2000

Effect of thyroid hormone on formation of the NSF fusion complex in normal and obese diabetic (db/db) mice

Felipe Barajas Galicia

EFFECT OF THYROID HORMONE ON FORMATION OF THE NSF FUSION COMPLEX IN NORMAL AND OBESE DIABETIC (*db/db*) MICE.

A Thesis

Presented to the

Faculty of

California State University,

San Bernardino

In Partial Fulfillment

of the Requirements for the Degree

Master of Science

in

Biology

---

by

Felipe Barajas Galicia

June 2000

EFFECT OF THYROID HORMONE ON FORMATION OF THE NSF FUSION COMPLEX IN NORMAL AND OBESE DIABETIC (*db/db*) MICE.

A Thesis

Presented to the

Faculty of

California State University,

San Bernardino

by

Felipe Barajas Galicia

June 2000

Approved by:

[redacted]
Richard Fehn, Chair, Biology — 6/14/00 Date

[redacted]
Colleen Talbot

[redacted]
David Polcyn

ABSTRACT

Diabetes mellitus is a syndrome characterized by high serum glucose levels. The inadequate control of serum glucose is due to insulin resistance by peripheral tissues such that the cells do not take up glucose. Glucose transporters in the peripheral tissues play an important role in the maintenance of glucose balance. The GLUT4 transporter, a glucose transporter found on the surface of skeletal muscle and adipose cells, acts as a transporter that allows these cells to take in glucose for normal cellular metabolism. One of the cellular defects associated with Non-Insulin Dependant Diabetes (NIDDM) is reduced GLUT4 expression in the plasma membrane of insulin responsive tissues.

As in all integral membrane proteins, new GLUT4 is added to the plasma membrane by insertion of vesicles, which occurs in two main steps. In the docking process, the GLUT4-containing vesicle binds to the plasma membrane, and in the fusion process, the vesicle is integrated into the plasma membrane. The fusion process requires the binding of NSF, which fuses the vesicular and plasma membranes through hydrolysis of ATP. The unique role of NSF in the fusion of vesicles, and the fact that it appears

as a soluble protein unless actively engaged in the fusion of vesicles, makes NSF a strong indicator for assessing vesicular trafficking and, more specifically, the amount of vesicular fusion that occurs in cells under the influence of putative trafficking regulators. In this study, the amount and location of NSF was assayed in skeletal muscle cytoplasm and plasma membrane to determine the efficacy of using thyroid hormone (T_3) to induce vesicular fusion in the C57BL/KsJ normal (*+/db*) or diabetic (*db/db*) mouse, a model for Non-Insulin Dependent Diabetes Mellitus (NIDDM). Animals were injected intraperitoneally with vehicle or 200 ng T_3/g BW for 9 days. Four treatment-phenotype combinations were constructed. Subcellular fractionation of skeletal muscle homogentates was performed by differential centrifugation followed by western blot analysis of NSF proteins in the cytoplasm and plasma membrane.

All animals treated with T_3 showed increases in metabolism that exceeded those of their vehicle-treated counterparts on day 10. Thyroid hormone treated animals have RQ values indicating greater fat catabolism compared to their vehicle treated counterparts. Serum glucose data showed that glucose levels in diabetic mice remained over

two times greater than normal and thus were unaffected by T_3 treatment. T_3 treatment induced an increase in total (plasma membrane + cytoplasmic fractions) NSF protein in skeletal muscle of both normal and diabetic animals. Data also indicate a lack of measurable plasma membrane associated NSF in vehicle treated diabetic mice. Consistent with previous studies which showed increases in plasma membrane associated GLUT4 of skeletal muscle in diabetic mice with T_3 treatment, plasma membrane associated NSF increased in diabetic mice after 9 days of T_3 treatment compared to diabetic mice treated with vehicle. These data suggest restoration of vesicular trafficking and specifically fusion of vesicles to the plasma membrane. Thyroid hormone increases cytoplasmic NSF concentrations in normal but not diabetic animals compared to their vehicle treated counterparts. The relative concentrations of plasma membrane and cytoplasmic NSF show that the majority of the NSF in T_3 treated diabetic animals is concentrated at the plasma membrane, which suggests a high level of vesicular fusion in this group. Furthermore, the quantities of NSF in T_3 treated normal and diabetic animals are similar indicating equal expression between phenotypes. Thus, the distribution of NSF between cytoplasm and plasma

membrane suggest that the deficiency in plasma membrane GLUT4 transporters is the result of trafficking defects in diabetic mice, rather than an absolute reduction in GLUT4 protein expression. It also suggests a role for T_3 in the regulation of skeletal muscle glucose uptake via GLUT4 transporters.

ACKNOWLEDGMENTS

I would like to express my most sincere gratitude and appreciation to my family for their everlasting support through some difficult and often trying scholastic years. For having blind faith in me, for reminding me of my humble beginnings, and even rescheduling my days to feed me, I will forever cherish my mother. A heartfelt thanks to my father for making sure that I was always taken care of and for being the safety net in my life. I am truly blessed with caring and understanding parents.

When I think of all the sleepless nights and agonizing assignments I can't help but wonder what mental state I'd be in if I didn't have a friend in Homer to remind me that there is always a future in raising llamas in Mexico. With all the money we'd make we could afford the luxuries of a boat and horse ranch, and still have leftover change for a CB radio. Thank you Brian for not missing a rent payment yet!!

Cathy and Adele Hutchinson, Dan Salcedo, Michael Mahoney, Dwight Gallo, and Carol Smith will forever occupy a special place in my heart for their time, patience, and consideration during those trying and difficult moments when I needed comforting, venting, a string or two to be

pulled, or even a copy card for a couple hundred pages of crap I no longer remember.

I would also like to thank my committee members for their time and effort toward the development my thesis, the first of a collection I'll be writing in the very near future. Dr. Richard Fehn is worthy of special consideration for showing me the extent of my potential. I'd also like to thank him for not only one, but three 12-hour marathon data collecting sessions, which still haunt my sleep.

Now that the final piece of this thesis is complete, I can meet my friend Brian with a beer in hand and reminisce as a large heap of papers maintain a steady burning bonfire, which will undoubtedly burn for quite some time. No worries, plenty of beer in the fridge and plenty of reminiscing to do.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER ONE: INTRODUCTION

Overview of Diabetes Mellitus

Diabetes mellitus is a syndrome characterized by high serum glucose concentrations which may be the result of inadequate insulin secretion by pancreatic β cells or insulin resistance by target tissues (Kahn and Weir, 1994). Two general types of diabetes mellitus are recognized. Insulin dependent diabetes mellitus (IDDM) is the most well studied form and is most prevalent in children; IDDM is also referred to as juvenile onset diabetes. IDDM individuals produce little or no insulin due to defects in pancreatic β cells, the site of insulin synthesis. Cellular defects causing IDDM can include defective insulin genes or improper processing of the insulin protein (Hadley, 1992). Because insulin stimulates cellular mobilization of glucose transporters toward the plasma membrane of certain cell types (Weinstein et al., 1991), the lack of insulin production by pancreatic β cells results in a chronic elevation of serum glucose levels due to ineffective glucose uptake and storage by the target tissues.

Non-insulin dependent diabetes mellitus (NIDDM) is the

second but most common form of diabetes, and accounts for 90-95% of all U.S. diabetes cases and is found in 5.9% of the U.S. Population (American Diabetes Association, 2000). It typically develops in older individuals and is also referred to as adult onset diabetes mellitus (Kahn and Weir, 1994). Peripheral tissue resistance to insulin in NIDDM individuals leads to a reduction in glucose transporter presentation in the plasma membrane of target cells, resulting in lower glucose uptake by these tissues. Consequently, an accumulation of extracellular serum glucose eventually renders an individual hyperglycemic. Increased sugar levels circulating in the body leads to excessive production of insulin, thereby causing the hyperinsulinemia, which also characterizes the NIDDM pathology. Diabetic individuals exhibit a host of other ailments, which include glucosuria, polyuria, polydipsia, polyphagia, dehydration, tissue wasting, ketosis, acidosis, obesity, as well as vascular and neuropathic complications (Seeley, 1992).

NIDDM may be caused by reduced numbers of insulin receptors on the target cell, defective target cell response due to poor insulin receptor function, a faulty signal

transduction pathway, or poor mobilization of glucose transporters to the plasma membrane. This last problem may possibly be due to a defect in the trafficking mechanism for vesicles, which position the transporter in the plasma membrane of cells (Hadley, 1992; Kahn, 1996). The analysis of potential vesicular trafficking defects in NIDDM is the focus of this project.

Diabetic Mouse Models, Leptin, and T_3

Diabetes is a mutation which occurs in the C57BL/KsJ (*db/db*) diabetic mouse, a model that displays characteristics of the metabolic disturbances resembling non-insulin dependent diabetes mellitus in humans (Hummel et al., 1966). The mice exhibit a severe disease syndrome with age-dependent onset of diabetes and a shortened life-span. Characteristics of the syndrome include hyperglycemia, hyperinsulinemia, insulin resistance, suppressed metabolism, reduced heat production, and obesity (Bray and York, 1979). Similar metabolic defects can be observed in the C57BL6J obese mouse (*ob/ob*) model (Pelleymounter et al., 1995). The *ob/ob* mice are deficient in leptin production, while the *db/db* mice have a defective leptin receptor (Pelleymounter

et al., 1995). Leptin is a protein hormone secreted primarily by white adipose tissue in direct relation to the amount of fat mass (Prolo et al., 1998). A potential role for leptin action through a hypothalamic-pituitary-adrenal axis mechanism has been suggested to explain leptin's function as a modulator of metabolic rate (Prolo et al., 1998). Studies have shown parallel metabolic and thermogenic responses to T_3 (Oh and Kaplan, 1995) and leptin (Pelleymounter et al., 1995) in *ob/ob* mice. Leptin does not have a direct effect on insulin stimulated glucose uptake in either soleus muscle of *ob/ob* or lean mice, or adipocytes of lean mice (Zierath et al., 1998). However, leptin has been shown to decrease β cell secretion of insulin (Emilsson et al., 1997). The *db/db* mouse also displays hyperthyroidism, a condition that has been determined to lower the expression of leptin in hyperthyroid rats (Fains, 1997). In contrast, rats with hypothyroidism increase expression of leptin mRNA and thus protein (Fains, 1997). Triiodothyronine or thyroid hormone (T_3) is the metabolically active form of thyroid hormone and is derived from tetraiiodothyronine or thyroxine (T_4), a hormone produced by the thyroid gland. T_3 normally

regulates metabolic activity in most cells (Hadley, 1992). Our lab is currently investigating the effects of exogenous T_3 on the metabolism of diabetic mice. Our lab has shown decreased adiposity and serum triglyceride concentrations, increased metabolism and heat production (thermogenesis), and some improvement in glycemic state in diabetic mice treated with supraphysiological doses of T_3 (Fehn et al., 1999). Thus, these animals show improvement of the pathology in response to T_3. The fact that complete euglycemia is not reached in diabetic mice treated with T_3 suggests that there is a defective glucose transport mechanism that is yet to be identified.

Glucose Transporters

Molecular cloning studies conducted over the past decade have led to the discovery of a family of glucose transporters. They are named GLUT1-7 for the order in which they were isolated. GLUT1-5, and 7 are functional sugar transporters, whereas GLUT6 is a pseudogene (Pessin and Bell, 1992). GLUT1 is found in all cells and is most abundant in cells that form blood-tissue barriers. It is expressed at high levels in fetal tissues, brain, and

placenta (Pessin and Bell, 1992). GLUT2 is primarily found in liver, small intestine, kidney, and insulin secreting β cells (Pessin and Bell, 1992). GLUT3 is an isoform found in all cells but is most abundant in the brain, kidney, and placenta (Pessin and Bell, 1992). Because of their ubiquitous distribution, GLUT1 and 3 are believed to be responsible for basal glucose uptake. GLUT4 is insulin regulatable and is found in skeletal muscle, cardiac muscle, and adipocytes (Pessin and Bell, 1992). GLUT5 is the small intestine isoform which is found primarily in the jejenal region and it is responsible for fructose transport (Sugawara-Yokoo, 1999). Finally, GLUT7 is found in intracellular organelles of hepatocytes and is believed to play a role in gluconeogenesis (Kahn, 1996). All of these transporters are required to facilitate glucose uptake for the various cell types that make up the tissues mentioned.

GLUT4 Studies

In the event of high serum glucose, pancreatic β cells will release insulin (Pessin and Bell, 1992). The basal rate of insulin secretion in humans is 0.5 to 1 units/hr, which can increase ten-fold after meals. Insulin has a

half-life of 5 to 8 minutes (Berne et al., 1998). The insulin released into the blood will then bind to insulin receptors on the surface of skeletal muscle and an intracellular message will be generated via a signal transduction cascade (Pessin and Bell, 1992). GLUT4 transporters are compartmentalized in vesicles within a cell. These GLUT4 containing vesicles form an intracellular pool of the transporter ready to be added to the plasma membrane. The insulin signal will lead to recruitment of GLUT4 containing vesicles from the intracellular pool to the inner surface of the plasma membrane where they will dock and then fuse, thus increasing the number of GLUT4 transporters in the plasma membrane. The increase in GLUT4 protein in the plasma membrane may be 10 to 40 times the basal level and these transporters will remain only 6 to 7 minutes after insulin removal, at which time they are recycled back into the cytoplasmic pool (Verhey et al., 1995). T_3 is known to augment glucose absorption from the gastrointestinal tract and increase glucose uptake from the blood, oxidation, and gluconeogenesis (Berne et al., 1998). This suggests a potential relationship between GLUT4 and T_3.

Reduced numbers of insulin receptors on the surface of cells, a faulty signal transduction pathway, or poor mobilization of GLUT4 containing vesicles due to a fusion defect can lead to Non-Insulin Dependent Diabetes Mellitus.

Glucose uptake into skeletal muscle and adipose tissue by GLUT4 is essential to the maintenance of serum glucose homeostasis (Kahn, 1996). In the past decade, intense research has examined the problem of lack of glucose uptake in NIDDM individuals and looked for potential defects that may be occurring in GLUT4 gene expression, transporter presentation, or function as it relates to diabetes with the purpose of understanding these defects and potentially developing therapies to prevent or control diabetes (Kahn, 1996).

Restoration of glucose transport across the plasma membranes of individuals having NIDDM is especially important in allowing these diabetics to improve their hyperglycemic condition. Recent studies have reported that thyroid hormone (T_3) treatments increase the number of membrane bound GLUT4 transporters in skeletal muscle of hypothyroid rats (Weinstein et al., 1994b) and rat

adipocytes (Mathaei et al., 1995). Estrada (1997) showed that diabetic (*db/db*) mice had lower levels of plasma membrane associated GLUT4 transporters compared to normal (*+/db*) mice. Restoration of GLUT4 transporters in skeletal muscle in response to thyroid hormone, has also been demonstrated in the *db/db* mouse (Estrada, 1997). This study showed a translocation of GLUT4 protein from an intracellular pool of GLUT4-containing vesicles to the plasma membrane. However, there is a question as to whether the transporters were successfully integrated or whether they were fully functional in that there was no detectable increase in the intracellular glucose concentration in subjects used in these studies (G. Shulman, personal communication). There may be the possibility of a regulated type of GLUT4 transporter having variable glucose transporting activity (Kahn, 1996). The GLUT4 transporter is proposed to have been successfully inserted in the plasma membrane, but not yet activated to proceed with glucose uptake. Activation of glucose uptake would be similar to allosteric regulation where an unspecified factor may elicit a conformational change in the GLUT4 transporter resulting

in activation of the transporter (Kahn, 1996). The uncertainty of GLUT4 association with the plasma membrane, whether it is a successfully inserted GLUT4 transporter or a GLUT4 transporter that is in a vesicle docked with the plasma membrane, has been the subject of concern and discussion in professional meetings due to the potential problems with docking and fusion of vesicles (G. Shulman, personal communication). Exofacial labeling of the plasma membrane of muscle cells in whole animals with antibodies for GLUT4 transporter protein has been attempted, but no successful applications of the technique have been reported (G. Shulman, personal communication). Exofacial labeling is highly important because it can it conclusively identify the GLUT4 transporters as being successfully inserted in the plasma membrane or confirm that they remain enclosed within a vesicle that has yet to fuse to plasma membrane. A successfully inserted transmembrane protein will display protein domains on the outer surface of the plasma membrane. In an intact animal, these domains can be immunostained with labeled antibodies, which are injected into the animal. In contrast, a protein enclosed within a docked vesicle would

not be presented on the outer face of the membrane and thus not labeled by the antibody. Tissue samples from these animals can then be viewed under microscopy to determine if antibodies are present on the outer surface of the plasma membrane. In the *db/db* mouse model it appears that GLUT4-containing vesicles have, at the very least, docked in response to T_3, but may not yet be fused to the plasma membrane (Weinstein et al., 1991; Mathaei et al., 1995; Estrada, 1997).

Glucose Toxicity

Many studies have been designed to gain better insight into the adverse effects of elevated serum glucose levels, a condition known as glucose toxicity. In a study conducted by researchers at the Harvard Medical School (Kahn et al., 1991), male Sprague Dawley rats were treated with streptozotocin to inhibit β cell secretion of insulin by the pancreas, thereby elevating serum glucose levels and mimicking IDDM. Their studies showed that hyperglycemia over a seven day period resulted in no significant difference of GLUT4 protein concentration in the hind limb muscle of these rats when compared to normal animals.

However, there was a 54% decrease in glucose uptake. After 14 days of hyperglycemia, these rats showed a 20% decrease in GLUT4 protein levels compared to normal animals. This decrease in GLUT4 was reversed with replacement injections of insulin, but glucose uptake remained largely suppressed. Because glucose transport decreased after 7 days while the concentration of GLUT4 transporters remained unaltered, this study suggested that either trafficking of GLUT4 transporters or transporter activity are affected by prolonged hyperglycemia and is thus a result of glucose toxicity. In IDDM individuals, insulin treatment prevents the onset of hyperglycemia and thus helps avoid complications associated with hyperglycemia (Rossetti et al., 1990). In a related study, Kurowski et al. (1999) used Sprague Dawley rat extensor digitorum longus muscle to study the effect of hyperglycemia on insulin action. Muscle tissues used in this experiment were pre-incubated in a medium containing glucose or glucose plus insulin at various concentrations for 4 hours. The tissues were then subjected to insulin after the pre-incubation phase for analysis of insulin action. Their results showed that muscles which

were pre-incubated in media containing both high concentrations of glucose and low concentrations of insulin displayed a 70% decrease in their ability to convert glucose to glycogen and a 30% decrease in their ability to take in glucose after insulin treatment when compared to muscle tissue pre-incubated in glucose-free media. In muscle tissue pre-incubated with high glucose and no insulin, the same trend was observed for insulin-stimulated glycogen synthesis. However, glucose uptake in these tissues remained unaltered after insulin addition. Further subcellular analysis of these tissues found a 60% decrease in activity of Akt/ Protein Kinase B (PKB), a protein involved in the insulin-signaling cascade. However, the reduced activity could not be attributed to low abundance of either Akt or PKB proteins. Furthermore, the activities of phosphatidylinositol (PI) 3-kinase, an upstream promotor for PKB and mitogen-activated protein (MAP) kinase, a parrallel signal, remained unaffected by the hyperglycemia. The results of that study indicated that hyperglycemia-induced insulin resistance was caused by inhibition of the insulin-signaling cascade due to a decrease in Akt/PKB activity.

Gibbs et al. (1995) used diabetic *db/db* mice overexpressing human-insulin regulatable glucose transporter (GLUT4) in skeletal muscle and adipose tissue to demonstrate the importance of hyperglycemia in the development of diabetes. Results from their study indicated that diabetic mice expressing an overabundance of human GLUT4 were better able to respond to insulin treatment, resulting in a milder hyperglycemic condition. In contrast, *db/db* mice not expressing human GLUT4 showed a severe hyperglycemic condition. Furthermore, *db/db* mice overexpessing human GLUT4 were able to tolerate and adequately dispose of large oral challenges of glucose, unlike their non-transgenic *db/db* counterparts. Together, these studies suggested a feed-forward effect of glucose toxicity on insulin stimulated glucose uptake, which affects at least muscle cells at various sub-cellular sites or mechanisms. That is to say, that an induced elevated state of serum glucose levels affects insulin signaling pathways (Kurowski et al., 1999), GLUT4 protein levels (Kahn et al., 1991), and activity of GLUT4 transporters (Kahn et al, 1991; Gibbs et al., 1995). These inhibited mechanisms in turn contribute

to the persistence of the hyperglycemic condition, while rendering the subject well beyond insulin treatment rescue (Kahn et al., 1991). Ultimately, these defects can potentially be the cause for many complications associated with diabetes such as glucosuria, polyuria, polydipsia, polyphagia, dehydration, tissue wasting, ketosis, acidosis, obesity, as well as vascular, and neuropathic complications (Seeley, 1992).

Vesicular Docking and Fusion

GLUT4 transporters are found in the plasma membrane of skeletal muscle and adipocytes, and allow the cells to passively take up glucose as a source of fuel. As with all integral membrane proteins, new GLUT4 is added to the plasma membrane by insertion of vesicles, a process which occurs in two main steps: 1) In the docking process, when the GLUT4-containing vesicle binds to the plasma membrane, and 2) the fusion process, in which the vesicle is integrated into the plasma membrane. The process consists of vesicular proteins binding with plasma membrane proteins to promote joining of the two membranes (Cain et al., 1992). The proteins involved in the docking mechanism include the tSNARE

proteins Syntaxin and SNAP-25 (Synaptosome Associated Protein MW of 25kd) and the vSNARE proteins Synaptobrevin or VAMP (Vesicular Associated Membrane Protein) and Synaptotagmin. αSNAP (Soluble NSF Attachment Protein) and an NSF (N-ethylmaliemide Sensitive Factor) tetramer protein are involved in the fusion mechanism. Syntaxin and SNAP-25 are located on the plasma membrane and are termed tSNAREs. VAMP and Synaptotagmin are located on vesicular membranes and are termed vSNAREs, while αSNAP and the NSF tetramer are soluble proteins involved in fusion of vesicles and found in the cytoplasmic pool (Sollner et al., 1993).

Syntaxin, SNAP 25, VAMP, and Synaptotagmin are each gene families with multiple isoforms within each family. For each different protein family, isoforms are found within distinct locations inside a cell (Bock and Scheller, 1997). These distinct locations include specific regions of the plasma membrane as well as the cis and trans faces of the golgi for vesicles passing through from the endoplasmic reticulum (Rothman, 1994). The significance of the existence of these gene families is that it allows various combinations of docking proteins (vSNARES and tSNARES) to

occur with varying degrees of binding affinities toward each other. Once a stable complex has been formed, complete docking and fusion may take place (Rothman, 1994). The details of the docking and fusion mechanism, which have primarily been studied in neuronal tissue and yeast, suggest that SNAP-25, Syntaxin, VAMP, and Synaptotagmin will dock the vesicle to the plasma membrane. The Syntaxin, SNAP 25, VAMP, and Synaptotagmin tetramer forms a v-tSNARE complex and is SDS (Sodium Dodecyl Sulfate) resistant (Hanson et al., 1997). An intermediate step, yet to be defined but believed to be Ca^{2+} dependent, will dislodge the Synaptotagmin via a conformational change in Synaptotagmin (Brose et al., 1992). Ca^{2+}-regulated removal of the Synaptotagmin, which has been blocking αSNAP binding to the v-tSNARE complex, will then allow the αSNAP to bind to the remaining three proteins (Rothman, 1994). Subsequent binding of NSF to αSNAP will produce a new complex termed the 20S fusion complex (Bark and Wilson, 1994). Upon ATP hydrolysis by NSF, the 20S complex dissociates due to a conformational change incurred by Syntaxin that also prevents reassociation of the v-tSNARE docking complex.

Once the complex has dissociated, fusion occurs. Studies using cell culture have shown that in the absence of ATP, NSF will fail to dissociate the 20S complex and thereby prevent fusion of the vesicle to the membrane (Barnard et al., 1997).

The NSF mediated fusion process allows a physical joining of GLUT-4 containing vesicles with the plasma membrane and subsequent presentation of the GLUT-4 transporter protein on the surface of cells. It is the intent of this study to provide further insight into the details of the fusion of GLUT4-containing vesicles with specific emphasis on the NSF fusion protein as it relates to T_3 induced translocation of GLUT4 containing vesicles to the plasma membrane in the *db/db* mouse model for NIDDM.

Proposed Effects of T_3 on NSF

NSF binding to the docking complex has proven to be crucial to the dissociation and removal of the docking complex in order to allow vesicular fusion to the plasma membrane to proceed. NSF can only associate with the v-tSNARE docking complex via binding to αSNAP in the docking complex. Therefore, detection of NSF in the plasma membrane

may suggest the presence of αSNAP and docked vesicles as well. This makes NSF an important indicator protein in the investigation of docking and fusion of vesicles. More importantly, the absence of NSF associated with the plasma membrane and docked vesicles in diabetic animals could indicate whether there is a problem in the fusion of GLUT4-containing vesicles conclusively. Low levels of NSF associated with the plasma membrane of skeletal muscle in diabetic animals would mean that low amounts of GLUT4-containing vesicles are fusing to the plasma membrane. This study could provide information that would help answer the current question surrounding GLUT4 integration into the plasma membrane and its activity.

Treatment of normal and diabetic mice with 200 ng T_3/g BW for 9 days has been shown to increase specific O_2 consumption, shift RQ values to indicate increased fat catabolism, and increase GLUT4 transporters on the plasma membrane of skeletal muscle (Fehn et al., 1999). Supraphysiological doses of T_3 can induce increased vesicular trafficking to the plasma membrane of skeletal muscle in diabetic mice (Estrada, 1997), which makes the

diabetic mouse model ideal for studying NSF in diabetes and in a whole organism. If T_3 increases the amount of NSF that can be available for the potential fusion of GLUT4-containing vesicles with the plasma membrane of skeletal muscle, then a decrease in serum glucose would be expected as a result of the insertion of GLUT4 transporters in the plasma membrane. With more glucose available to skeletal muscle cells, an increase in the glycolytic pathway should follow. Increases in total and specific O_2 consumptions should occur as a result of glycolysis.

This project is designed to gain a better perspective on the functional aspects of vesicular fusion and its potential regulation by T_3. The purpose of my research is to determine: 1) whether there is a detectable difference in NSF quantities associated with the plasma membrane of untreated normal and diabetic mice, 2) to determine whether there is a relationship between T_3 and NSF that would lead to an increase in NSF associated with the plasma membrane with T_3 treatment, and 3) whether NSF's relationship with T_3 parallels that observed between T_3 and GLUT4 from previous studies.

In this study, NSF was assayed to determine the efficacy of using thyroid hormone to induce vesicular fusion in the C57BL/KsJ (*db/db*) mouse, a model for NIDDM. In light of the fact that our lab has shown upregulation of plasma membrane GLUT4's of skeletal muscle in response to 9 days treatment with 200 ng T_3/g BW, we should expect that if T_3 indeed has restored docking of vesicles to the plasma membrane of skeletal muscle in diabetic mice, then there should be a comparable increase in plasma membrane NSF. The unique role of NSF in the fusion of vesicles and the fact that it appears as a soluble protein unless actively engaged in the fusion of vesicles, makes NSF a strong candidate for assessing the likelihood and amount of vesicular fusion that occurs in skeletal muscle cells under the influence of thyroid hormone.

CHAPTER TWO: MATERIALS and METHODS

Animals

Female C57BL/KsJ heterozygous normal (+/*db*) and homozygous diabetic (*db/db*) 8-10 week old mice were obtained from Jackson Laboratories (Bar Harbor, Maine). Females were used to maintain consistency with previous studies in which an increase in GLUT4 in the plasma membrane was observed (Estrada, 1997). Four different treatment/phenotype groups were constructed. Each treatment group consisted of 5 (+/*db*) or 5 (*db/db*) animals for a total of 20 animals for this study. The animals were housed in shoebox cages containing hardwood chip bedding and maintained at 23°C, 40-60% humidity and 14L: 10D lighting (lights were on from 6 am to 8 pm). Water and Teklab 4% chow mix were provided *ad libidum*. T_3 (Sigma) was prepared every morning by dissolving 1 mg of lyophilized T_3 in 10 ml of 0.5mM of NaOH. The solution was then covered with foil until used. Every morning, between 7:30 am and 9:30 am, animals were injected intraperitoneally with vehicle or 200 ng T_3/g BW for 9 days.

Metabolic Rate Determinations

Metabolic rates were determined from 6:00 am to 7:30 am

for each animal on days 1, 5, and 10. Rates of oxygen consumption and carbon dioxide production were measured using an open flow respiration system with an Ametek S-3 A/II Oxygen Analyzer and an Ametek CD-3A Carbon Dioxide Analyzer, respectively. A 10 second steady state reading was recorded for each animal at a constant flow rate of 400 ml/min (as determined by Gilmont Flowmeter, model D-665) to determine metabolic responses. Data are reported as total O_2 (ml $O_2 \cdot min^{-1}$), and specific oxygen consumption (ml $O_2\ g^{-1} \cdot min^{-1}$). Metabolic substrate utilization was assessed by comparing Respiratory Quotients (RQ) from the O_2 and CO_2 values obtained.

Serum Glucose Determination

Animals were anesthetized with CO_2 and blood samples obtained via cardiac puncture for serum glucose determinations. After blood samples were collected, animals were sacrificed by CO_2 asphyxiation. Red blood cells were separated from serum and the serum was stored at -20°C in 1.5 ml microtiter tubes until assayed. Serum glucose concentrations were determined with a glucose assay kit (Sigma #115A) using 20 µl of serum from each animal, which

was compared against a glucose standard curve. The assay produced a colorimetric response proportional to the serum glucose concentration and was measured at 520 nm using a microplate reader (BIO-Tek instuments, Model EL 312e). These data are reported in mg/dL.

Tissue Sample Preparations

Gastrocnemius skeletal muscle samples were collected. Gastrocnemius skeletal muscle (0.05 g) was excised and homogenized in 1ml STM/PMSF buffer (0.32 M sucrose, 3 mM $MgCl_2$, 0.5% Tween 20, and 1.0 mM Phenylmethylsulfonyl Fluoride) using a motorized glass homogenizer at room temperature for 45 seconds. Homogenates were then centrifuged at 14,000 g for 5 minutes at 4°C to produce a supernatant containing the cytoplasmic vesicles and organelles and a pellet containing the plasma membrane with docked vesicles (Herman et al., 1994). The supernatant containing cytoplasmic vesicles was then transferred to a fresh microcentrifuge tube and stored at -20°C for further determination of protein and NSF content of the cytoplasmic fraction. In order to solubilize membrane-bound proteins in the pellet, 5 ml of STM buffer was added and the mix was in

turn sonicated (Bronwill Biosonik model) for 1 minute at 80% power. The homogenate was centrifuged at 14,000 g for 5 minutes at 4°C to remove membranous residues of the plasma membrane and vesicles (Herman et al., 1994). The supernatant containing protein from the plasma membrane fraction was stored at -20°C until use. Protein concentrations of each fraction were determined using the Coomassie Brilliant Blue Method (Mathaei et al., 1995) with Bovine Serum Albumin (Sigma) used to generate a standard curve.

Western Blots

Each fraction was tested for the presence and quantity of NSF. One sample was assayed from each of the 5 representative animals in each treatment group. 10 µg of total protein was loaded per well on a 10% SDS laemmli gel and run on a Mighty Small SE250 electrophoretic assembly (Hoefer Scientific Instruments) for 1 hr at 35 mA at 4°C. After the proteins were separated, they were transferred electrophoretically to 0.2 µm pore size nitrocellulose membrane (Midwest Scientific) at 4°C for 3 hrs at 500 mA using a Hoefer Semiphor Transfer Apparatus (Model TE70).

The nitrocellulose membrane was then blocked with BLOTTO (5% Carnation non-fat milk in TBS (20 mM Tris, 500 mM NaCl, and 0.0001% merthiolate, pH 7.5)) for 30 min. at room temperature with shaking. The BLOTTO was removed and replaced with 5 µl Rabbit-anti Rat NSF polyclonal-antibody (Chemicon, Lot # 59397136) in 25 ml BLOTTO (1:5,000 dilution) and the membrane was incubated overnight at room temperature with shaking. The NSF antibody in BLOTTO was removed and the membrane rinsed three times for 10 min each with BLOTTO. A secondary biotinylated Goat-anti Rabbit IgG antibody was added (10 µl in 25 ml BLOTTO) and the membrane left to incubate at room temperature for 3 hrs with shaking. The nitrocellulose membrane was washed 3X with TBS for 10 min. 20 µl of avidin-HRP (Horseradish Peroxidase, Sigma) in 20 ml of TBS was added to the membrane and allowed to incubate for 2 hrs at room temperature with shaking. After rinsing 3X with TBS, a Western Blotting Detection Reagent (ECL, Amersham Life Sciences) was used to indicate the presence of NSF as follows: The membrane was incubated in ECL reagent for 1min, blotted dry and then placed in an 8X10 exposure cassette (Sigma). Sheets of 8X10 Kodak- OMAT Film

(Sigma) were exposed for 1 min, 30 seconds, or 10 seconds to obtain a full range of band intensities. The quantity of the NSF present in the sample was determined using a NSF protein standard, 3 µl (150 ng) of NSF were loaded along with the protein samples and a Biorad Model 620 densitometer to quantify the intensity of the band on the film. The optical density (O.D.) readings were converted to ng NSF using the positive control as an internal standard on each gel. Samples from each of the five animals in each treatment group were used to test for the presence of the NSF protein in the fusion complex. The total NSF content was determined by adding the cytoplasmic and plasma membrane NSF (ng) fractions together.

Statistics

The results of the serum glucose assay and quantity of NSF were statistically analyzed using 2-Way Analysis of Variance and Duncan's Multiple Range Test (Mason, 1989). Metabolic Data (Total O_2 Consumption, Specific O_2 Consumption, and RQ values) were analyzed using Repeated Measures Analysis of Variance and Duncan's Multiple Range Test. All data are reported as mean and standard deviation.

Significant difference is taken as $P < 0.05$ in all cases unless otherwise stated.

CHAPTER THREE: RESULTS

Body Weights and Metabolism

Diabetic mice were significantly.heavier than normal counterparts irrespective of treatment. Diabetic animals showed greater average body weights compared to normal animals on day 1. Body weights for all animals did not change significantly over the 9 days of treatment (Figure 1). After thyroid hormone treatment normal animals did not show significant differences in body weight compared to normal vehicle-treated mice (20.24 g± 2.33 g vs. 21.56 g± 1.67 g, respectively). T_3-treated diabetic animals did not show a significant difference in body weight compared to the vehicle-treated diabetic group (40.88 g± 0.95 g vs. 41.7 g± 0.80 g, respectively). However, by day 10 diabetic T_3-treated animals show near significant decreases in body weights compared to diabetic vehicle-treated animals (36.38 g± 0.70 g vs. 44.88 g± 1.00 g, respectively, $0.05 < P < 0.10$).

Metabolic data for days 1 and 5 were not used for statistical analysis due to erratic data collected on these days. Day 10 data were used for phenotype/treatment

comparisons instead. Vehicle-treated diabetic animals have higher total oxygen consumption rates compared to the normal vehicle-treated animals on day 10 (449.6± 43.2 ml $O_2 \cdot min^{-1}$ vs. 387.2± 27.6 ml $O_2 \cdot min^{-1}$, respectively; Figure 2). However, specific O_2 consumption shows that diabetic vehicle-treated animals displayed lower specific O_2 consumption rates compared to normal vehicle-treated animals (10.0± 1.0 ml $O_2 \cdot g^{-1} \cdot min^{-1}$ vs. 18.7± 1.8 ml $O_2 \cdot g^{-1} \cdot min^{-1}$, respectively). Thyroid hormone-treated normal animals had greater increases in their total oxygen consumption compared to their vehicle-treated counterparts on day 10 (478.4± 20.5 ml $O_2 \cdot min^{-1}$ vs. 387.2± 27.6 ml $O_2 \cdot min^{-1}$, respectively). Additionally, thyroid hormone-treated normal animals show higher specific oxygen consumption compared to normal vehicle-treated animals (22.532± 1.753 ml $O_2 \cdot g^{-1} \cdot min^{-1}$ vs. 18.788± 1.823 ml $O_2 \cdot g^{-1} \cdot min^{-1}$, respectively). Similar increases for both total and specific O_2 consumption are observed in thyroid hormone-treated diabetic animals when compared to their vehicle-treated counterparts (540.0± 5.27 ml $O_2 \cdot min^{-1}$ vs. 449.6± 43.22 ml $O_2 \cdot min^{-1}$, respectively) and (14.852± 1.476 ml $O_2 \cdot g^{-1} \cdot min^{-1}$ vs. 10.026± 1.021 ml $O_2 \cdot g^{-1} \cdot$

min^{-1}, respectively).

RQ Determination

On day 10, diabetic vehicle-treated animals have higher RQ values compared to normal vehicle-treated animal, indicating greater carbohydrate catabolism in diabetic vehicle-treated animals (Table 1). There was no change in RQ value with thyroid hormone treatment in normal animals. However, diabetic animals treated with thyroid hormone showed reductions in RQ values to levels observed for vehicle and T_3-treated normal animals.

Table 1. RQ for vehicle-treated and T_3-treated normal (+/*db*) and diabetic (*db/db*) mice from day 10.

	Normal, Vehicle	Normal, T_3	Diabetic, Vehicle	Diabetic, T_3
Day 10	0.65± 0.10 [a]	0.67± 0.03 [a]	0.78± 0.66 [b]	0.68± 0.06 [a]

Values are mean ± SD, n=5. Values with different superscripts are statistically distinguishable at $P \leq 0.05$.

Serum Glucose Determination

Normal vehicle-treated and normal thyroid hormone-treated animals show similar serum glucose concentrations after 9 days of treatment (Table 2). The diabetic mice receiving vehicle had glucose concentrations two times

greater than the levels observed in the normal groups. Diabetic mice treated with T_3 showed no change in serum glucose levels.

Table 2. Day 10 serum glucose concentrations of vehicle treated and T_3-treated normal (*+/db*) and diabetic (*db/db*) mice.

	Vehicle	Thyroid Hormone
Normal	226.54 mg/dL± 64.31 [a]	216.0 mg/dL± 30.0 [a]
Diabetic	589.0 mg/dL± 12.0 [b]	605.0 mg/dL± 38.0 [b]

Values are mean± SD, n=5. Values having different superscripts are statistically distinguishable at $P \leq 0.05$.

Total (Plasma Membrane and Cytoplasmic) Cellular NSF Content

The total (sum of plasma membrane and cytoplasmic fractions) amount of cellular NSF in diabetic vehicle-treated animals was less than that of normal vehicle-treated animals (9.2± 4.3 ng vs. 61.5± 18.2 ng, respectively; Figure. 4). Within both phenotypes, thyroid hormone treatment leads to increases in total NSF levels. T_3 treatment induced NSF in diabetic mice to levels that are equivalent to those observed in normal T_3-treated mice (108.4± 41.0 ng vs. 95.3± 32.3 ng, respectively).

NSF Content of the Cytoplasmic Fraction

Cytoplasmic NSF fractions were equivalent in vehicle-treated normal and diabetic animals (Figure 5). Thyroid hormone-treated normal animals have a greater quantity of NSF in their cytoplasm compared to normal vehicle-treated animals, diabetic vehicle-treated animals, and diabetic T_3-treated animals (49.2± 12.5 ng vs. 24.0± 9.5 ng, 9.2± 4.3 ng, and 18.4± 11.6 ng, respectively).

NSF Content of the Plasma Membrane Fraction

The basal level of NSF in skeletal muscle plasma membrane of normal vehicle-treated animals is 37.4± 14.9 ng NSF (Figure 6). NSF was not detectable in plasma membrane fractions of diabetic, vehicle-treated animals (Figures 6 and 7). Normal animals treated with thyroid hormone had no change in plasma membrane-associated NSF compared to their vehicle-treated counterparts (46.1± 23.0 ng vs. 37.4± 14.9 ng). However, in diabetic animals, thyroid hormone caused a large increase in plasma membrane NSF (90.03± 39.4 ng), to values greater than those observed in normal animals treated with either T_3 or vehicle (46.1± 23.0 ng or 37.4± 14.9 ng, respectively).

CHAPTER FOUR: DISCUSSION

N-ethylmaliemide sensitive factor (NSF) is the only protein known to be involved in the initiation of fusion of vesicles to the plasma membrane to date. Another associated protein which has received considerable attention is αSNAP. This protein serves as a bridge between a docked vesicle and NSF, but does not itself induce fusion of vesicles (Colombo et al., 1998). The importance of NSF in this role has been largely documented in yeast studies (Sollner et al., 1993), giant squid axon studies (Llinas et al., 1981; Bark and Wilson, 1994), as well as in intracellular studies of vesicular trafficking through organelles (Rothman, 1994). The unique role of NSF in the fusion of vesicles and the fact that it appears as a soluble cytoplasmic protein unless actively engaged in the fusion of vesicles makes NSF a strong candidate for assessing the likelihood and amount of vesicular fusion that occurs in skeletal muscle cells under the influence of thyroid hormone. In this study, the amount and location of NSF was assayed to determine the potential role of T_3 in promoting vesicular fusion in the C57BL/KsJ *db/db* mouse, a model for NIDDM. T_3 treatment induced an

increase in total (sum of plasma membrane+ cytoplasmic fractions) NSF protein quantity in both normal and diabetic animals (Figure 7). However, it is difficult to assess whether these increases were due to increased expression of the NSF gene, or an increased production of protein from already existing RNA or even reduced degradation. Other studies have suggested an increased level of expression of GLUT4 transporter protein due to an increase in GLUT4 mRNA levels in T_3-treated mice and rats (Shimokawa et al., 1997; Torrence et al., 1997). Considering the increase in GLUT4 mRNA, translocation of GLUT4-containing vesicles to the plasma membrane from the cytoplasmic pool (Estrad, 1997), and the necessity for NSF to promote fusion of vesicle to the plasma membrane, it is evident that there is a potential relationship between vesicles containing GLUT4 protein and NSF to facilitate fusion of those vesicles to the plasma membrane.

Previous research has shown up-regulation of plasma membrane associated GLUT4 in skeletal muscle of diabetic mice in response to T_3 (Weinstein et al., 1994b; Brozinick et al., 1996; Estrada, 1997). The association of GLUT4

transporters with the plasma membrane fraction established that the vesicles were, at the very least, docked to the plasma membrane. However, whether the vesicles successfully fused and installed functional transporters into the plasma membrane remains to be answered. In light of these results, if T_3 indeed restored docking of vesicles to the plasma membrane, then an increase in NSF associated with the plasma membrane should be seen provided that fusion of those vesicles is also occurring. This hypothesis assumes that in the diabetic mouse models NSF is structurally sound and thus capable of successfully presenting glucose transporters, though there is no evidence at this time to suggest that a malfunction in NSF is occurring. Vesicles containing GLUT4 contain v-SNARE proteins. Vesicle associated membrane protein-2 (VAMP2) and Synaptogamin have been identified in rat skeletal muscle and rat cardiomyocytes (Zorzano et al., 1997). This suggests that skeletal muscle GLUT4-containing vesicles are dependent upon the SNARE proteins, which allow the vesicle to dock itself to the plasma membrane. Once docked, the vesicle should subsequently be fused to the plasma membrane by NSF hydrolysis of ATP.

The current study found no statistical difference in the amount of NSF associated with the plasma membrane between normal groups treated with vehicle or T_3 (Figure 6). This suggests that in normal mice there may be an established rate of vesicular trafficking (fusion and internalization) partially controlled through the levels of NSF available to facilitate fusion of vesicles. Thus, T_3 is not able to influence this intracellular regulatory mechanism. It was also found that vehicle treated diabetic mice had a complete lack of measurable plasma membrane associated NSF (Figures 6 and 9). Thus, the expression level was below the detection limits of the assay. There most likely is some NSF associated with the plasma membrane in these cells. Presumably NSF appears too crucial to a cell to not be present in the diabetic mice. This is important in determining whether or not GLUT4 containing vesicles are being fused to the plasma membrane of diabetic mice. Little or no fusion of GLUT4-containing vesicles, as suggested by the lack of plasma membrane NSF, suggests the resulting lack of glucose uptake may be one of the major causes of hyperglycemia in type II diabetes. Perhaps an

absence of functional NSF at the plasma membrane is an initial step in the development of hyperglycemia. Plasma membrane associated NSF increased in diabetic mice after 9 days of T_3 treatment, suggesting restoration of vesicular trafficking and specifically, fusion of vesicles. These findings are consistent with previous studies where increases in plasma membrane associated GLUT4's were observed with thyroid hormone treatment of normal and diabetic animal subjects (Weinstein et al., 1991; Estrada, 1997). Together these findings suggest that with T_3 treatment GLUT4 containing vesicles are mobilized from an intracellular pool, then docked and fused to the plasma membrane. Alternatively, it may be that T_3 induces a generalized increase in fusion of vesicles, which may include GLUT4-containing vesicles. The absence of plasma membrane-associated NSF in diabetic vehicle-treated groups suggests that there would be very little GLUT4 protein being inserted into the plasma membrane in these animals.

In the cytoplasm there is no difference in NSF levels between normal and diabetic animals receiving vehicle treatment. Thyroid hormone increases cytoplasmic NSF in

normal animals compared to their vehicle-treated counterparts. However, it does not increase cytoplasmic NSF levels in diabetic mice compared to their vehicle-treated counterparts. It is important to point out that residual organelle fragments in the cellular fractionation process may have contaminated the cytoplasmic NSF fractions.

When cytoplasmic NSF data is compared to the plasma membrane NSF data, it appears that the majority of the NSF protein in T_3-treated diabetic animals is concentrated at the plasma membrane, which suggests a high level of vesicular fusion (Figure 7). Furthermore, the total amount of NSF for T_3-treated normal and diabetic animals is not different (Figure 4). It is the partitioning of the NSF between the plasma membrane and the cytoplasm that varies. The ratio of plasma membrane to cytoplasmic NSF (Table 3) is lower in diabetic vehicle-treated animals compared to normal vehicle-treated animals. T_3 treatment does not decrease the ratio in normal animals but markedly increases it in diabetic animals. This suggests T_3-induced trafficking occurring in diabetic animals. Together, these findings indicate that there is, in fact, intracellular regulation of

the amount of NSF available to allow fusion of vesicles with the plasma membrane.

Table 3. Ratios of Plasma Membrane vs. Cytoplasm NSF protein for vehicle-treated and T_3-treated normal (*+/db*) and diabetic (*db/db*) mice.

	Normal, Vehicle	Normal, T_3	Diabetic, Vehicle	Diabetic, T_3
Plasma Membrane vs. Cytoplasm Fraction	1.85 ± 1.31^{a}	0.93 ± 0.33^{b}	0 ± 0^{b}	4.15 ± 2.06^{a}

Values are mean ± SD, n=5. Values with different superscripts are statistically distinguishable at $P \leq 0.05$.

A possible reason that could explain the decreased amount of vesicular fusion occurring in T_3-treated, normal animals compared to diabetic animals receiving similar treatment is that normal animals had at some point greater amounts of vesicular fusion than what was observed in T_3-treated, diabetic animals. However, over time, T_3-treated skeletal muscle cells of normal animals may have reached a peak amount of vesicular fusion events, after which down-regulation of vesicular fusion occurs. This proposal assumes that the regulatory mechanism is faulty or slower in diabetic, T_3-treated animals that display a larger proportion of NSF in the plasma membrane relative to the

cytoplasm. Perhaps at one point normal T_3-treated animals also had a large proportion of NSF located at the plasma membrane, but, with the continuance of the study, these decreased back to normal levels, and what we see in diabetic T_3-treated animals, is the product of a delayed effect.

A comparison of the total NSF with the plasma membrane fraction of NSF shows what occurs in all treatment groups when total NSF increases (Figure 8). The plasma membrane NSF vs. total NSF shows that as total NSF increases, a larger quantity of NSF will be associated in the plasma membrane fraction. With low total NSF levels one will find low levels of NSF in the plasma membrane. Since NSF is found free-floating in soluble form within the cytoplasm, its presence in the plasma membrane only increases as a cell's demand for vesicular fusion increases, thus increasing the likelihood of finding NSF in higher amount in the plasma membrane.

Metabolic data for days 1 and 5 were not used for statistical comparisons due to erratic readings collected on these days. Results for day 10 indicate that normal animals have a greater specific oxygen consumption compared to

diabetic animals irrespective of the treatment. Additionally, an increase in specific oxygen consumption was induced in T_3-treated normal and diabetic mice compared to their vehicle-treated counterparts. This suggests an increase in metabolism in the T_3-treated mice because body weights did not change significantly over the course of the study. However, on day 10 a likely decrease in the average body weight of T_3-treated animals compared to diabetic vehicle-treated animals was observed (36.38 g± 0.70 g vs. 44.88 g± 1.00 g, respectively, $0.05 \leq P \leq 0.10$). This indicates a trend toward weight reduction and or reduced weight gain with T_3 treatment in diabetic animals. Furthermore, it supports the notion of increased metabolic activity with thyroid hormone treatment. Both control mice and diabetic mice were expected to have shown increased metabolic activity with thyroid hormone treatment when compared to their respective normal and diabetic vehicle treated counterparts. Other studies using mouse models have shown greater metabolic activity from mice treated with thyroid hormone compared to their vehicle-treated counterparts (Clark, 1995; Oh and Kaplan, 1994). In

addition, based on this previous research, a lower amount of metabolic activity was expected from diabetic mice treated with vehicle compared to normal animals treated with vehicle than what was observed. However, it is possible that the daily injections of an alkaline vehicle were eliciting a stress response that translated into increased metabolism. The fact that the animals were subject to daily injections over 9 days might have produced anticipation in the mice. Even though animals were injected after metabolic readings were recorded, their fear toward the injection may have caused them to become excited, which increased their total O_2 consumption enough to be misrepresented as an elevation in specific O_2 consumption. Previous studies allowed a 5 to 7 day period of animal handling between the arrival time of the mice and the initiation of the study. This method would minimize erratic data, which might otherwise be attributed to elevated metabolic activity associated with transportation and adaptation to the new environment. This study only allowed a 2 day period of animal handling due to time constraints, thus the baseline data may have been artificially elevated. The age of these mice might have

also contributed to the observed metabolic increases. If these mice were approaching their pubescent ages, then it is very possible that they may have experienced other hormonal influence, which increased their metabolism. Jackson Laboratories estimates their mice to be 8-10 weeks old ± 3 days upon arrival. Thus it is possible that they were closer to being 10 weeks old or 7.5 weeks old and still growing at the beginning of the study.

Diabetic animals that received thyroid hormone treatment showed a marked shift toward fat catabolism when compared to their vehicle treated counterparts for day 10. Previous studies in our lab have shown RQ shifts indicating increased fat catabolism in diabetic animals treated with thyroid hormone when compared to vehicle-treated animals after 9 days of treatment (Estrada, 1997). This study supports those observations.

In order to assess the effect of T_3 on the diabetic animals' ability to manage serum glucose levels, a glucose assay was conducted using serum collected before sacrificing the animals. If T_3 indeed successfully induced presentation of the GLUT4 protein on the outer face of the plasma

membrane, then functional GLUT4 should contribute to the development of a euglycemic state in diabetic mice by increasing glucose uptake by skeletal muscle. Certainly, plasma membrane NSF levels observed for diabetic animals treated with T_3 seem to support the successful presentation of GLUT4. However, serum glucose assays of the animals show that the diabetic mice have much higher serum glucose levels than their normal counterparts regardless of treatment. These results suggest that T_3 increased the metabolism of normal and diabetic mice and restored an active fusion of vesicles as indicated by increased NSF at the plasma membrane. However, T_3 was not able to normalize the hyperglycemia in diabetic mice. Studies have reported that there is an induction of hepatic glucose release through gluconeogenesis from rats and mice treated with T_3 termed thyrotoxicity (Comte et al., 1990; Weinstein et al., 1994a). This would explain the slight, though non- significant increase in serum glucose levels observed in the diabetic animals treated with T_3 as compared to their vehicle treated counterparts. If in fact more GLUT4 was inserted into the plasma membrane, as other studies have shown, the

persistence of elevated serum glucose levels in diabetic mice suggests one of two explanations. The first is that of a defective GLUT4, which, despite being inserted in the plasma membrane, is still not able to allow glucose uptake. The second is one that fits a current theory that a GLUT4 transport protein that requires further activation as a second step after insertion into the plasma membrane (Kahn, 1996). As stated earlier, the most effective procedure to determine with a greater certainty whether the GLUT4 is presented on the outer face of the plasma membrane in diabetic animals is *in vivo* exofacial labeling. However, this procedure has been difficult to perfect. Therefore the most recent literature surrounding the controversy between poor insertion of the glucose transporter in the plasma membrane and the inactive glucose transporter will remain speculative at best in trying to resolve this dilemma.

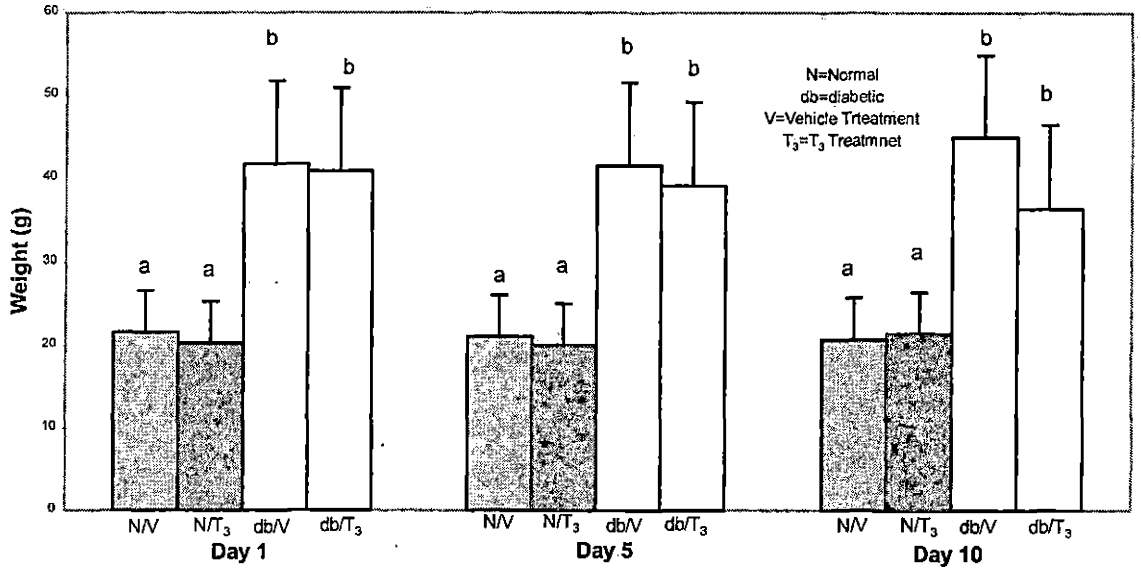

Figure 1. Body weights of normal (+/*db*) and diabetic (*db/db*) mice treated with either vehicle (0.5mM NaOH) or T_3 (200 ng/g BW) on 9 consecutive days. Values are mean ± SD. Groups having different superscripts are statistically distinguishable at $P \leq 0.05$. Initial body weights of diabetic animals are greater than normal animals. No significant weight changes were observed during the study. On day 10 diabetic T_3-treated animals show near significant decreases in body weights compared to diabetic vehicle-treated animals ($0.05 \leq P \leq 0.10$).

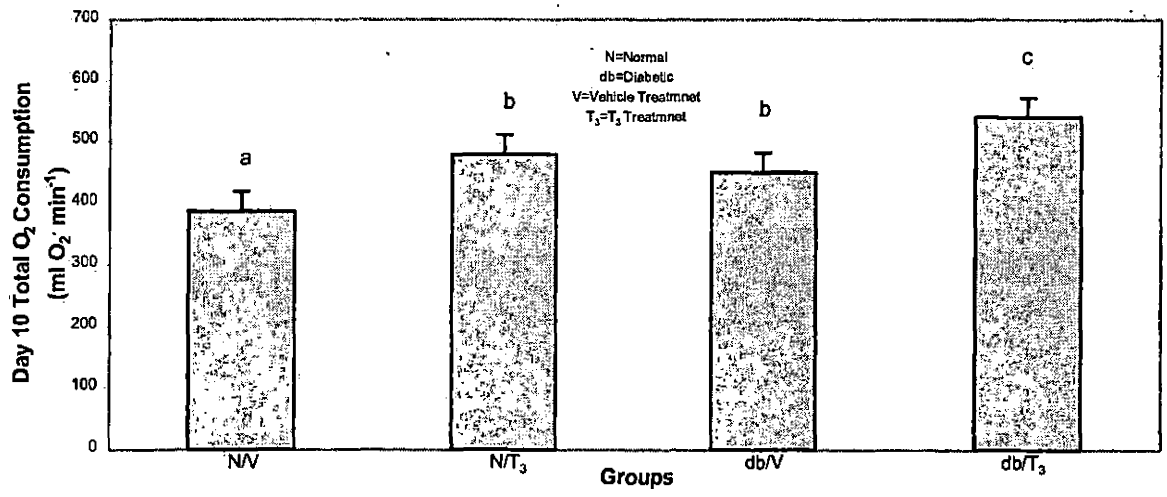

Figure 2. Total O_2 consumption rates for day 10 of normal (+/*db*) and diabetic (*db/db*) mice treated with either vehicle (0.5mM NaOH) or T_3 (200 ng/g BW) on 9 consecutive days. Values are mean ± SD. Groups having different superscripts are statistically distinguishable at $P \leq 0.05$. Diabetic animals have greater O_2 consumptions compared to normal animals. T_3 stimulates metabolism in both normal and diabetic animals with diabetic animals showing similar change.

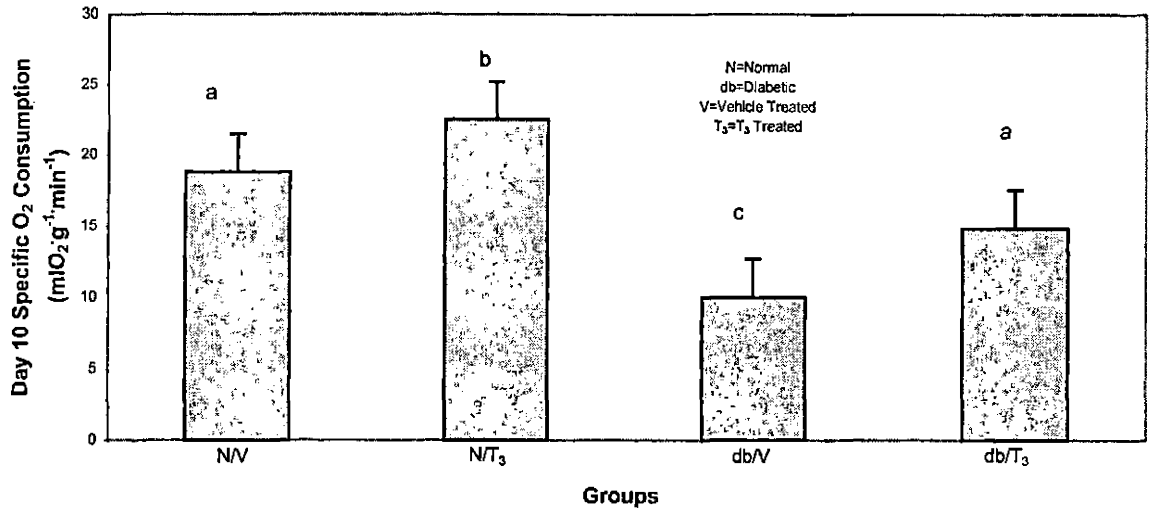

Figure 3. Specific O_2 consumption rates of normal (+/*db*) and diabetic (*db/db*) mice treated with either vehicle (0.5mM NaOH) or T_3 (200 ng/g BW) on 9 consecutive days. Values are mean ± SD. Groups having different superscripts are statistically distinguishable at $P \leq 0.05$. Vehicle-treated diabetic animals show approximately half the specific oxygen consumption of that observed in normal animals due to greater proportional body fat. T_3 increased specific O_2 consumption in both phenotypes with diabetic animals assuming a metabolic rate equivalent to vehicle-treated normal animals.

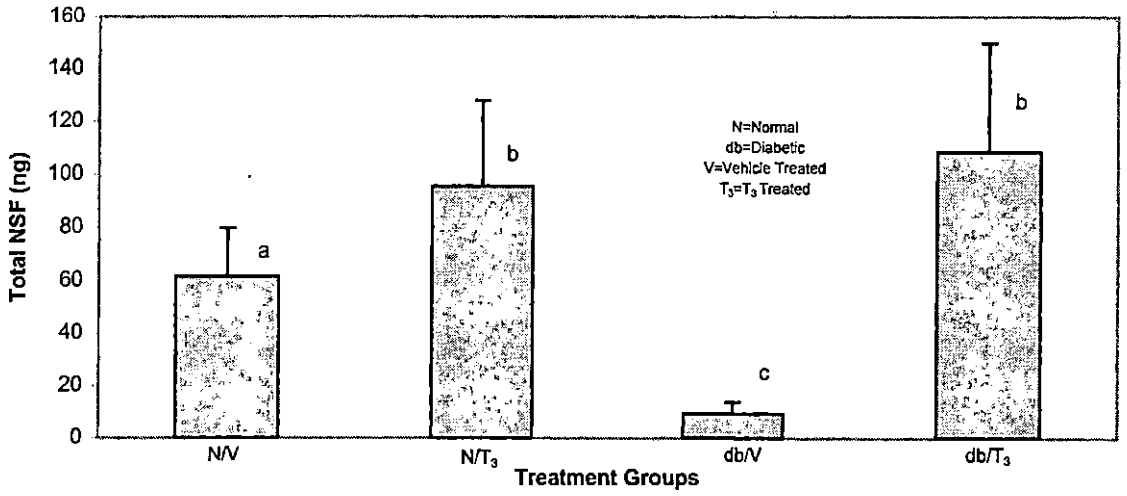

Figure 4. NSF total protein content for normal (*+/db*) and diabetic (*db/db*) mice treated with vehicle (0.5mM NaOH) or T_3 (200 ng/g BW) (Values are mean ± SD, n=5; $P \leq 0.05$). Vehicle-treated diabetic animals have greatly reduced NSF levels vs. vehicle-treated normal animals. T_3 increased total NSF expression in both phenotypes with diabetic animals attaining expression levels equivalent to those observed in normal animals.

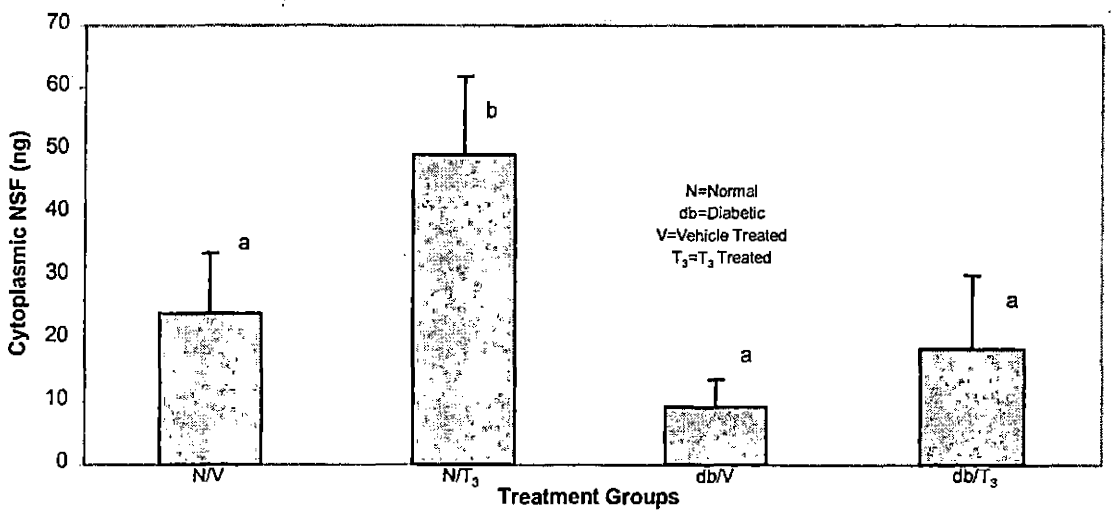

Figure 5. Cytoplasmic NSF protein content in normal (*+/db*) and diabetic (*db/db*) mice treated with vehicle (0.5mM NaOH) or T_3 (200 ng/g BW) (Values are mean ± SD, n=5; $P \leq 0.05$). Cytoplasmic NSF levels of diabetic vehicle-treated animals are not different from those in normal vehicle-treated animals. T_3 does not increase NSF in the cytoplasm of diabetic animals and markedly increases cytoplasmic NSF levels in normal animals.

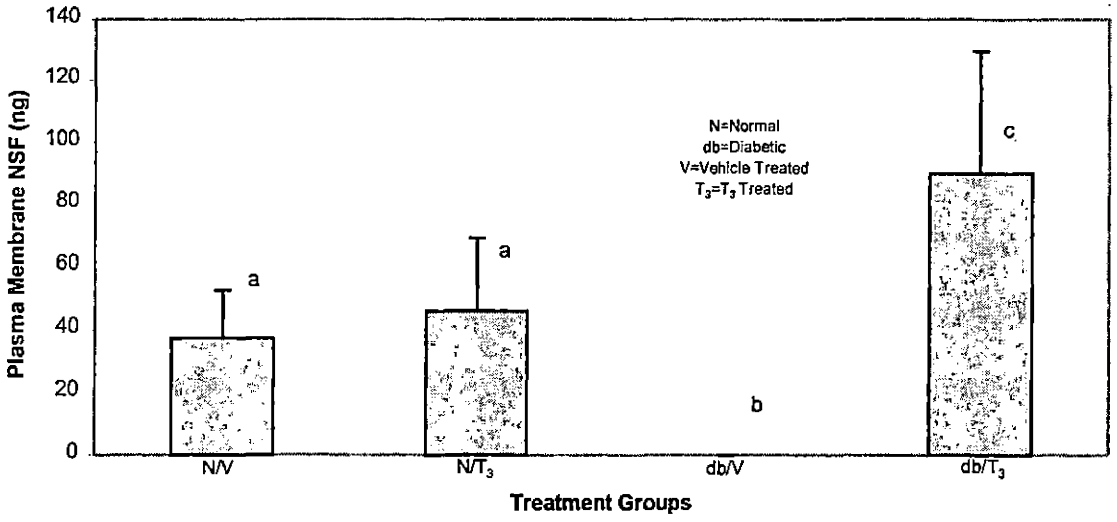

Figure 6. Plasma membrane-associated NSF protein content in normal (+/*db*) and diabetic (*db/db*) mice treated with vehicle (0.5mM NaOH) or T_3 (200ng/g BW) (Values are mean ± SD, n=5; P≤ 0.05). Vehicle-treated diabetic animals do not show measurable NSF levels. T_3 has no effect on normal animals, but markedly increases diabetic animal NSF levels above those observed in diabetic vehicle-treated and normal animals.

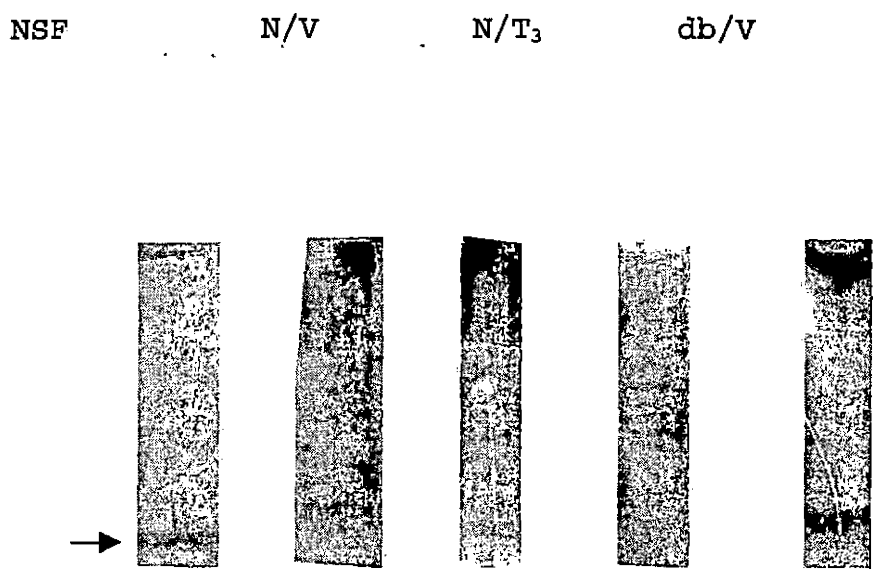

Figure 7. Fluorograms of western blots showing immunoreactive NSF of the plasma membrane. The NSF band is indicated by the arrow.

(NSF) = internal NSF control.

(N/V) = normal vehicle-treated animal.

(N/T_3) = normal T_3-treated animal.

(db/V) = diabetic vehicle-treated animal.

(db/T_3) = diabetic T_3-treated animal.

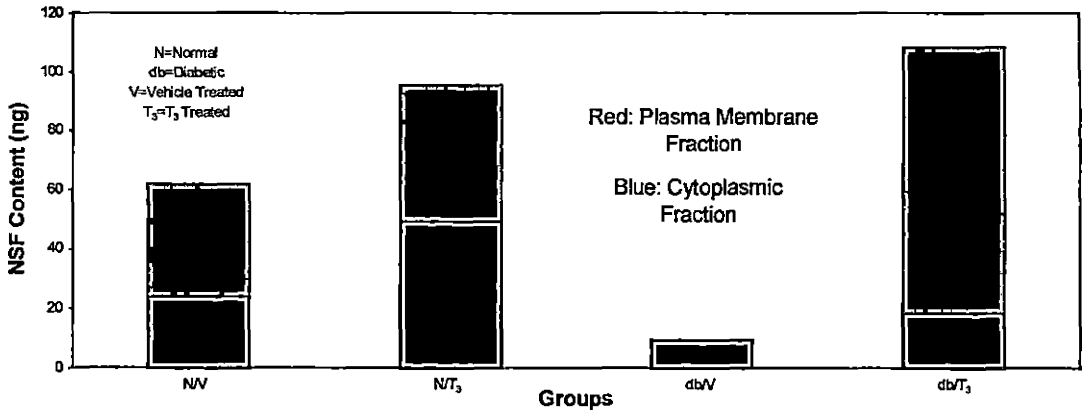

Figure 8. Plasma Membrane and Cytoplasmic NSF protein in vehicle treated (0.5mM NaOH) and T_3 treated (200ng/g BW) normal (+/*db*) and diabetic (*db/db*) mice. Combined (total) expression is less in *db*/V compared to N/V. T_3 increases total expression in normal animals with most being retained in the cytoplasmic fraction. T_3 induced NSF in diabetic animals with the vast majority appearing in the plasma membrane.

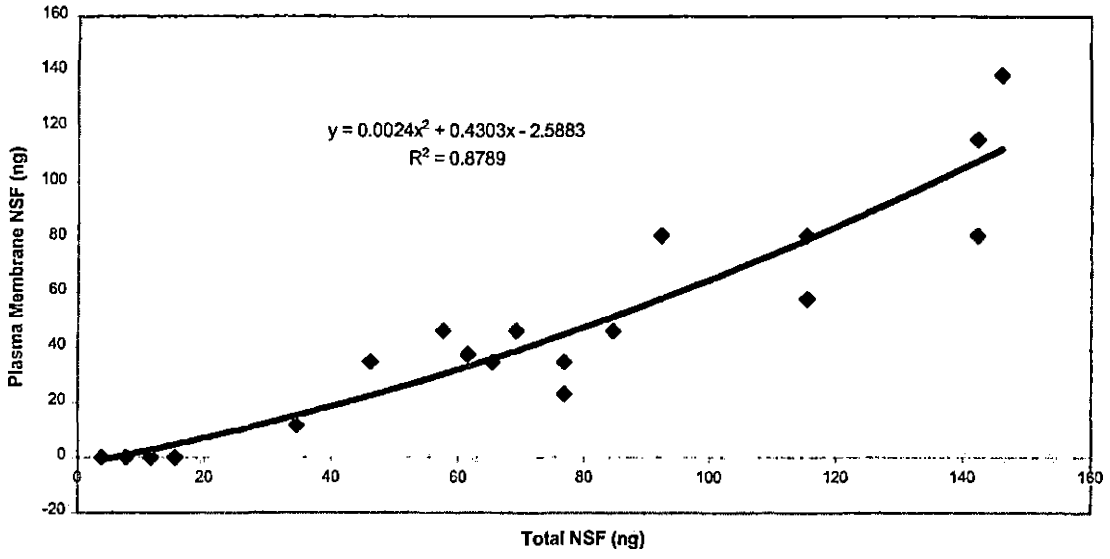

Figure 9. Second order regression polynomial curve for plasma membrane vs. total (PM and Cyto) NSF protein for all treatment groups combined. The plasma membrane fraction is an approximate fixed quantity of the Total NSF.

APPENDIX A

Total O_2 consumption rates for days 1, 5, and 10 of normal (+/*db*) and diabetic (*db/db*) mice treated with either vehicle or T_3 on 9 consecutive days.

	Normal, Vehicle	Normal, T_3	Diabetic, Vehicle	Diabetic, T_3
Day 1	124.00±57.13	88.00±10.95	116.80±33.39	210.40±39.85
Day 5	328.00±35.77	392.80±39.93	292.00±61.12	424.80±18.41
Day 10	387.20±27.62	478.40±20.51	449.60±43.22	540.00±50.27

Values are mean± SD, n=5. Groups having different superscripts are statistically distinguishable at $P \leq 0.05$.

On day one of treatment, animals received intraperitoneal injections of vehicle or thyroid hormone before oxygen consumption and carbon dioxide liberation had been recorded. As a result, values obtained for total and specific oxygen consumption calculations were compromised by stress incurred by the animals due to the additive effects of handling and injections. Therefore, day 1 data were not included in analyses. Since both days 5 and 10 data show the same total oxygen consumption patterns in each treatment group, then day 10 data was used to show the effect of T_3 in this study. Additionally, O_2 and CO_2 values were evaluated

and determined to have increased for O_2 and decreased for CO_2 over the 10 day study. Thus eliminating a flow rate malfunction. Despite the fact that specific oxygen consumptions increased in progressive fashion over the course of ten days for all animals, all animals treated with T_3 showed an increase in metabolism that exceeded those of their vehicle treated counterparts (Appendix A and B).

Appendix B

Specific O_2 consumption rates for days 1, 5, and 10 of normal (*+/db*) and diabetic (*db/db*) mice treated with either vehicle or T_3 on 9 consecutive days.

	Normal, Vehicle	Normal, T_3	Diabetic, Vehicle	Diabetic, T_3
Day 1	3.76±0.96	3.72±0.64	2.4±0.89	3.35±1.78
Day 5	15.70±1.91	19.81±2.55	7.05±1.46	10.86±0.60
Day 10	18.78±1.82	22.53±1.75	10.02±1.02	14.85±1.47

Values are mean± SD, n=5. Groups having different superscripts are statistically distinguishable at P≤ 0.05.

APPENDIX C

RQ values for days 1, 5, and 10 of normal (*+/db*) and diabetic (*db/db*) mice treated with either vehicle or T_3 on 9 consecutive days.

	Normal, Vehicle	Normal, T_3	Diabetic, Vehicle	Diabetic, T_3
Day 1	0.75± 0.10	0.85± 0.09	0.85± 0.63	0.89± 0.03
Day 5	0.71± 1.05	0.72± 0.03	0.79± 0.03	0.75± 0.03
Day 10	0.65± 0.10	0.68± 0.03	0.78± 0.66	0.68± 0.06

Values are mean± SD, n=5. Groups having different superscripts are statistically distinguishable at P≤ 0.05.

RQ data indicated that all animals displayed a tendency to shift their metabolic substrate usage toward fat catabolism as the study progressed. Vehicle treated normal animals displayed progressive decreases in RQ values through day 10 (Table 2). Day 1 and 5 data were not used for comparisons due to erratic readings recorded on these days.

REFERENCES

American Diabetes Association. Diabetes Facts and Figures.http://diabetes.org/ada/facts.asp#profile. May 19, 2000.

Bark, C., and M. Wilson. Regulated vesicular fusion in neurons: snapping together the details. *Proceedings of the National Academy of Science* USA 91: 4621-4624, 1994.

Barnard R., A. Morgan, R. Burgoyne. Stimulation of NSF ATPase activity by alpha-SNAP is required for SNARE complex disassembly and exocytosis. *Journal of Cell Biology* 139: 875-883, 1997.

Berne R., M. Levy, B. Koeppen, B. Stanton. Physiology., Mosby, Inc., 1998, p. 830.

Bock J., and R. Scheller. Protein transport: A fusion of new ideas. *Nature* 387: 133-135, 1997.

Bray, G. A. and York, D.A. Hypothalamic and genetic obesity in experimental animals: An autonomic and endocrine hypothesis. *Physiological Reviews* 59: 719-809, 1979.

Brose, N., A. Petrenko, T. Suhof, R. Jahn. Synaptogamin: A calcium sensor on the synaptic vesicle surface. *Science* 256: 1021-1025, 1992.

Brozinick, J., B. Yaspelkis, C. Wilson, K. Grant, E. M. Gibbs, S Cushman, and J. Ivy. Glucose transport and GLUT4 protein distribution in skeletal muscle of GLUT4 transgenic mice. *Biochemical Journal* 313: 133-140, 1996.

Cain, C., W. Trimble, G. Lienhard. Members of the VAMP family of synaptic vesicle proteins are components of glucose transporter-containing vesicles from rat adipocytes. *The Journal of Biological Chemistry* 267: 11681-11684, 1992.

Clark, C. Thyroid hormone influence on oxygen consumption rates, body mass, and lipid metabolism in mice with non-insulin dependent diabetes mellitus. M.S. Thesis, California State University San Bernardino, 1995.

Colombo, M., S. Gelberman, S. Whiteheart, and P. Stahl. N-ethelymaleimide-sensitive factor-dependent α-SNAP release, an early event in the docking/fusion process, is not regulated by Rab GTPases. *The Journal of Biological Chemistry* 273: 1334-1338, 1998.

Comte, B., H. Vidal, M. Laville, J. Riou. Influence of thyroid hormones on gluconeogenesis from glycerol in rat hepatocytes: A dose response study. *Metabolism* 39: 256-263, 1990.

Emilsson, V., Y. Liu, M. Cawthorne, N. Morton, M. Davenport. Expression of the functional leptin receptor mRNA in pancreatic islets and direct inhibitory action of leptin on insulin secretion. *Diabetes* 46: 313-316, 1997

Estrada P. The effect of triiodothyronine on GLUT4 protein expression in skeletal muscle and adipose tissue of obese-diabetic *(db/db)* mice. M.S. Thesis, California State University San Bernardino, CA., 1997.

Fains, J., E. Coronel, M. Beauchamp, S. Bahouth. Expression of leptin and β_3-adrenergic receptors in rat adipose tissue in altered thyroid states. *Journal of Biochemistry* 322: 145-150, 1997.

Fehn, R., C. Clark, P. Estrada. Thyroid hormone restores metabolism in leptin-resistant *db* mice. Dept. of Biology, California State University San Bernardino, CA. 92407, FASEB Journal, 12(4), 1999, p. A430.

Fleming, K., T. Hohl, R. Yu, S. Muller, B. Wolpensinger, A. Engel, H. Englehardt, A. Brunger, T. Sollner, P. Hanson. A revised model for the oligomeric state of the N-ethylmaleimide-sensitive fusion protein NSF. *The Journal of Biological Chemistry* 273: 15675-15681, 1998.

Gibbs, M.E., J. Stock, S. McCoid, H. Stukenbrok, J. Pessin, R. Stevenson, A.J. Milici, and J. McNeish. Glycemic improvement in diabetic *db/db* mice by overexpression of the human insulin-regulateable glucose transporter (GLUT4). *Journal of Clinical Investigation* 95: 1512-1518, 1995.

Griffin, M., M. Marcucci, G. Cline, K. Bell, N. Barucci, D, L. Goodyear, E. Kraegen, M. White, and G. Shulman. Free fatty acid-induced insulin resistance is associated with activation of protein kinase C θ and alterations in the insulin signaling cascade. *Diabetes*, 48: 1270-1274, 1999.

Hadley, M. Endocrinology., New Jersey, Prentice Hall, 1992, p. 292- 296, 344- 346, and 352- 353.

Hanson, P., R. Robyn, H. Morisaki, J. Reinhard, and J. Heuser. Structural and conformational changes in NSF and its membrane receptor complexes visualized by quick-freeze-etch electron microscopy. *Cell* 90: 523-532, 1997.

Herman, G., F. Bonzelius, A. Cieutat, R. Kelly. A distinct class of intracellular storage vesicles identified by expression of the glucose transporter GLUT4. *Proceedings of the National Academy of Science* 91: 12750-12754, 1994.

Hummel, K., M. Dick, and K. Coleman. *Diabetes*, a new mutation in the mouse. *Science* 153: 1127-1128, 1966.

Kahn, B. B. Glucose transport: Pivotal step in insulin action. *Diabetes* 45: 1644-1654, 1996.

Kahn B., L. Rossetti, H. Lodish, M. Charron. Decreased *in vivo* glucose uptake but normal expression of GLUT1 and GLUT4 in skeletal muscle of diabetic rats. *Journal of Clinical Investigation* 87: 2197-2206, 1991.

Kahn, C. and G. Weir. Diabetes Mellitus, 13th ed., Philadelphia, Lea and Febiger, 1994, p. 193- 197, 320-321, and 586- 595.

Kurowski, T., Y. Lin, Z. Lou, P. Tsichlis, M. Buse, S. Heydrick, N. Ruderman. Hyperglycemia inhibits insulin activation of Akt/protein kinase B but not phosphotidylinositol 3-kinase in rat skeletal muscle. *Diabetes* 48: 653-658, 1999.

Llinas, R., I. Steinberg, K. Walton. Relationship between presynaptic calcium current and postsynaptic potential in squid giant synapse. *Biophys Journal*. 33: 323-351, 1981.

Mason, R. Statistical Design and Analysis of Experiments., New York, John Wiley and Sons, 1989, p. 301 and 307.

Mathaei, S., B. Trost, A. Hamann, C. Krausch, H. Benecke, H. Greten, W. Hopper, H. Klein. Effect of *in vivo* thyroid hormone status on insulin signaling and GLUT1 and GLUT4 glucose transport systems in rat adipocytes. *Journal of Endocrinology* 144: 347-357, 1995.

Oh S., and M. Kaplan. Early treatment of obese (*ob/ob*) mice with triiodothyronine increases oxygen consumption and temperature and decreases body fat content. *Proceedings of the Society for Experimental Biological Medicine* 207: 260-267, 1994.

Oh, S., and M. Kaplan. Early treatment of obese *ob/ob* mice with triiodothyronine increases oxidative metabolism in muscle but not in brown adipose tissue or liver. *Journal of Nutrition* 125: 112-124, 1995.

Pelleymounter, M., M.Cullen, M. Baker, R. Hecht, D. Winters, T. Boone, F. Collins. Effects of the *obese* gene product on body weight regulation in *ob/ob* mice. *Science* 269: 540-543, 1995.

Pessin J.E. and G. Bell. Mammalian facilitative glucose transporter family: Structure and molecular regulation. *Annual Review of Physiology*, 54: 911-930, 1992.

Prolo, P., M. Wong, J. Licinio. Leptin. *The International Journal of Biochemistry and Cell Biology* 30: 1285-1290, 1998.

Rossetti, L., A. Giaccari, R. DeFronzo. Glucose Toxicity. *Diabetes Care*. 13: 610-630, 1990.

Rothman J.E. Mechanisms of intracellular protein transport. *Nature* 372: 55-63, 1994.

Seeley, R., T. Stephens, P. Tate. Anatomy and Physiology 2nd ed., St. Louis, Moseby-Year Book, Inc., 1992, p. 567- 570.

Shimokawa, T., M. Kato, K. Shioduka, J. Irie, O. Ezaki. Effect of triiodothyronine on muscle cell differentiation and blood glucose level in hyperglycemic KK mice. *Biochemical and Biophysical Research Communications*, 235: 790-793, 1997.

Sollner, T., M. Bennett, S. Whiteheart, R. Scheller, J. Rothman. A protein assembly-disassembly pathway *in vitro* that may correspond to sequential steps of synaptic vesicle docking, activation, and fusion. *Cell* 75: 409-418, 1993.

Sugawara-Yokoo, M. Presence of fructose transporter GLUT5 in the S3 proximal tubes in the rat kidney. *Kidney International* 56: 1022-1028, 1999.

Torrence, C., J. Devente, J. Jones, G. Dolhms. Effects of thyroid hormone on GLUT4 glucose transporter gene expression and NIDDM in rats. *Endocrinology*, 138: 1204-1214, 1997.

Verhey, K., J. Yeh, M. Birnbaum. Distinct signals in the GLUT4 glucose transporter for internalization and for targeting to an insulin-responsive compartment. *The Journal of Cell Biology*, 130: 1071-1079, 1995.

Weinstein, S., E. O'Boyle, R. Haber. Thyroid hormone increases basal and insulin-stimulated glucose transport in skeletal muscle: The role of GLUT4 transporter expression. *Endocrinology*, 129: 4554-4564, 1991.

Weinstein, S., E. O'Boyle, M. Fisher, R. Haber. Regulation of GLUT2 glucose transporter expression in liver by thyroid hormone: Evidence of hormonal regulation of the hepatic glucose transport system. *Endocrinology* 135: 649-654, 1994a.

Weinstein, S., J. Watts, R. Haber. Thyroid hormone increases muscle/fat glucose transporter gene expression in rat skeletal muscle. *Diabetes* 43: 1185-1189, 1994b.

Zierath, J., E. Frevert, J. Ryder, P. Berggren, B. Kahn. Evidence against a direct effect of leptin on glucose transport in skeletal muscle and adipocytes. *Diabetes* 47: 1-4, 1998.

Zorzano, A., L. Sevilla, E. Tomas, A. Guma, M. Palacin, Y. Fischer. GLUT4 trafficking in cardiac and skeletal muscle: Isolation and characterization of the distinct intracellular GLUT4-containing vesicle populations. *Biochemical Society Transactions* 25: 968-974, 1997.