causes the increase in the enzyme by coordinately increasing the mRNAs of both the α- and β-subunits of the enzyme. Horowitz et al. (1990) found a decrease in the α subunit of the enzyme in the skeletal muscle of hypothyroid rats. When T_3 was administered to the rats, synthesis of the enzyme increased, leading to increased enzyme activity.

The activity of the sodium, potassium pump is thought to be a major determinant of basal metabolic rate. Up to 20-45% of basal oxygen consumption in mammalian tissues

has been attributed to the sodium, potassium pump (Edelman and Ismail-Beigi 1970). The large amount of oxygen consumption attributed to the sodium, potassium pump introduces the possibility that some types of obesity may be related to decreased sodium, potassium pump activity. The only publication describing measurement of the activity of the sodium, potassium pump in obese Zucker rats, a very brief report comparing Na^+,K^+-ATPase activity in liver homogenates from hypothyroid obese and lean T_3-treated Zucker rats, reported no differences in the level of enzyme in the obese and lean T_3-treated Zucker rats (Bray et al. 1978). Untreated obese and lean rats were not studied.

Decreased numbers of Na^+,K^+-ATPase pump units have been found, however, in skeletal muscle, kidney, and liver tissues of the genetically obese *ob/ob* mouse (Lin et al. 1979). The *ob/ob* mouse is not hypothyroid but has an impaired peripheral response to thyroid hormones (Guernsey and Morishige 1979).

Methods in the Literature

Several methods have been used to measure the activity of Na^+,K^+-ATPase. One method measures the hydrolysis of ATP to ADP and inorganic phosphate (P_i) by Na^+,K^+-ATPase in the presence and absence of the specific inhibitor ouabain. Ouabain (g-strophanthin) is a cardiac glycoside commonly used because of its water solubility and its specificity for the sodium, potassium pump, which is indicated by ouabain's lack of effect on other membrane nucleotide di- and triphosphatases (Hootman and Ernst 1988). Ouabain binds to the phosphorylated intermediate form of Na^+,K^+-ATPase, which is known as the E_2P configuration (Hootman and Ernst 1988). The binding of ouabain to

the Na^+,K^+-ATPase enzyme disrupts the normal interactions between subunit sites (Askari et al. 1988). The activity of the enzyme can be determined by subtracting the inorganic phosphate yield from the hydrolysis of ATP in the absence of ouabain from the inorganic phosphate yield from the hydrolysis of ATP in the presence of ouabain.

Several investigators, including Bray et al. (1978), have measured Na^+,K^+-ATPase activity in whole tissue homogenates. Since the enzyme is a complex of integral plasma membrane proteins, it makes more sense to measure the enzyme activity in isolated plasma membranes. Also, since data are reported as μmoles inorganic phosphorous (P_i) released/hr/mg protein, using membranes decreases the measurement of protein that is not part of the enzyme complex. The best aspect of this method is that it measures actual enzyme activity as quantitated by P_i release, not merely the presence of Na^+,K^+-ATPase pump units.

Another method of measuring the enzyme is the [^{3}H]-ouabain binding site method. In this method the number of working enzyme units is determined by incubating tissue samples with [^{3}H]-ouabain in the presence of vanadate and then counting the radioactivity of the samples (Clausen et al. 1987). Vanadate facilitates ouabain binding to Na^+,K^+-ATPase by interacting with the phosphate site on the enzyme, allowing binding of cardiac glycosides to the enzyme in the absence of ATP (Hansen 1979).

In this study both of the methods described above were developed and used to measure basal and T_3-stimulated Na^+,K^+-ATPase in rats.

CHAPTER 2

MATERIALS AND METHODS

Development of the Na^+,K^+-ATPase Activity Method

This technique was adapted from a method developed by Esmann (1988). It is based on the ouabain-sensitive hydrolysis of ATP in the presence of Na^+, K^+, and Mg^{2+}. Kidneys were used because plasma membranes could be isolated using available equipment and kidneys have a high concentration of Na^+,K^+-ATPase (Jorgensen 1974).

Preparation of Plasma Membranes. The rats were killed by decapitation and the kidneys immediately removed and placed in an ice-cold solution containing 0.24 M sucrose-30 mM histidine (sucrose-HIS). The kidneys were minced and homogenized in 10 volumes of sucrose-HIS using a Polytron (Virtis Research Equipment, Gardiner, New York).

The homogenates were centrifuged in an International Equipment Company B-20 refrigerated centrifuge (IEC, Needham Heights, Massachusetts) for 15 minutes at 6000 x g. The fat layer was removed and the supernatants were transferred to clean tubes and centrifuged for another 15 minutes at 6000 x g. The 6000 x g supernatants were transferred to ultracentrifuge tubes and placed on ice. The supernatants were centrifuged in a refrigerated Beckman L5-65 ultracentrifuge (Beckman Instruments Inc., Fullerton, California) for 30 minutes at 60,000 x g. The pellets were resuspended in the original volume of sucrose-HIS and centrifuged an additional 30 minutes at 60,000 x g. The final pellets were resuspended in one-half the original volume of sucrose-HIS and kept on ice.

Protein Assay. An estimate of the protein concentration of each of the membrane suspensions was made using the Sigma Protein Kit P5656 (Sigma Chemical Co., St. Louis, Missouri). If samples had a protein concentration greater than 4 mg/ml of suspension, they were diluted to 4 mg/ml with sucrose-HIS.

Preincubation with Deoxycholic Acid (DOC). One ml of each suspension was incubated at room temperature for 30 minutes with 1.5 mg of DOC to prevent the membranes from forming vesicles. Vesicle formation decreases the Na^+,K^+-ATPase activity that can be detected because only one side of the membrane is accessible (Jorgensen 1974). The optimal amount of DOC was determined by preincubation of representative one ml samples of membrane suspension with 0.5, 1.0, 1.5, 2.0, or 2.5 mg of DOC. The greatest Na^+,K^+-ATPase activity was observed using 1.5 mg, so a DOC concentration of 1.5 mg/ml of isolated membrane suspension was used in later experiments.

Incubation. The incubation buffer was prepared as described by Esmann (1988). The final concentrations in the incubation were NaCl - 130 mM, KCl - 20 mM, $MgCl_2$ - 4 mM, NaN_3 - 5 mM, ATP - 3 mM, histidine - 30 mM, EGTA - 0.2 mM. To 800 μl of incubation buffer was added 100 μl of either ouabain (final concentration 1 mM) or water.

The tubes were placed in a 37°C water bath for a two minute preincubation. After two minutes, 100 μl of diluted membrane suspension was added and the solution mixed. The blanks were immediately mixed with 250 μl of 20% ice-cold trichloroacetic acid (TCA) and placed on ice to stop the reaction. The other tubes were returned to the bath

for a five minute incubation. At the end of the five minutes, 250 μl of ice cold 20% TCA was added to each tube, and the tubes were mixed and placed on ice. All the tubes were centrifuged for 20 minutes at 3000 rpm at 4°C in a Servall GLC-1 centrifuge (Ivan Sorvall Inc., Norwalk, Connecticut).

Assay of Inorganic Phosphorus. Inorganic phosphorus (P_i) in the supernatants from the incubations was measured using the Sigma Inorganic Phosphorus Kit 670 (Sigma Chemical Co., St. Louis, Missouri).

Calculations. The amount of inorganic phosphorus generated by the Na^+,K^+-ATPase enzyme during the incubation was calculated as follows. The P_i concentrations of the blanks were subtracted from those of the incubation tubes to correct for the ATP that nonspecifically degraded in solution. The P_i concentrations of the tubes containing ouabain were then subtracted from those of the tubes without ouabain. As ouabain is a specific inhibitor of the sodium, potassium pump, the resulting value represented P_i generated by the sodium, potassium pump. The μmol P_i was multiplied by 12 (to calculate the P_i released per hour) and divided by the protein concentration to yield μmol P_i/hr/mg protein.

Test of the Method. Two experiments with Sprague-Dawley rats were completed to test the method. First, male Sprague-Dawley rats (Charles Rivers Breeding Laboratory, Wilmington, Massachusetts) weighing approximately 220 g were injected subcutaneously with either thyroxine (T_4) (15 μg/ 100 g of body weight) or vehicle (5 mM NaOH). One group was injected with T_4 24 h before sacrifice, the other 48 h and 24 h before sacrifice. Statistical analysis by the Student-Newman-Keuls test determined there were

no significant differences ($p>0.05$) in sodium, potassium pump activity among the control and T_4-treated groups (Table 1).

In the second experiment, male Sprague-Dawley rats weighing approximately 350 g were injected intraperitoneally with either triiodothyronine (T_3) (100 μg/ 100 g of body weight) or vehicle (0.9% saline). One group was injected with T_3 24 h before sacrifice, the other 48 h and 24 h before sacrifice. No increase in the Na^+,K^+-ATPase activity in kidney tissue from the T_3-treated rats was found (Table 2). The P_i release method was abandoned for the planned studies of Zucker rats because it did not to show the expected increase in Na^+,K^+-ATPase activity in response to thyroid hormones. This increase in response to thyroid hormone treatment in rats has been reported by others including Ismail-Beigi and Edelman (1971), Lin and Akera (1978), Lin et al. (1979), McDonough et al. (1988), using a variety of methods.

There are several possible reasons why the P_i release method was not successful in this study. First, the membrane isolation procedure was such an involved process, it is possible that the individual membrane preparations were not comparable. It was expected that this problem would be even more severe when membrane isolates from obese and lean Zucker rats were studied.

Second, there were technical problems with the protein assay. The protein assay solutions became cloudy in the test tubes before the assay time was up and could not be read accurately in the spectrophotometer. Without an accurate protein determination, individual P_i values could not be compared.

Lastly, the lengthy and complex method required 16 hours per experiment and

Table 1. Na^+,K^+-ATPase Activity in Kidney Tissue of T_4-treated Sprague-Dawley Rats (n=3).

Group	Enzyme activity (μmol P_i/h/mg protein)[b]
Control	18.7 ± 1.2
T_4-Treated	
24 h[a]	19.6 ± 1.3[c]
48 h[a]	21.8 ± 2.0[c]

[a] 15 μg /100 g body weight 24 h before sacrifice or 48 and 24 h before sacrifice

[b] ± S.E.M.

[c] Not significantly different from control, $p > 0.05$

Table 2. Na^+,K^+-ATPase Activity in Kidney Tissue of T_3- treated Sprague-Dawley Rats (n=3).

Group	Enzyme activity (μmol P_i/h/mg protein)[b]
Control	14.0 ± 1.5
T_3-Treated	
24 h[a]	16.4 ± 1.6[c]
48 h[a]	14.4 ± 1.5[c]

[a] 100 μg /100 g body weight 24 h before sacrifice or 48 and 24 h before sacrifice

[b] ± S.E.M.

[c] Not significantly different from control, $p > 0.05$

involved a trip out-of-state to use an ultra-centrifuge. The long hours of concentrated effort increased the chances for human error.

Development of [^{3}H]-Ouabain Binding Method

This technique was adapted from Norgaard et al. (1983 and 1984) and Kjeldsen et al. (1986). Its basis is the specific binding of [^{3}H]-ouabain to Na^+,K^+-ATPase in the presence of vanadate. Skeletal muscle was used because the method was designed for use with skeletal muscle. Skeletal muscle accounts for up to 6% of the basal metabolic rate (Clausen and Hansen 1982).

Tissue Collection. The rats were sacrificed by decapitation. The soleus and extensor digitorum longus (edl) muscles were removed from both hindlimbs, and pieces of muscle with wet weights of 5-10 mg were used in the assay.

[^{3}H]-Ouabain Binding. [^{3}H]-ouabain was purchased from DuPont New England Nuclear Research Products (Boston, Massachusetts). It had a specific activity of 29.0 Ci/mmol and a concentration of 1.0 mCi/ml. All washes and incubations were carried out in 2 ml buffer per sample per incubation. The buffer contained 1 mM vanadate, 3 mM $MgSO_4$, 10 mM Tris HCl, and 250 mM sucrose, pH 7.3 (Norgaard et al. 1984).

The muscle samples were placed in nylon mesh bags (Nitex, 363 μm mesh size) approximately 1 cm X 1.5 cm. The samples were washed in a Tris-vanadate buffer for 2 X 10 min at 4°C to reduce the concentration of Na^+ and K^+ to levels that would not interfere with the binding of [^{3}H]-ouabain (Norgaard et al. 1984).

The samples were then incubated for 2 X 60 min at 37°C in vanadate buffer containing 1 μCi/ml [^{3}H]-ouabain and unlabeled ouabain added to a final total ouabain

concentration of 1×10^{-6} M (Kjeldsen et al. 1986). Nonspecific binding (NSB) samples were incubated in the same solution but with unlabeled ouabain added to a final total ouabain concentration of 1×10^{-3} M. Following the incubation, the samples were washed in unlabeled vanadate buffer 4 X 30 min at 4°C. The samples were placed in scintillation vials containing 0.5 ml of 0.3 M trichloroacetic acid and soaked overnight (Kjeldsen 1986). Scintillation mixture (Budget Solve, Research Products International, Mount Prospect, Illinois) was added to the vials in 15 ml aliquots and the ^3H activity was measured in a Packard Tri-Carb Model 1600CA Liquid Scintillation Analyzer (Packard Instrument Co., Downers Grove, Illinois).

Calculations. The counting efficiency of the machine was determined by counting 5 μl stock [^3H]-ouabain. The calculated efficiency (50.4%) was then used to correct the cpm (counts per minute) of the samples to dpm (disintergrations per minute). Two 10-minute counts for each sample were used to obtain an average from which the background was subtracted. The dpm value was divided by the muscle wet weight in g to ascertain dpm/g. The dpm/g of the NSB samples were subtracted, and the dpm/g was converted to pmole/g. The final figure, expressed as pmol ouabain bound/g wet weight, was determined by correcting the pmole/g tissue value for the percent [^3H]-ouabain in solution.

Test of the Method. As a test of the method, female Sprague-Dawley rats (Charles River Breeding Laboratory, Wilmington, Massachusetts) weighing approximately 275 g were used. Female rats were acquired because the Zucker rats also would be female. The rats were injected intraperitoneally with either T_3 (25 μg/ 100 g body wt) or vehicle

(0.9% saline) at 12 h intervals for 36 h (four injections). Twelve hours after the last injection the rats were killed by decapitation and the [^{3}H]-ouabain binding to soleus and edl muscles was measured. The values in this experiment were not corrected for nonspecific binding.

A significant increase in [^{3}H]-ouabain binding to the soleus muscle in the T_3-treated rats was found (Table 3). There was no apparent increase in binding to edl muscle in T_3-treated rats.

Zucker Rat Experiment

The basal and T_3-stimulated [^{3}H]-ouabain binding to skeletal muscle in lean and obese Zucker rats was studied. Female lean (approximately 200 g) and obese (approximately 350 g) Zucker rats 11 weeks of age obtained from Dr. Roy Martin (University of Georgia, Athens, Georgia) were injected intraperitoneally with either T_3 (25 μg/100 g body wt) or vehicle (0.9% NaCl) at 12 h intervals for 36 h (4 injections). The binding of [^{3}H]-ouabain to soleus and edl muscles was measured and corrected for nonspecific binding. Sera from the rats were sent to Dr. Lewis Braverman, University of Massachusetts Medical Center, Worcester, MA, for T_3 and T_4 assay.

Statistical Analysis. Analysis of variance was used to determine statistical significance (CSS Statistica, Statsoft, Tulsa, Oklahoma).

Table 3. [^{3}H]-ouabain Binding to Soleus and Extensor Digitorum Longus (edl) Muscle in T_3-treated Sprague-Dawley Rats (n=5).

Group	pmol/g wet wt[b]	
	soleus	edl
Control	121 ± 19	124 ± 16
T_3-treated[a]	188 ± 14[c]	115 ± 28[d]

[a] 25 μg/100 g body weight at 12 h intervals for 48 h, with the last injection 12 h before sacrifice

[b] ± S.E.M.

[c] Significantly different from control, $p < 0.05$

[d] Not significantly different from control, $p > 0.05$

CHAPTER 3

RESULTS

Analysis of the sera showed no differences in T_3 and T_4 concentrations between the obese and lean control groups, but it did show that the method of injection had been successful (Table 4). Serum T_3 concentrations were elevated in the T_3-injected rats. Serum T_4 concentrations decreased in the same animals, probably because of negative feedback. There were no significant differences ($p>0.05$) in either basal or T_3-stimulated [^{3}H]-ouabain binding (pmol/g tissue) to soleus or edl muscles between the lean and obese rats. Both the obese and the lean rats showed significant increases over basal for binding to soleus ($p=0.0007$), but not to edl ($p>0.05$), when the rats were treated with T_3 (Table 5).

Table 4. Body Weight and Serum T_3 and T_4 Concentrations of Control and T_3-treated Zucker Rats[b] (n=4).

Group	Weight	T_3 (ng /dl)[a]	T_4 (μg /dl)[a]
Control Lean	195 ± 5	171 ± 13	5.07 ± 0.59
Control Obese	347 ± 13	142 ± 31[c]	4.15 ± 0.23[c]
T_3-treated Lean	197 ± 7	> 1000	1.34 ± 0.04[d]
T_3-treated Obese	349 ± 10	> 1000	2.65 ± 0.25[d]

[a] 25 μg/100 g body weight at 12 h intervals for 48 h, with the last injection 12 h before sacrifice

[b] ± S.E.M.

[c] Not significantly different from control, $p > 0.05$

[d] T_3-treated lean and obese are both different from control lean and obese, $p < 0.05$; T_3-treated obese and lean are significantly different, $p < 0.05$

Table 5. [^{3}H]-ouabain Binding to Soleus and Extensor Digitorum Longus (edl) Muscle in T_3-treated Zucker Rats.

Group	pmol/g wet wt[b]	
	soleus	edl
Control Lean	144 ± 14	152 ± 22
Control Obese	128 ± 16	176 ± 36
T_3-treated Lean[a]	203 ± 3[c]	132 ± 12[d]
T_3-treated Obese[a]	197 ± 23[c]	200 ± 14[d]

[a] 25 μg/100 g body weight at 12 h intervals for 48 h, with the last injection 12 h before sacrifice

[b] ± S.E.M.

[c] Significant difference from control, $p < 0.05$

[d] Not significantly different from control, $p > 0.05$

CHAPTER 4

DISCUSSION

Discussion of the Data

The thyroid aids regulation of metabolic rate; therefore, impaired thyroid function can affect metabolism. Since T_3 increases Na^+,K^+-ATPase activity, a major metabolic determinant, the question of the role of T_3 in the regulation of the sodium, potassium pump in genetically obese animals arose. This study was to determine whether the sodium, potassium pump in the obese Zucker rat has the same basal level as in the lean rat and whether the amount of enzyme detected changes in response to T_3 in the same manner in the obese rat as in the lean Zucker rat.

There were no differences in T_3 and T_4 serum concentrations between control lean and control obese Zucker rats (Table 4). Findings of no significant difference in circulating levels of T_3 in female lean and obese controls are in accord with reports in the literature. Young et al. (1980) and Autissier (1980) reported no significant differences in circulating T_3 in the serum of obese female Zucker rats but did report lower serum concentrations of T_4 in the obese female rats. Young et al. (1980) found a decrease in circulating levels of both T_3 and T_4 in male obese Zucker rats; Autissier (1980) used only female rats, as did this study. The lack of a difference in T_4 concentrations between control lean and obese rats in this study is probably due to two factors. First, the study groups may have been too small (n=4) to detect a statistically significant difference. Second, the rats used in this study were females and the difference is more pronounced in males (Young et al. 1980).

T_3 injections caused an increase in serum T_3 concentrations in both lean and obese Zucker rats to levels that were beyond the range of the assay (10-1000 ng/dl). Serum T_4 concentrations decreased in both treated groups due to negative feedback. The major hormone secreted by the thyroid gland is T_4. When levels of T_3 (the active form of the hormone) are low, T_4 is converted to T_3 by the enzyme 5'-deiodinase in a process known as deiodination. When thyroid hormone levels are low, thyrotropin releasing hormone (TRH) is secreted by the hypothalamus, stimulating the secretion of thyroid stimulating hormone (TSH) by the adenohypophysis. TSH stimulates the thyroid to secrete thyroid hormones (mainly T_4). High thyroid hormone concentrations in the blood block the secretion of TRH (Eckert 1978). The results in the present study show that the injection method was successful and that the obese Zucker rats have functioning negative feedback loops for thyroid hormones. The serum T_4 concentrations in the T_3-treated obese rats were, however, higher than those of the T_3-treated lean rats (Table 5, $p<0.05$). This may be indicative of a sluggish feedback loop.

There were no significant differences in basal or T_3-stimulated [^{3}H]-ouabain binding (pmol/g tissue) to soleus or edl muscles between the lean and obese rats. Both groups showed increases over basal for binding to soleus, but not for edl, when treated with T_3. Similar results, using liver homogenates, were reported by Bray et al. (1978). Incongruencies may arise, however, when comparing two different tissues. Campion et al. (1987) reported that results vary by muscle and by level of energy intake in studies of skeletal muscle metabolism in lean and obese Zucker rats. It is hazardous to draw broad conclusion using data from only two muscles, but these two muscles were carefully

selected in the present study to represent two major types of skeletal muscle. The soleus is a slow twitch muscle with a high aerobic capacity while the edl is a fast contracting muscle with a high anaerobic capacity.

Comparison with *ob/ob* Mouse

Another obese rodent that has been studied extensively is the *ob/ob* mouse. There are many similarities between the Zucker "fatty" rat and the *ob/ob* mouse, but several dissimilarities as well. Similar to the obese Zucker rat, the *ob/ob* mouse is hyperinsulinemic, has increased adipocyte size, and shows reduced oxygen consumption (York et al. 1977). The *ob/ob* mouse, however, has normal levels of circulating thyroid hormones suggesting normal thyroid function (Guernsey and Morishige 1979). The reports on sodium, potassium pump activity in *ob/ob* mice conflict. Clausen and Hansen (1982) reported no differences in enzyme activity in *ob/ob* mice while Lin et al. (1981) and Guernsey and Morishige (1979) did find a difference. Both the study by Clausen and Hansen (1982) and that by Lin et al. (1981) used skeletal muscle and measured [^{3}H]-ouabain binding as was done in this study. Guernsey and Morishige (1979) used skeletal muscle and liver and measured tissue respiration. *Ob/ob* mice do show an increase in [^{3}H]-ouabain binding in skeletal muscle in response to T_3 treatment (Lin et al. 1979).

Guernsey and Morishige (1979) and Lin et al. (1981) have suggested that decreased Na^+,K^+-ATPase activity in the *ob/ob* mouse in the presence of normal levels of T_3 could be due to reduced nuclear binding of T_3. The obesity of the *ob/ob* mouse was once thought to be due to a decrease in the number of sodium, potassium pump units (Bray et al. 1978, York et al. 1978) but it is now attributed to an increased energy efficiency

and decreased thermogenesis caused by low activity of the brown adipose tissue (Clausen and Hansen 1982, Hughes and York 1983). The thermogenic response is so low in *ob/ob* mice that they are unable to regulate body temperature under cold stress (York et al. 1977). This is not the case with the obese Zucker rat. The Zucker "fatty" rat reacts much the same as its lean littermate in response to thermogenic stimuli except when the stimulus is food. The specific dynamic action of food is reduced in the obese Zucker rat compared to the lean (Rothwell et al. 1981). The obese rat also does not increase thermogenesis in response to a low protein-high carbohydrate diet as does its lean littermate (Young et al. 1980). In most cases, such as cold stress, the obese Zucker rat responds as does the lean Zucker rat and is able to increase thermogenesis (Armitage et al. 1984).

Comparison of the Methods

Both the Na^+,K^+-ATPase activity method and the [^{3}H]-ouabain binding method have been used in research with the Zucker "fatty" rat and the *ob/ob* mouse. The Na^+,K^+-ATPase activity method measures the amount of inorganic phosphorus released in the hydrolysis of ATP by the enzyme, but may detect only 10% of the enzyme's activity (Kjeldsen et al. 1988). The [^{3}H]-ouabain binding site method measures the radioactivity of tissue samples incubated with [^{3}H]-ouabain and can theoretically detect 100% of the total enzyme activity (Kjeldsen et al. 1988). The ouabain binding sites found by this method have been shown to be working enzyme units (Clausen et al. 1987). When the increase in the enzyme is small, the [^{3}H]-ouabain binding site method detects the difference more easily.

Na^+,K^+-ATPase and Obesity

In 1991, Clausen et al. reported that the oxygen consumption and heat production which could be attributed to Na^+,K^+-ATPase activity accounted for only 2-6% of the energy used by rat and mouse tissue. This is much smaller than the difference of 40% reported by Ismail-Beigi and Edelman (1970). If the amount of energy used by the pump is found to be as small as suggested by Clausen et al., the possible effect on the obesity of the *ob/ob* mouse and the Zucker "fatty" rat would be very small (Hughes and York 1983), although even a small amount of energy could add up over the life of the mouse or rat and contribute significantly to the energy balance of the animal. If the original determination is correct, the sodium, potassium pump could very likely be a major factor in the development of the obesity in these animals (Lin et al. 1978, Lin et al. 1979, Guernsey and Morishige 1979). Further research using additional tissues and younger animals is needed to clarify the role, if any, of Na^+,K^+-ATPase in the onset of obesity in genetically obese animals. Throughout the extensive amount of research done in the area of animal obesity, one fact is evident: the obesity of these animals is almost certainly multifactorial. There may be a defect in the central nervous system that includes a dysfunctional hypothalamus (Argiles 1989). Johnson et al. (1991) discuss many possible additional factors, including low neuropeptide Y, growth hormone, and adipsin levels, as well as high lipoprotein lipase levels. Discovering the defect in each process that contributes to the animal's obesity is an area where much work needs to be done.

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