1997

The effect of triiodothyronine on GLUT4 protein expression in skeletal muscle and adipose tissue of obese-diabetic (db/db) mice

Paula Joanne Estrada

THE EFFECT OF TRIIODOTHYRONINE ON GLUT4 PROTEIN EXPRESSION IN SKELETAL MUSCLE AND ADIPOSE TISSUE OF 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

Paula Joanne Estrada

June 1997

THE EFFECT OF TRIIODOTHYRONINE ON GLUT4 PROTEIN EXPRESSION IN SKELETAL MUSCLE AND ADIPOSE TISSUE OF OBESE-DIABETIC (*db/db*) MICE

A Thesis

Presented to the

Faculty of

California State University,

San Bernardino

---

by

Paula Joanne Estrada

June 1997

Approved by:

[redacted]

Richard Fehn, Chair, Biology — 6/9/97 Date

[redacted]

David Polcyn

[redacted]

Michael Loik

ABSTRACT

Thyroid hormone (T_3) induces GLUT4 transporters and restores normal membrane-bound GLUT4 quantities in adipocytes and myocytes, induces thermogenesis, improves glycemic status, and normalizes the metabolism of T_3-resistant *db/db* mice. The effect of T_3 on GLUT4 transporter expression in *+/db* and *db/db* mice was investigated by comparing the cytoplasmic and membrane bound GLUT4 in adipocytes and myocytes from vehicle-treated and T_3-treated animals. Total GLUT4 in adipocytes of vehicle-treated *db/db* mice was 50% of *+/db* mice (0.886±0.31 vs. 1.99±0.37 OD units, $p \leq 0.01$) but could be restored to normal (1.928±0.21 OD units) with 500 ng T_3/g BW. Cytoplasmic GLUT4 content of adipocytes in vehicle-treated *db/db* mice was 50% less than *+/db* animals (0.624±0.22 vs. 1.488±0.36 OD units, $p \leq 0.01$). However, T_3 treatment (500 ng/g BW) increased cytoplasmic GLUT4 in *db/db* mice beyond that of *+/db* mice (1.50±0.22 vs. 1.04±0.26 OD units, $p \leq 0.01$). The GLUT4 plasma membrane fraction in *db/db* mice was also 50% less than *+/db* animals (0.262±0.09 vs. 0.502±0.06 OD units, $p \leq 0.05$) but approximated that of *+/db* mice with 500 ng T_3/g BW dose (0.428±0.28 vs. 0.406±0.20 OD units, $p > 0.05$). A disproportionate depletion of cytoplasmic GLUT4 in *db/db* mice and a concomitant increase in plasma membrane GLUT4 indicates that T_3 regulates GLUT4 translocation from the cytoplasm to the plasma membrane. Diabetic mice have reduced GLUT4 expression in the plasma membrane which likely influences glucose uptake and contributes to hyperglycemia in this model. However, thyroid hormone increases GLUT4 expression and translocation in adipose tissue of *db/db* mice.

Total myocyte GLUT4 contents are similar in *+/db* and *db/db* mice. T_3 treatment increased these quantities in both *+/db* (0.58±0.07 vs. 0.744±0.04 OD units, $p \leq 0.05$) and *db/db* (0.572±0.06 vs. 0.656±0.04 OD units, $p \leq 0.05$) mice. The cytoplasmic GLUT4 quantity in vehicle-treated *db/db* mice was greater than *+/db* mice (0.326±0.05 vs. 0.268±0.05 OD units, $p \leq 0.05$) while the membrane quantity in *db/db* mice was less than *+/db* mice (0.246±0.03 vs. 0.312±0.06 OD units, $p \leq 0.05$). Vehicle-treated *db/db* mice possess total GLUT4 quantities equivalent to *+/db* mice, but have reduced plasma membrane quantities which indicate disproportionate distributions of the GLUT4 transporter. However, T_3 treatment (500 ng/g BW) in *db/db* mice decreased cytoplasmic GLUT4 content and increased plasma membrane fraction which suggests that T_3 promotes GLUT4 translocation.

T_3-induced thermogenesis occurred in both *+/db* and *db/db* mice and the temperature increase in *db/db* mice paralleled the appearance of GLUT4 in the plasma membrane of myocytes. A 48% reduction in serum glucose concentrations was also found in *db/db* mice in response to T_3 (592.0±126 vs. 334.9±62.9 mg glucose/dL, $p \leq 0.01$). Respiratory quotient (RQ) values indicate, however, a shift to fat catabolism with T_3 treatment in *db/db* mice (0.74±0.03). The apparent metabolic substrate in T_3-treated *db/db* mice is not consistent with increased membrane-bound GLUT4 transporters facilitating skeletal muscle glucose uptake which would lead to decreased serum glucose concentrations. Enhamced glucose uptake by another tissue is suggested. This is the first study to show that T_3 restores GLUT4 translocation in *db/db* animals and may explain the glucose transport defect underlying NIDDM.

ACKNOWLEDGEMENTS

This project would not have been accomplished without the help and support of many people. Although I cannot thank every person, I would like to extend my gratitude to a few people. I thank my thesis committee Dr. Richard Fehn, Dr. Dave Polcyn, and Dr. Michael Loik for their project guidance and assistance in preparing the thesis manuscript. I especially would like to thank Dr. Richard Fehn, my major advisor, for all the time and effort he invested in me throughout the years. I sincerely appreciate his guidance, encouragement, and mentoring, but most of all his friendship.

Very special thanks go to John Tometich for his continued love and support during my graduate years. I sincerely thank him for his continued encouragement and patience throughout the course of my thesis project and thesis writing. I appreciate all that he has done however, there are not enough words to describe my gratitude.

Dr. Stuart Sumida and Dr. Beth Rega each deserve special thanks for their friendship and support throughout my graduate training as well as for continued use of the photography equipment to make those "last-minute" slides.

I cannot thank Dr. Colleen Talbot enough for serving as an adjunct member of my committee and for the invaluable assistance with preparation of my thesis. I would like to thank both Dwight Gallo and Mike Mahoney for their technical support since they were the ones who always ensured that the equipment was functioning and that my supplies were ordered. In addition, I thank Carol Smith for her continued assistance throughout the years.

My parents, brother, sister, and grandmother also deserve very special thanks for their continued love, prayers, and financial support throughout my education. I appreciate

all of their encouraging words during this time of my life as well understanding the way I am. I could not have done this without them. Thank you so much for everything. I am most grateful to all of these individuals and many others who assisted with this project.

This project was partially funded by CSUSB ASI graduate research funds, the California Pre-Doctoral Fellowship, the CSUSB Graduate Equity Fellowship, and the National Hispanic Scholarship Fund.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER ONE: INTRODUCTION

Overview of Diabetes Mellitus

Diabetes mellitus is a major health problem that appears in the written literature dating back approximately two thousand years (Kahn and Weir 1994). According to the American Diabetes Association, 16 million people are affected in the United States and an additional 500,000 to 700,000 individuals are diagnosed each year. It is the fourth leading cause of death by disease among adults in the U.S. and it is expected that 178,000 individuals per year will die from complications due to diabetes. It is a chronic disease that does not yet have a cure.

Diabetes mellitus is a syndrome characterized by high concentrations of serum glucose (hyperglycemia) due to the inadequate control of glucose levels. This results from the lack of glucose uptake by the skeletal muscle, adipose tissue, and liver, and is also associated with the inability to secrete or appropriately respond to insulin (Kahn and Weir 1994). Because insulin is the normal hormonal signal for cellular glucose uptake, an individual who has this syndrome is in a chronic hyperglycemic state. The cause of diabetes is still unknown, however both environmental and genetic factors have been implicated.

There are two general classifications of diabetes mellitus. Type I diabetes mellitus is insulin-dependent diabetes mellitus (IDDM) and is most widely understood. It is the form of diabetes most prevalent in children (juvenile onset). IDDM individuals are characteristically thin and have extremely low insulin levels. They produce minute quantities or no insulin due to dysfunctional pancreatic β-cells (the cells which are responsible for

insulin production), defects in the insulin gene, and/or defects in the processing of the insulin protein (Hadley 1992) which result in low functional levels of insulin. Because the lack of insulin prevents glucose uptake by muscle and adipose tissue, these individuals have elevated serum glucose concentrations and elevated ketone body levels due to increased fat catabolism. Excess lipolytic activity occurs in order to provide the necessary energy from fat stores in the absence of glucose uptake (Kahn and Weir 1994). IDDM comprises 10% of all diabetes mellitus cases and is the form of diabetes that is most familiar to people. Further discussion of this form is not within the scope of this project.

Type II diabetes mellitus refers to non-insulin-dependent diabetes mellitus (NIDDM) and it is the most common form of diabetes. NIDDM comprises 90% of all diabetes mellitus cases (Kahn and Weir 1994), and it is the subject of this study. It is typically adult-onset, at first asymptomatic, and rather slow in developing. Individuals are characteristically obese and produce excessive insulin, thus they are hyperinsulinemic. However, the tissues in NIDDM individuals do not respond to insulin appropriately. Therefore, NIDDM individuals are unable to effectively control glucose uptake because of insulin resistance. NIDDM may be caused by defects in the target cell such as a reduced number of receptors on the target tissue surface (DeFronzo *et al.* 1992), post-receptor defects, altered function in the insulin transduction mechanism due to reduced kinase activity (Stephens and Pilch 1995), or cellular glucose transporter defects. All of these can cause hyporesponsiveness to insulin (Hadley 1992) which would contribute to a hyperglycemic state (DeFronzo *et al.* 1992; Zorzano *et al.* 1996). Whereas nearly eighty-five percent of those individuals classified as having NIDDM are also obese, the link between diabetes and obesity remains to be

determined (DeFronzo *et al.* 1992). It has been proposed that obesity may be diet-induced (excessive intake of calories), may result from decreased energy expenditure, or may be due to altered lipid metabolism (Guerre-Millo 1996; Beck 1995). However, defective glucose transport seems to be central to the hyperglycemic aspect of the syndrome. High serum glucose levels persist since glucose is not properly handled by the insulin-hyporesponsive skeletal muscle and adipose tissue, yet, glucose uptake by the liver continues to take place and this substrate subsequently is converted to and stored as fat. This is also observed in the adipocytes and may lead to obesity because the excess glucose taken up by adipocytes can be converted into fat via lipogenesis and stored as triglycerides. Skeletal muscle from NIDDM individuals, however, is not able to take up glucose as readily by other mechanisms therefore the insulin-responsive glucose transport mechanism warrants further investigation.

Diabetic Mouse Model

In order to study non-insulin-dependent diabetes mellitus, the C57BL/KsJ diabetic (*db/db*) mouse is employed as the working animal model for this syndrome. The *diabetes* mutation arose spontaneously in the C57BL/KsJ strain and the *db* gene is autosomal recessive (Hummel *et al.* 1966; reviewed in Bray and York 1979). As in humans, this mouse model displays high serum glucose concentrations and elevated insulin concentrations, as well as age-dependent development of obesity (Hummel *et al.* 1966; reviewed in Bray and York 1979). This animal also displays decreased membrane-bound GLUT4 in adipocytes (Gibbs *et al.* 1995). Its tissues display insulin resistance and the syndrome is the result of a genetic defect. The mechanism for NIDDM in the *db/db* mouse

model yet remains to be determined. Recently, Chua *et al.* (1996) reported that the *db* gene on chromosome 4, which gives rise to the *diabetes* mutation in the C57BL/KsJ mouse, is also the gene that codes for the leptin receptor. The "weight-reducing" hormone, leptin, is said to be the protein product of the *ob* gene which is secreted by adipose tissue in proportion to the fat content (Pelleymounter *et al.* 1995; Halaas *et al.* 1995). Leptin treatment in *ob/ob* mice has been shown to decrease body weight, decrease adiposity, increase metabolic efficiency, and decrease voluntary feeding (Pelleymounter *et al.* 1995; Halaas *et al.* 1995). However, *db/db* mice have abnormally high serum leptin levels and when treated with exogenous leptin do not respond to treatment (Pelleymounter *et al.* 1995; Halaas *et al.* 1995) thus suggesting a defect in the OB-receptor, also known as the leptin receptor (Chua *et al.* 1996). The receptor may represent one component of the afferent signalling system (leptin action at the brain) but a defect at this level still does not completely explain obesity in this animal model. The efferent signal to the target tissue remains to be identified. This efferent signalling system may play a directive role in specifying target tissue responses or in regulating tissue responsiveness to other hormones.

The C57BL/KsJ diabetic (*db/db*) mouse, a model for NIDDM and obesity, has been shown to exhibit resistance to thyroid hormone (T_3). Obese-diabetic (*db/db*) mice display excess adiposity, high serum T_3 concentrations, decreased serum T_4 concentrations (Fehn *et al.* 1988), low metabolic rates (specific oxygen consumption rates) (Clark 1995), and lower body temperatures (Hummel *et al.* 1966) which suggest that these animals are resistant to thyroid hormone. The animals are therefore considered to be functionally "hypothyroid." It has been proposed that, as a result of being T_3 resistant, the obese-

diabetic mice accumulate fats due to decreased energy utilization and decreased mobilization of fat stores (Fehn *et al.* 1988). Our laboratory has shown that treatment with supraphysiological doses of T_3 can overcome the target tissue resistance leading to enhanced metabolic response, activation of fat mobilization from adipocytes, restored normal metabolic rates, and increased thermogenesis. This indicates that the T_3-resistance exhibited by the *db/db* animals is not absolute but relatively refractory. Thus, the model allows for the study of T_3 effects on physiological events, such as metabolism and glucose uptake.

Facilitative Glucose Transporters

Obesity is a common health problem in industrialized societies and it is characterized by increased adipose tissue mass (Guerre-Millo 1996). Obesity may be caused by over-eating, lack of physical activity, or may be genetically determined. It is attributed to energy imbalance, meaning that more calories are taken in as food than are expended to maintain the basal metabolic rate or to engage in physical activity. In humans, obesity is strongly associated with the development of NIDDM (Considine *et al.* 1995), and was believed to be caused by defects in the *ob* gene (Pelleymounter *et al.* 1995; Halaas *et al.* 1995). However, recent studies have found no mutations in either the *ob* gene or the *ob receptor* gene in humans, thus they are not the cause of obesity (Considine *et al.* 1996). Alternative explanations are required to explain this pathology, and may include a defect in the glucose transporter or in the vesicular translocation/trafficking mechanism. Although this defect would not explain the entire basis for obesity, it might explain the redistribution of glucose away from the muscle to liver and adipose tissues. Since the skeletal muscle cannot take up

glucose efficiently in NIDDM individuals, high serum glucose levels prevail which result in glucose uptake that predominantly occurs within the liver and adipose tissue. Glucose can be taken up in adipose tissue by way of glucose transporters that are expressed independent of insulin action and in the liver via the GLUT2 transporter (which will be discussed later). Excess glucose taken up by these cells would then be converted into fats which would contribute to increased adiposity.

Six proteins have been identified as being responsible for the facilitated diffusive transport of glucose down its concentration gradient across cell membranes and these have been designated as facilitative glucose transporters: GLUT1, GLUT2, GLUT3, GLUT4, GLUT5 (Pessin and Bell 1992; Mueckler 1994), and GLUT7 (Mueckler 1994), whereas GLUT6 (Stephens and Pilch 1995) has been identified as a pseudogene with no specific function. This transport is driven exclusively by the chemical gradient and not the hydrolysis of ATP, nor is it coupled to either sodium or proton gradients.

GLUT1 is expressed in the brain, kidney, colon, and red blood cells and is responsible for basal glucose uptake. This transporter is constituitively expressed and has a Km value of approximately 6.9 mM. This means that it has a high affinity for glucose but it saturates at physiological glucose concentrations. It is responsible for basal glucose transport and it plays an important role in glucose transport across the blood brain barrier as well as other barrier tissues (Mueckler 1994; Livingstone *et al.* 1996). GLUT2, a protein predominantly expressed in the liver, is involved in hepatic uptake and release of glucose and it apparently functions as part of a glucose sensor. This transporter has a high Km for glucose (approximately 13.2 mM), has a low affinity, and is not saturated within normal physiologic

glucose concentrations (Livingstone *et al.* 1996). Therefore, it transports glucose in quantities directly proportional to serum glucose concentrations. GLUT3 is widely distributed in human tissues (Mueckler 1994) but is predominantly expressed in the brain and is responsible for constitutive neuronal glucose uptake (Pessin and Bell 1992). GLUT3, unlike the other transporters, has a very low Km for glucose (1.8 mM) and a high affinity for glucose (Mueckler 1994) but it saturates at low glucose levels. This transporter serves as a fail-safe mechanism for glucose transport across the blood-brain-barrier in order to ensure that the brain is constantly supplied with glucose, even during hypoglycemic states. The GLUT4 glucose transporter is an insulin-regulatable glucose transporter that is found in adipose tissue, cardiac muscle, and skeletal muscle. This transporter will be discussed in more detail below. GLUT5 is expressed in the jejunum. It has been characterized as being responsible for intestinal absorption of fructose and possibly other hexoses (Pessin and Bell 1992; Mueckler 1994). GLUT6 has been identified as a pseudogene having no specific target tissue or function (Stephens and Pilch 1995). GLUT7 transporter is expressed in intracellular membranes of hepatocytes and possibly other gluconeogenic tissues. This transporter allows glucose to diffuse freely from the endoplasmic reticulum of gluconeogenic tissues in response to the action of glucose-6-phosphatase on glucose-6-phosphate (Mueckler 1994; Stephens and Pilch 1995). However, the actual significance of this transporter's function remains unclear. The six different glucose transporter isoforms have distinct characteristics and functions that are dependent on the tissue in which they are expressed. As such, the glucose transport rates of these glucose transporter isoforms are characteristic of the function of the target tissue.

The GLUT4 glucose transporter protein, the focus of this project, is expressed in skeletal muscle, cardiac muscle, and adipose tissue. It has a Km value of approximately 4.6 mM, it is positively responsive to insulin, and is involved in insulin-stimulated glucose uptake (Pessin and Bell 1992, Stephens and Pilch 1995). This transporter is responsible for a rapid increase in glucose uptake in response to increased serum insulin concentrations (Mueckler 1994; Stephens and Pilch 1995). When stimulated by insulin, GLUT4 concentrations in the plasma membrane of cells normally increase approximately 20-fold (Pessin and Bell 1992). It has been reported that the plasma membrane GLUT4 fraction in adipose tissue (Stephens and Pilch 1995) and skeletal muscle (Zorzano *et al.* 1996) of obese NIDDM patients is reduced. This would have an overall impact on glycemic regulation since glucose cannot be readily taken up by the target tissues. Thus, it would be of importance to gain a better understanding of the GLUT4 regulatory mechanisms and expression in NIDDM. Since NIDDM individuals have impaired glucose uptake, alterations in the structure, function, or regulation of the glucose transporters, specifically GLUT4, are good candidate mechanisms for transport defects associated with this syndrome. The GLUT4 glucose transporter is an integral membrane component found in insulin responsive tissues. Little is known of the regulatory mechanisms which control GLUT4 transporter expression and distribution in the skeletal muscle and adipose tissue. This will be the central focus of the study.

GLUT4 Vesicular Trafficking

The GLUT4 transporter facilitates glucose uptake across the plasma membrane

between the serum and cell cytoplasm and normally increases in number with increased insulin concentrations. The GLUT4 transporter protein is processed and translocated like other proteins within the cell by the following mechanisms. Proteins are synthesized at the endoplasmic reticulum and move toward the Golgi complex for further processing and modification within a spike-coated vesicle as part of a process known as vesicular trafficking (Morris and Frizzell 1994; Gauter *et al.* 1994). The vesicle fuses with the *cis*-face of the Golgi complex, and subsequently buds from the organelle. The vesicle then fuses with the *medial*-face of the Golgi complex and after further modification and processing, the vesicle buds. The budded vesicle then approaches and fuses with the *trans*-face of the Golgi complex. Within the *trans*-face of the Golgi complex, the vesicle is labelled with mannose-6-phosphate receptors and these identify the vesicle as one which will reside in the cytoplasm. The vesicle is removed from the secretory pathway and is localized in a region of the Golgi complex where the vesicle becomes clathrin-coated. Such processing is characteristic of lysosomal vesicles. However, if the vesicle is to be secreted, it remains uncoated, as is the case with GLUT4-containing vesicles (Stephens and Pilch 1995). Subsequently, these vesicles with their protein contents are then transported towards the plasma membrane (translocation) where the vesicles will dock and fuse with the plasma membrane and incorporate their contents into the membrane as integral transmembrane proteins (Appendix 1).

Vesicles are believed to be transported through the cytoplasm driven by kinesin along microtubules (Morris and Frizzell 1994). A vesicle attaches to kinesin, a molecule that serves as the motor in the translocation process, and this molecule is activated by Ca^{2+}. The

intracellular activity of kinesin is regulated by the influx of Ca^{2+} into the cell or Ca^{2+} release from the endoplasmic reticulum (or the sarcoplasmic reticulum if the process is occurring within the skeletal muscle). This proportedly stimulates movement of kinesin along a microtubule, which transports the vesicle towards the surface of the cell (Morris and Frizzell 1994). The vesicle then comes in contact with various docking proteins at the membrane surface. These proteins enable the vesicle to dock and fuse thereby integrating into the membrane the vesicular protein components (Appendix 2).

Vesicular translocation, docking, and fusion with the plasma membrane involve a series of steps regulated by several key proteins. The vesicle membrane contains the proteins synaptobrevin and synaptotagmin (12 kDa and 65 kDa integral membrane proteins, respectively). These form a 7S docking complex with a 25 kDa protein SNAP-25 (Synaptosomal Associated Protein-25), and syntaxin (32 kDa), both of which are located in the plasma membrane of the cell (Scheller 1995; Bark and Wilson 1994). As α-snap (Soluble NSF Associated Protein) associates with the 7S complex synaptotagmin is dislodged. A 20S complex is formed when cytoplasmic NSF (N-ethylmaleimide-Sensitive Factor) associates with α-snap on the 7S complex. NSF protein binds ATP and, as a result of ATP hydrolysis, NSF and α-snap dissociate from the 20S complex. Syntaxin, which is tightly associated with the integral membrane proteins Neurexins and Ca^{2+} channel proteins, is thus exposed and is able to associate with synaptotagmin on the vesicle. The association stimulates a conformational shift of the associated complex causing an influx of Ca^{2+}. Synaptotagmin, which is sensitive to Ca^{2+}, binds Ca^{2+} and this causes another conformational shift in the associated proteins. This shift subsequently causes SNAP-25 and the syntaxin-

synaptotagmin fusion complex to separate along the cell membrane thereby creating a "gap" in the cell membrane which enables the proteins from the vesicle to become embedded within the membrane (Bark and Wilson 1994; Scheller 1995). Stephens and Pilch (1995) state that the docking-fusion mechanism of GLUT4-containing vesicles is similar to that of the neurotransmitter secretion model (as described above), differing only in a few docking proteins that are specific for GLUT4. Although the GLUT4 translocation mechanism may be a point for potential defects within NIDDM, non-functional docking proteins on the vesicle or plasma membrane as well as lack of fusion and integration of vesicular contents are also potential candidates. The current study will address translocation and docking of the GLUT4 glucose transporters in tissues from normal and obese-NIDDM subjects.

GLUT4 Studies

Studies in transgenic mice, into which the functional GLUT4 gene has been transfected from humans, and diabetic animal models have consistently shown that insulin increases the quantity of GLUT4 at the plasma membrane of adipose tissue and leads to a concomitant decrease in cytoplasmic GLUT4 quantity. Zorzano *et al.* (1996) reported that insulin treatment similarly caused the GLUT4 quantity in adipose tissue and skeletal muscle to increase at the plasma membrane and decrease in the cytoplasm. Other studies using transgenic animals showed that tissues from transgenic animals (skeletal muscle and/or adipose tissue) had significantly greater GLUT4 present in the plasma membrane in comparison to their non-transgenic counterparts (Brozinick *et al.* 1996; Gibbs *et al.* 1995; Olson and Pessin 1995; and Hansen *et al.* 1995). Upon insulin stimulation, there was a

more dramatic increase in membrane bound GLUT4 transporter quantity in the transgenic mice in comparison to non-transgenic mice. There was a concomitant decrease in the GLUT4 quantity in the cytoplasm indicating that GLUT4-containing vesicles translocated from the cytoplasm to the plasma membrane. Gibbs *et al.* (1995) utilized a hGLUT4 *db/db* transgenic mouse that carries a functional human GLUT4 gene which leads to GLUT4 transporter overexpression. This study showed that the glucose transport defect (reduced plasma membrane GLUT4 content) in transgenic *db/db* mice could be compensated for by inducing extra GLUT4 transporters which correct for insulin resistance. This indicated that insulin insensitivity in this model may be at the level of vesicular translocation, docking, or fusion, since upregulation of transporter expression could overcome the glucose transport defect. The transgenic *db/db* mice showed a marked decrease in hyperglycemia due to increased glucose transport via the GLUT4 glucose transporter in comparison to the non-transgenic *db/db* mice. The target tissues, skeletal muscle and adipose tissue, in these mice are resistant to insulin's action on GLUT4 induction and/or translocation which suggests that GLUT4 translocation may be regulated by another factor or another hormone since translocation took place in the absence of insulin signalling. The translocation defect can be compensated by GLUT4 overexpression but it can also be potentially corrected by the direct action of another hormone other than insulin on elements involved in the translocation process. If this is the case, an increased number of GLUT4 transporters at the plasma membrane could facilitate increased glucose uptake and could improve the glycemic status of the NIDDM subjects. Since the process of GLUT4 transporter protein expression in the plasma membrane and translocation is insulin-responsive, the regulation of the GLUT4

transporter is a likely candidate for altered function in non-insulin-dependent diabetes mellitus and warrants further investigation.

NIDDM and Obesity

Several factors that may contribute to the onset of obesity include overeating, metabolic defects, hypothalamic disorders, increases in carbohydrate, fat, or protein intake, and decreased physical activity (Beck 1995; Guerre-Millo 1996). Adiposity can manifest in general categories of presentation which reflect either cellular hypertrophy or hyperplasia, or both. Enlargement of the adipocytes may be due to an increase in cell volume which is associated with excessive fat storage and is characteristic of hypertrophy. Alternatively, an increase in the overall number of adipocytes is characteristic of hyperplasia which results from excessive mitotic divisions (Hadley 1992). Adipocytes within adipose tissue serve as an efficient energy repository by housing fat droplets which contain two times the amount of potential energy per unit of weight compared to either proteins or carbohydrates. However, the prevalence of obesity in relationship to non-insulin-dependent diabetes mellitus is not yet understood.

Eighty-five percent of individuals with NIDDM are also characteristically obese. Studies have reported increased adipocyte volumes and decreased membrane-bound GLUT4 in adipocytes of NIDDM subjects (Fabres-Machado and Saito 1995) and obese-*db/db* animals (Estrada unpublished observations). The adipocytes in subjects with NIDDM generally increase in lipid content, cell number, and/or cell size (Bjorntorp 1987), thus accounting for the obesity associated with the syndrome. Obesity is also associated with

insulin resistance such that insulin-sensitive tissues (primarily skeletal muscle and adipose tissue) are not able to respond to insulin appropriately and thus have impaired glucose uptake. Since the majority of NIDDM individuals are obese, perhaps obesity is also associated with alterations in facilitated glucose transport via defective GLUT4 transporter function. Onset of obesity in NIDDM subjects, including the *db/db* mouse, is associated with decreases in both specific O_2 consumption, as well as total metabolic rate (Clark 1995; Jequier 1987). These responses are also observed in a phenotypically-related model, the *ob/ob* mouse (Boissonneault *et al.* 1978; Oh and Kaplan 1994). The reduced metabolism of NIDDM subjects apparently conserves energy, which is then stored causing increased adiposity. The mechanism that results in a decreased metabolic rate and increased adiposity (potentially leading to the onset of NIDDM) however, still remains undetermined.

Glucoregulatory and Thyroid Hormones

There are many hormonal signals that are involved in regulating glycemic status, such as glucagon, insulin, and the catecholamines (specifically epinephrine). Glucagon stimulates the release of glucose from the liver via glycogenolysis (in order to maintain short-term levels of blood glucose in a well-fed animal) or gluconeogenesis (in order to maintain glucose levels under prolonged fasting or exercise periods). Insulin is secreted by the Islets of Langerhans in the pancreas, specifically the β-cells, in response to elevated serum glucose levels. Insulin enhances glucose uptake by the hepatocytes, myocytes, and adipocytes where it is metabolized, stored as glycogen, or used as a substrate to synthesize proteins or fats. Epinephrine, however, inhibits glucose uptake by adipocytes during periods of stress. This

ensures that during times of such activity, glucose is taken up more readily by the liver, skeletal muscle, or brain instead of being stored away. Thus, serum glucose levels are not regulated solely by one hormone but rather regulation involves the action of multiple hormones that are dependent on the physiological status of the individual.

While a variety of causative agents (mentioned above) have been proposed in the regulation of the glycemic status within an individual, another hormone, triiodothyronine (T_3), may be indirectly involved in regulating the glucose levels. Thyroid hormone plays a role in the regulation of growth, development, and metabolism (Glass and Holloway 1990). Thyroid hormone controls the baseline metabolic rate of animals and is also permissive to the action of other hormones such as insulin (Hadley 1992). This means that thyroid hormone action is required for insulin to act on the insulin-sensitive tissues and elicit a response. However, NIDDM individuals are resistant to insulin and their tissues cannot adequately take up glucose. Thus, thyroid hormone's permissive action to insulin should increase insulin sensitivity at the target tissues and thyroid hormone should be accounted for as a potential mediator in NIDDM.

Thyroid hormone is regulated at several levels and by several tissues. The hypothalamus regulates pituitary gland secretion of thyroid stimulation hormone (TSH) via thyrotropin releasing-hormone (TRH). The pituitary gland produces and releases Thyroid-Stimulating Hormone (TSH) which, in turn, stimulates the thyroid gland to produce thyroxine (T_4), the metabolically inactive form of thyroid hormone. T_4 is converted to the metabolically active triiodothyronine (T_3) via 5'-monodeiodinase in the liver and kidney. T_3 is responsible for regulating metabolic activity within most cells (Oppenheimer 1994). It

acts by binding to nuclear receptors located on the DNA where it forms dimeric hormone-receptor complexes which are responsible for regulating gene transcription and the expression of gene products. This hormone is responsible for regulating metabolism at the tissue level. It potentiates lipogenesis (fat synthesis) in the liver by inducing the synthesis of hepatic lipogenic enzymes, lipolysis (fat catabolism) in adipose tissue by inducing the lipolytic enzymes (Glass and Holloway 1990), and thermogenesis (heat production) in skeletal muscle by inducing Na^+/K^+-ATPase (Glass and Holloway 1990; Oppenheimer 1994). It is, therefore, an important regulator of overall energy balance.

The *db/db* mice are resistant to thyroid hormone (T_3) which means that the target tissues are relatively unresponsive to the action of thyroid hormone. While these animals have high serum T_3 levels which make them technically hyperthyroid, they are functionally hypothyroid since they are hyporesponsive to T_3. The target tissue resistance can be overcome with supraphysiological doses which results in an enhanced metabolic response, activation of fat mobilization from adipocytes, restoration to normal metabolic rates, and increased thermogenesis (Clark 1995; Estrada unpublished results). These are responses that are potentially linked to glucose transport since an increase in glucose uptake can provide the substrate to be utilized in metabolic and thermogenic processes. Since *db/db* animals are T_3 resistant, thyroid hormone cannot elicit its permissive action to insulin within the animal's tissues. This may contribute to the insulin resistance characteristically seen in NIDDM subjects. Thus, the insulin-responsive GLUT4 transporter is a likely candidate for thyroid hormone regulation.

Thyroid hormone has been reported to regulate GLUT4 expression in adipose tissue (Matthei *et al.* 1995) and skeletal muscle (Weinstein *et al.* 1994; Weinstein *et al.* 1991; Casla *et al.* 1990). A pilot study (Estrada unpublished results) on the effect of T_3 on GLUT4 expression has also shown that T_3 regulates the GLUT4 transporter in the *db/db* mouse. Thyroid hormone treatment regulates the redistribution (translocation) of the GLUT4-containing vesicles within the cytoplasm of adipocytes towards the plasma membrane and may regulate the docking-fusion mechanism (Estrada unpublished results). However, no published information exists on the role of thyroid hormone on GLUT4 glucose transporter regulation in *db/db* mice.

Proposed Effect of Thyroid Hormone on GLUT4 expression

In the present study, the effect of T_3 on GLUT4 glucose transporter expression will be assessed in lean C57BL/KsJ *+/db* and obese-diabetic C57BL/KsJ *db/db* mice. This will be done by comparing the content of GLUT4 transporters in adipose tissue and skeletal muscle from vehicle-treated normal and obese-diabetic mice with the tissues obtained from normal and diabetic mice treated with exogenous T_3 for nine days. Because adipose tissue and skeletal muscle in *db/db* mice are resistant to insulin (reviewed in Bray and York 1979; Hummel *et al.* 1966), the quantity of GLUT4 present in the plasma membrane is expected to be below normal since insulin action is needed to induce GLUT4 mRNA expression (Sinha *et al.* 1991). The quantity of GLUT4 in the cytoplasmic fraction (the result of gene transcription and translation) and plasma membrane fraction (the result of protein transport, docking, and fusion) will be evaluated.

The target tissues in individuals with NIDDM are resistant to insulin leading to impaired glucose uptake and hyperglycemia. In insulin- and T_3-resistant *db/db* mice, the insulin response may be restored via overcoming the thyroid hormone resistance with supraphysiological doses of T_3. This could then result in regulation of the GLUT4 transporter via T_3 treatment. Under the assumption that T_3 may play a role in GLUT4 expression and/or vesicular trafficking, treatment with thyroid hormone should increase GLUT4 transcription. This, in turn, should lead to increased translation of the GLUT4 transporter protein. Subsequently, an increase in GLUT4-containing vesicles should be observed, as well as increased vesicle fusion with the plasma membrane of adipocytes and myocytes of *db/db* mice.

There are five potential sites of action for regulation of the GLUT4 glucose transporter if the GLUT4 quantity at the plasma membrane increases in response to T_3 treatment. 1) T_3 may regulate gene expression at the transcriptional level because thyroid hormone has nuclear receptors on the DNA. This could potentially regulate gene transcription of mRNA for the GLUT4 transporter or various elements involved in the GLUT4 trafficking mechanism; 2) This event could alternatively lead to increased translation of the GLUT4 transporter protein by enhancing translational efficiency; 3) or by stabilizing the GLUT4 transporter mRNA; 4) Triiodothyronine might also increase incorporation of GLUT4 transporters into vesicles via regulation of processing occurring within the Golgi complex; 5) Thyroid hormones (T_4 in this case) have been shown to regulate actin polymerization and this may play a role in GLUT4 vesicle transport and fusion with the plasma membrane (Leonard 1997). An assessment of total GLUT4 protein expression and

cellular distribution was performed in a pilot project to narrow these possibilities.

The pilot project of GLUT4 expression in adipocytes was conducted using an experimental design similar to that proposed for this study except that the tissues were not homogenized fresh but rather after storage at -20°C. Normal (*+/db*) and diabetic (*db/db*) mice were treated with exogenous T_3 (0 ng/g BW or 500 ng/g BW) for ten days and each treatment group consisted of five animals. Animals were euthanized on day 10 of study with CO_2. Adipose tissue was extracted and homogenized in order to separate the cytoplasmic and plasma membrane fractions. Homogenates (0.5 µg protein/100 µl) were loaded onto nitrocellulose paper using a dot blot apparatus for immunodetection of the GLUT4 transporter. A commercial polyclonal GLUT4 antibody (rabbit-anti-insulin regulatable glucose transporter, East Acres Biologicals) was used to identify the GLUT4 transporter and was visualized using chemilumenescence detection (Amersham RPN 2108). GLUT4 transporters were quantified using a Bio-Rad video densitomter and data were reported as OD units.

Total GLUT4 expression in *+/db* mice increased with T_3 treatment which suggested GLUT4 induction in response to T_3. In *db/db* mice, total GLUT4 expression decreased with T_3 treatment (Table 1). The quantity of GLUT4 in the cytoplasm of *+/db* mice increased with T_3 treatment (0.158±0.07 vs. 0.196±0.05, repectively; $p \leq 0.05$) whereas in *db/db* animals, the cytoplasmic quantity decreased in response to T_3 (0.284±0.04 vs. 0.178±0.07, respectively; $p \leq 0.05$). The quantity of membrane-bound GLUT4 transporter in *+/db* animals did not change with T_3 treatment (0.168±0.05 vs. 0.170±0.02, respectively; $p > 0.05$) which suggested T_3 had no effect and represented normal turnover. However, in *db/db*

TABLE 1. OD readings of adipocyte cytoplasmic and membrane-bound GLUT4 proteins in vehicle-treated (0 ng T_3/g BW) and T_3-treated (500 ng /g BW) normal (*+/db*) and diabetic (*db/db*) mice. (Values± SEM, n=5).

Phenotype/Treatment Group	Cytoplasm	Membrane-Bound	Total GLUT4
+/0	0.158 ±0.068	0.168 ±0.053	0.326±0.16
db/0	0.284 ±0.040	0.062 ±0.058	0.346±0.13
+/500	0.196±0.047	0.170 ±0.020	0.366±0.23
db/500	0.178 ±0.071	0.090 ±0.024	0.268 ±0.12

animals, the GLUT4 quantity increased at the plasma membrane with T_3 as a concomitant decrease in cytoplasmic GLUT4 was observed. These were indications that GLUT4 transporters were being redistributed from the cytoplasm to the plasma membrane in *db/db* animals. The intent of the pilot study was to determine if thyroid hormone altered GLUT4 transporter expression in *db/db* mice. Based on this study, it was concluded that thyroid hormone was not likely to be affecting GLUT4 transporter mRNA transcription in the insulin-resistant *db/db* mice because a pool of cytoplasmic GLUT4 transporters existed in vehicle-treated *db/db* mice.

Adipocytes were evaluated in the pilot study as a reference point so as to provide a framework on the role of thyroid hormone in regulating GLUT4 expression. This could then be used to formulate hypotheses in reference to the action of thyroid hormone on GLUT4 expression in another insulin responsive tissue, skeletal muscle, and the impact it would have on metabolic responses observed in other studies. In the current study, GLUT4 protein expression and subcellular distributions will be assessed in normal and diabetic mice to see if T_3 influences GLUT4 transporter trafficking phenomena. This will be done by quantifying the total GLUT4 transporter present in the cell cytoplasm and plasma membrane

under various thyroid hormone treatments to determine if GLUT4 transporter translocation from the cytoplasm to the plasma membrane occurs. The total GLUT4 quantity will be indicative of the induction process and the distribution of the cytoplasmic and membrane-bound GLUT4 fractions will be representative of the trafficking mechanism. The implication of increased numbers of membrane-bound GLUT4 transporters is that it would allow for increased glucose transport into skeletal muscle and adipose tissue. This would then be utilized by the skeletal muscle as a fuel source for thermogenesis. If an increase in heat production does occur, the body temperatures of *db/db* mice should increase and an increase in oxygen consumption should be observed.

CHAPTER TWO: MATERIALS AND METHODS

Animals and Tissues

C57BL/KsJ heterozygous normal (*+/db*) and homozygous diabetic (*db/db*) mice were obtained from Jackson Laboratories (Bar Harbor, Maine). The animals were housed at approximately 23 °C under a 14:10 light:dark cycle. Lights were on from 6 am until 8 pm daily, and five animals were housed per cage in 11" long X 7" wide X 5" tall plastic cages with wood chip bedding and white tissue. Water and Teklab 4% rodent chow were available *ad libitum*.

Female *+/db* and *db/db* mice, 8-10 week old, were preconditioned 1 week prior to study by handling, exposure to metabolic chambers, and being subjected to temperature reading procedures. After preconditioning, animals received daily intraperitoneal injections of thyroid hormone (L-3,5,3' triiodothyronine; Sigma #T2877) in doses of 0, 100, 200, and 500 ng T_3/g body weight for nine consecutive days (Kalousek 1986; Clark 1995). T_3 was prepared daily using 100 μg/ml in 0.5 mM NaOH and was protected from light via wrapping the injection bottle with aluminum foil. The control animals received intraperitoneal injections of 0.5 mM NaOH in proportional volumes (2 μl/g body weight). Each treatment group consisted of five *+/db* and five *db/db* animals. Injections were administered between 8 am and 10 am after daily body weights, metabolic readings, and body temperatures were recorded. On day 10, animals were euthanized by CO_2. Blood was collected from each animal by cardiac puncture. Tissues (adipose tissue and skeletal muscle) were excised and processed (see below) to obtain homogenates for GLUT4 analysis.

Metabolic Rate Determinations and Temperature Studies

Oxygen consumption and carbon dioxide production rates of the mice were measured for 3 minutes each day for 10 days using an Ametek S-3A/II Oxygen Analyzer and an Ametek CD-3A Carbon Dioxide Analyzer. An open flow system using room air was utilized and was calibrated daily prior to use with gases of known concentrations. Readings were taken at a constant flow rate of 400 ml per minute (as determined by Gilmont Flowmeter, model D-665) in order to determine relative metabolic responses. Daily rectal body temperatures for normal and diabetic mice were recorded using a temperature probe (Sensortek Thermocouple, model BAT-12) and radiant heat was measured on days 7-10 with a Microprocessor Thermometer (Type J-K-T Thermocouple, model HH21). Data are reported as respiratory quotient (RQ, ml CO_2/ ml O_2) and temperature (°C).

GLUT4 Fractionation Studies

Omental adipose tissue (0.25 g) and gastrocnemius skeletal muscle (0.05 g) were excised and homogenized in 1 ml of STM/PMSF buffer (0.32M sucrose, 3mM $MgCl_2$, and 0.5% Tween 20, 0.1M Phenylmethylsulfonyl Fluoride) using a motorized glass homogenizer at room temperature for 45 seconds. Homogenates were centrifuged at 14,000 g for 5 minutes at 4°C (Herman *et al.* 1994) to produce a supernatant containing the cytoplasmic vesicles and a pellet containing the plasma membrane with docked vesicles. The supernatant (approximately 1 ml) was transferred to a fresh microcentrifuge tube. In order to solubilize membrane-bound proteins in the pellet (docked vesicles and fused transporters), 5 ml of STM buffer was added to the pellet and this was subsequently sonicated (Bronwill Biosonik

model) for 1 minute at 80% power and the resultant homogenate centrifuged at 14,000 g for 5 minutes at 4°C (Herman *et al.* 1994) to remove remnants of the plasma membrane and vesicles. The homogenates were stored at -20°C until analyzed.

GLUT4 Quantification

The Coomassie Brilliant blue method (Bradford 1976; Matthaei *et al.* 1995) using a Bovine Serum Albumin (BSA, Sigma #A7906) standard was utilized to quantify the protein content of the supernatants and the pellets.

A 100 µl aliquot of homogenate (approximately 0.313 µg) was subjected to dot blotting and immunodetection using a polyclonal antibody against GLUT4 (Rabbit Anti-Insulin Regulatable Glucose Transporter, East Acres Biologicals) (Burcelin 1993). A 96-well microtiter plate was prepared that contained various dilutions of the supernatant and pellet samples resulting in 0.313 µg total protein/100 µl for dot blot procedures. A 100 µl aliquot of each sample was loaded into individual wells of the dot blot manifold. Samples were loaded on to the nitrocellulose membrane (0.2 µm, Protran #BA83) by vacuum and each well was rinsed twice with 100 µl TBS (20 mM Tris, 500 mM NaCl, and 0.0001% merthiolate, pH 7.5). The nitrocellulose paper was blocked with BLOTTO (5% Carnation non-fat milk in TBS, Tris-buffered Saline) for 30 minutes at room temperature, while shaking. A 1:10,000 dilution of GLUT4 polyclonal antibody (5 µl in 50 ml BLOTTO) was added to the blot which was incubated in a covered polyethylene dish while shaking overnight at room temperature. This was followed with 3 BLOTTO rinses for 10 minutes each at room temperature. After the third rinse, 10 µl of the secondary antibody

(biotinylated goat-anti-rabbit IgG, Sigma#B-9642) in 25 ml BLOTTO (a 1:2500 dilution) was added and incubated at room temperature for 3 hours while shaking. Following this incubation, blots were rinsed in TBS 3 times at room temperature for 10 minutes each. Twenty microliters of avidin-horse radish peroxidase (AV-HRP, Bio-Rad) in 20 ml TBS (a 1:10,000 dilution) was added and incubated at room temperature for 1 hour while shaking, followed by 3 rinses in TBS. The blot was then subjected to chemiluminescence detection (Amersham #RPN2108) using Kodak RP X-Omat film. The GLUT4 fluorograms were quantified using a Bio-Rad model 620 video densitometer (Herman *et al.* 1994). GLUT4 protein data are reported as Optical Density (O.D.) units.

Serum Glucose Determination

Serum glucose concentrations were determined with a commercial glucose assay kit (Sigma #115A) using 0.4 µl of serum from each animal. This assay produces a colorimetric response measured at 520 nm using a microplate reader (BIO-TEK instruments, Model EL 312e) that is proportional to the serum glucose concentrations. Data are reported as mg/dL.

Statistical Analysis

Data represeneting body mass, temperature, metabolic studies, GLUT4 transporter protein characterization, and the serum glucose assay were subjected to multifactorial analysis of variance (ANOVA) and Duncan's Multiple Range Test (Mason *et al.* 1989). Data are reported as means ± SEM and n=5 in all cases. A single asterisk (*) denotes significance at $p \leq 0.05$ and two asterisks (**) denote significance at $p \leq 0.01$.

CHAPTER THREE: RESULTS

GLUT4 Fractionation Characterization

The effect of T_3 on GLUT4 transporter expression in *+/db* and *db/db* mice was investigated by comparing the total GLUT4 transporter expression, cellular distribution of GLUT4 transporter (cytoplasmic GLUT4 protein fraction and membrane bound GLUT4), and the fraction of GLUT4 transporters associated with the plasma membrane in adipocytes and myocytes obtained from vehicle-treated and T_3-treated animals. Tissue homegenates consisted of primarily myocytes (skeletal muscle) and adipocytes (adipose tissue).

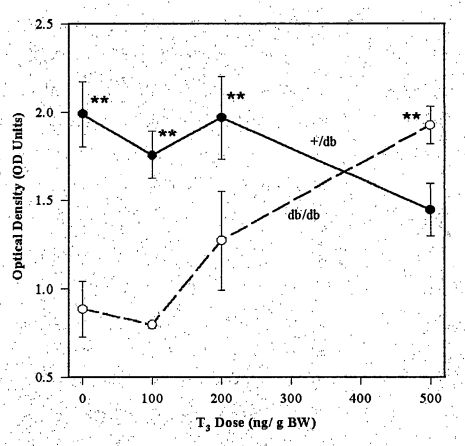


Figure 1. Total GLUT4 transporter content in adipocytes of normal (*+/db*) and diabetic (*db/db*) mice treated with T_3 (0, 100, 200, and 500 ng/g BW). Comparisons are between phenotypes at the given dose (Values are ± SEM, n=5).

Total GLUT4 transporter quantity (cytoplasmic fraction and membrane-associated fraction) was assessed in adipocytes of *+/db* and *db/db* mice in response to T_3 (Figure 1).

The total GLUT4 quantity in adipocytes of *+/db* animals that were vehicle-treated or treated with 100 or 200 ng T_3/g BW were not altered. In the mice that received 500 ng T_3/g BW dose, the total GLUT4 quantity decreased in comparison to the vehicle-treated animals (1.758±0.15 vs. 1.99±0.19 OD units, respectively; $p \leq 0.01$). These data suggest that within physiological concentrations T_3 treatment does not alter the number of GLUT4 glucose transporters and apparently does not regulate induction. In *db/db* mice, the total GLUT4 quantity in adipocytes of vehicle-treated animals was approximately 50% less than normal, vehicle-treated animals (0.886±0.15 vs. 1.99±0.19 OD units, respectively; $p \leq 0.01$). However, increasing doses of T_3 increased total GLUT4 transporter expression in *db/db* mice (Figure 1) with maximal expression occurring at 500 ng T_3/g BW. At this dose, the total quantity of GLUT4 transporters was greater than vehicle-treated *db/db* mice (1.928±0.11 vs. 0.886±0.15 OD units, respectively; $p \leq 0.01$) and approached the quantity observed in vehicle-treated, *+/db* animals (1.928±0.11 vs. 1.99±0.19 OD units, respectively; $p > 0.01$). This suggests that GLUT4 transporter induction occurred in response to T_3-treatment which resulted in normalization of total GLUT4 transporter quantities.

The quantity of GLUT4 transporters in the cytoplasm of adipocytes was assessed in *+/db* and *db/db* mice (Figure 2). The baseline expression of GLUT4 transporter protein in the cytoplasm of adipocytes of vehicle-treated, *+/db* animals was 1.488±0.18 OD units and no change was observed at 100 and 200 ng T_3/g BW doses. Treatment with 500 ng T_3/g BW, however, caused a decrease in quantity (1.488±0.18 vs. 1.04±0.13 OD units, respectively; $p \leq 0.01$). The baseline quantity of cytoplasmic GLUT4 transporters in

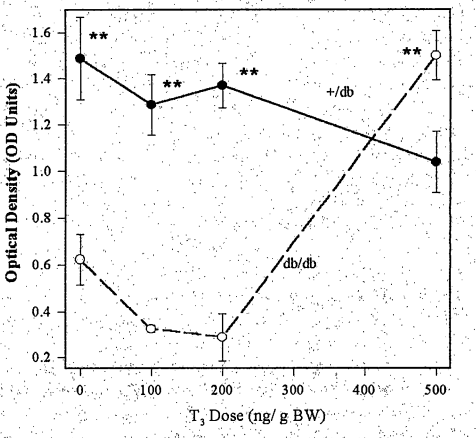


Figure 2. Cytoplasmic GLUT4 transporter content in adipocytes of normal (*+/db*) and diabetic (*db/db*) mice treated with T_3 (0, 100, 200, and 500 ng/g BW). Comparisons are between phenotypes at the given dose (Values are ± SEM, n=5).

adipocytes of *db/db* mice was approximately 50% less than normal, vehicle-treated animals (0.624±0.11 vs. 1.488±0.18 OD units, respectively; $p \leq 0.01$). In diabetic mice, the cytoplasmic quantity of GLUT4 transporters at the dose of 100 ng T_3/g BW decreased significantly to 0.326±0.02 OD units ($p \leq 0.05$) and again at the physiological hyperthyroid dose of 200 ng T_3/g BW (0.288±0.10 OD units; $p \leq 0.05$) in comparison to the vehicle-treated *db/db* mice. However, the quantity of GLUT4 transporter within the cytoplasm at the supraphysiological dose (500 ng T_3/ g BW) increased more than 2-fold in comparison to the vehicle-treated, *db/db* animals (1.55±0.22 vs. 0.624±0.22 OD units, respectively; $p \leq 0.01$), and approached values of normal, vehicle-treated animals (1.50±0.22 vs. 1.488±0.36, respectively; $p > 0.05$). These data suggest that T_3 treatment plays a role in normalizing the cytoplasmic quantity of GLUT4 transporters in *db/db* mice.

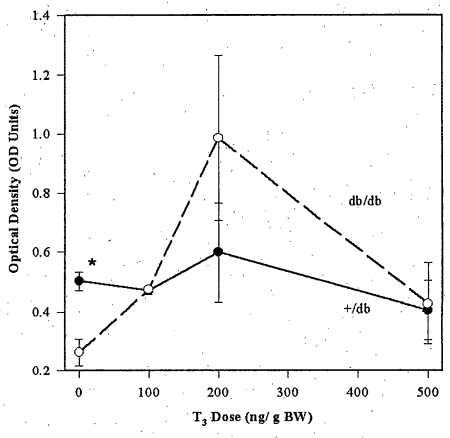


Figure 3. GLUT4 quantity associated with plasma membrane of adipocytes of normal (*+/db*) and diabetic (*db/db*) mice treated with T_3 (0, 100, 200, and 500 ng/g BW). Comparisons are between phenotypes at the given dose (Values are ± SEM, n=5).

The baseline GLUT4 quantity associated with the plasma membrane of adipocytes in vehicle-treated *+/db* mice, shown in Figure 3, was approximately 0.502±0.03 OD units; T_3 did not change this value at any dose (0.470±0.01, 0.600±0.17 OD units, and 0.406±0.10 OD units respectively, $p>0.05$). These values represent the normal expression levels of GLUT4 transporters at the plasma membrane. The baseline expression of the GLUT4 transporters associated with the plasma membrane of adipocytes from vehicle-treated, *db/db* mice was approximately 50% less than normal mice (0.262±0.05 vs. 0.502±0.03 OD units, respectively; $p\leq0.05$). The replacement dose of 100 ng T_3/g BW restored membrane-bound GLUT4 levels to those of normal, vehicle-treated animals (0.474±0.01 vs. 0.502±0.03 OD units, respectively; $p>0.05$). At the hyperthyroid dose (200 ng T_3/g BW), the quantity of GLUT4 transporter associated with the plasma membrane increased dramatically in

comparison to vehicle-treated *db/db* mice (0.262±0.05 vs. 0.986±0.55 OD units, respectively; $p \leq 0.05$) and exceeded that of *+/db* mice treated with the same dose (0.986±0.28 vs. 0.600±0.17, respectively; $p \leq 0.05$). However, the GLUT4 quantity in *db/db* mice at the supraphysiological dose was indistinguishable from that of normal animals (0.428±0.14 vs. 0.406±0.10 OD units, respectively; $p > 0.05$). The membrane-bound GLUT4 quantity of *db/db* mice increased with the replacement and hyperthyroid doses in conjunction with decreases in cytoplasmic GLUT4 transporter content. This redistribution suggests that T_3 may play an important role in supporting vesicular translocation.

The proportion of total GLUT4 transporters associated with the plasma membrane was assessed in adipocytes of *+/db* and *db/db* mice in order to determine whether T_3 might play a role in regulating GLUT4 transporter cellular distributions and are reported as a

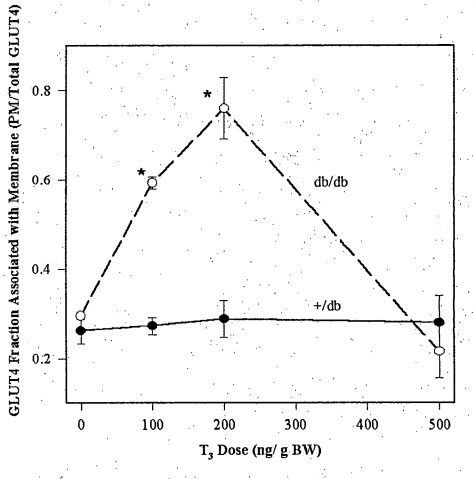


Figure 4. Fraction of GLUT4 transporters associated with plasma membrane of adipocytes of normal (*+/db*) and diabetic (*db/db*) mice treated with T_3 (0, 100, 200, and 500 ng/g BW). Comparisons are between phenotypes at the given dose (Values are ± SEM, n=5).

percentage (Figure 4). The plasma membranes of vehicle-treated *+/db* mice contained 26±3% of the total GLUT4 quantity. The GLUT4 transporter percentage associated with the membrane remained unchanged at all doses of T_3 in comparison to vehicle-treated animals. These data suggest that the distribution of GLUT4 transporters between the cytoplasmic fraction and plasma membrane is not under control by T_3, at least with respect to physiological doses present in *+/db* animals. In vehicle-treated *db/db* mice, the percentage of GLUT4 transporters associated with the plasma membrane of adipocytes was 29.5±1% (Figure 4). In mice that received 100 ng T_3/g BW dose, the percentage of GLUT4 increased approximately 2-fold in comparison to vehicle-treated *db/db* animals (59±2% vs. 29.5±1% respectively, $p \leq 0.01$) and subsequently increased again with 200 ng T_3/g BW to 76±7% ($p \leq 0.01$). With 500 ng T_3/g BW, the percentage of GLUT4 associated with the membrane significantly decreased in comparison to *db/db* mice receiving hyperthyroid doses (21.6±6% vs. 76±7%, respectively; $p \leq 0.01$), and approximate values observed in *+/db* mice (21.6±6% vs. 26±3%, respectively; $p > 0.05$). In vehicle-treated animals, the fraction of total GLUT4 associated with the plasma membrane is equal in both *+/db* and *db/db* mice suggesting a proportionate distribution. T_3 treatment does not alter GLUT4 redistribution in *+/db* animals. But, in *db/db* mice which are T_3 resistant and functionally hypothyroid, T_3 stimulated a redistribution with proportionately more GLUT4 residing in the plasma membrane. This represents the basal effect of T_3 on GLUT4 trafficking because *db/db* mice are "normalized" at supraphysiological T_3 doses. These data show that T_3 treatment increases the percentage of GLUT4 transporters associated with the plasma membrane of adipocytes in *db/db* mice. Thus, T_3 plays a role in GLUT4 transporter translocation.

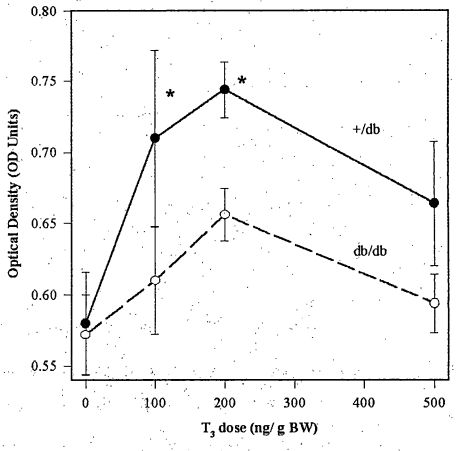


Figure 5. Total GLUT4 transporter content in myocytes of normal (*+/db*) and diabetic (*db/db*) mice treated with T_3 (0, 100, 200, and 500 ng/g BW). Comparisons are between phenotypes at the given dose (Values are ± SEM, n=5).

Skeletal muscle is the major thermogenic tissue and the most metabolically active. It is insulin-responsive and accounts for 85% of serum glucose uptake in the body. Therefore it is important to examine the GLUT4 transporter profile in myocytes. The total GLUT4 quantity was determined in the myocytes of *+/db* and *db/db* mice (Figure 5). The total GLUT4 transporter quantity in vehicle-treated *+/db* animals was 0.58±0.04 OD units and this subsequently increased with thyroid hormone treatment with the maximal response occurring at 200 ng T_3/g BW (0.744±0.02 OD units). However, at the supraphysiological dose, the total GLUT4 transporter quantity significantly decreased relative to mice receiving 200 ng T_3/g BW (0.664±0.04 vs. 0.744±0.02 OD units, respectively; $p \leq 0.05$). Although not significant at $p \leq 0.05$, the total GLUT4 quantity observed in animals receiving 500 ng T_3/g BW was apparently greater than vehicle-treated *+/db* mice ($p \leq 0.10$). The increase in

total GLUT4 transporter quantity suggests that T_3 induces GLUT4 transporter expression in normal animals.

The total GLUT4 transporter quantity in myocytes of vehicle-treated *db/db* animals was similar to that of vehicle-treated normal animals (0.572±0.03 vs. 0.580±0.04 OD units, respectively; $p>0.05$). Treatment with 100 or 200 ng T_3/g BW caused the total GLUT4 quantity to increase. At the supraphysiological dose (500 ng T_3/g BW), the total GLUT4 transporter quantity in *db/db* mice approached values observed in vehicle-treated *+/db* animals (0.594±0.02 vs. 0.58±0.04 OD units, respectively; $p>0.05$). This suggests that some GLUT4 induction at 100 and 200 ng T_3/g BW doses occurred with thyroid hormone treatment and T_3 had a normalization effect on the total GLUT4 transporter quantities. Also, attenuation responses were observed with supraphysiological T_3 treatment. These responses in *db/db* mice paralleled the pattern observed in *+/db* animals but always to a lesser degree.

The distributions of GLUT4 transporters in the cytoplasm and plasma membrane were also determined in skeletal muscle (Figures 6 and 7) and an expression pattern similar to adipose tissue was observed. The baseline expression of cytoplasmic GLUT4 transporter in myocytes of vehicle-treated (0 ng T_3/g BW) *+/db* animals was 0.268±0.03 OD units (Figure 6). The cytoplasmic GLUT4 transporter quantity in myocytes of *+/db* mice increased linearly with treatments of 100 and 200 ng T_3/g BW. Treatment with 500 ng T_3/g BW also increased cytoplasmic GLUT4 but to a lesser degree (0.402±0.03 OD units).

In *db/db* mice, the baseline expression of cytoplasmic GLUT4 in myocytes was

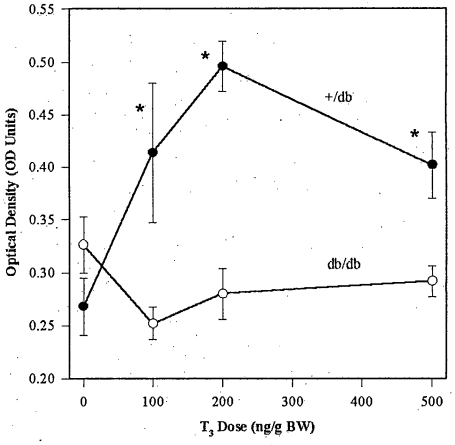


Figure 6. Cytoplasmic content of GLUT4 transporters in myocytes of normal (*+/db*) and diabetic (*db/db*) mice treated with T_3 (0, 100, 200, and 500 ng/g BW). Comparisons are between phenotypes at the given dose (Values are ± SEM, n=5).

0.326±0.03 OD units (Figure 6). The cytoplasmic GLUT4 quantity in vehicle-treated *db/db* mice was not significantly different than *+/db* animals but the quantity of cytoplasmic GLUT4 in *db/db* mice decreased with all doses of thyroid hormone ($p \leq 0.05$). However, the cytoplasmic GLUT4 content transporters of *db/db* mice approximated values of vehicle-treated *+/db* mice when treated with 500 ng T_3/g BW.

The baseline GLUT4 transporter expression in the plasma membrane of vehicle-treated *+/db* mice was 0.312±0.03 OD units (Figure 7). The quantity of GLUT4 glucose transporters associated with the plasma membrane in these animals decreased with thyroid hormone treatment ($p \leq 0.05$). In vehicle-treated *db/db* mice, the baseline GLUT4 transporter quantity was less than vehicle-treated *+/db* mice (0.246±0.02 vs. 0.312±0.03 OD units, respectively; $p \leq 0.05$). In contrast to normal animals, thyroid hormone treatment

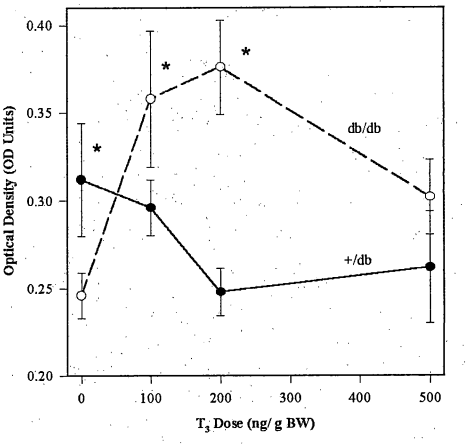


Figure 7. GLUT4 quantity associated with plasma membrane of myocytes of normal (*+/db*) and diabetic (*db/db*) mice treated with T_3 (0, 100, 200, and 500 ng/g BW). Comparisons are between phenotypes at the given dose (Values are ± SEM, n=5).

increased the quantity of GLUT4 transporters associated with the plasma membrane in *db/db* mice. The GLUT4 quantity with supraphysiological T_3 doses approached values of normal, vehicle-treated animals (0.302±0.02 vs. 0.312±0.03 OD units, $p>0.05$) which suggests that T_3 normalized *db/db* mice. The observed increases in GLUT4 transporters associated with the plasma membrane as the cytoplasmic pool decreases suggests that in *db/db* mice vesicular translocation occurs in response to thyroid hormone administration.

To further evaluate the possibilities of T_3-induced vesicular translocation in myocytes, the quantity of GLUT4 transporters associated with the plasma membranes were assessed and reported as a proportion of the total cellular GLUT4 content. In vehicle-treated *+/db* animals, the percentage of GLUT4 was approximately 54±4% (Figure 8). Normal mice showed dose-dependent decreases in the GLUT4 transporter percentage associated with the

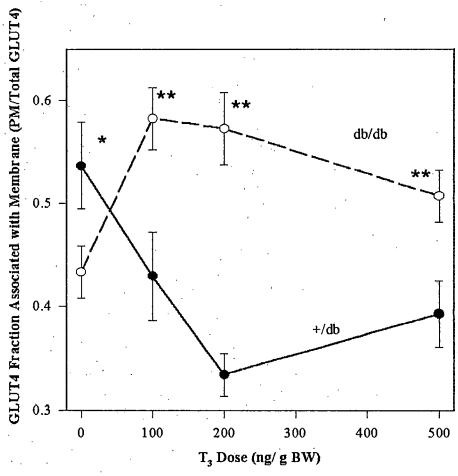


Figure 8. Fraction of GLUT4 associated with plasma membrane of myocytes of normal (*+/db*) and diabetic (*db/db*) mice treated with T_3 (0, 100, 200, and 500 ng/g BW). Comparisons are between phenotypes at the given dose (Values are ± SEM, n=5).

plasma membrane up to 200 ng T_3/g BW. The percentage of GLUT4 transporters associated with the plasma membrane of myocytes in vehicle-treated *db/db* animals was less than the vehicle-treated *+/db* mice (43.3±3% vs. 54±4%, respectively; $p \leq 0.05$). Treatment with thyroid hormone increased the percentage of GLUT4 transporters associated with the plasma membrane at all doses in *db/db* animals and surpassed the percentage of GLUT4 transporters associated with the plasma membrane of *+/db* mice at the 100 ng T_3/g BW dose (58.2±6% vs. 43±9%, $p \leq 0.01$). However, at the supraphysiological dose, the percentage of GLUT4 transporters associated with the membrane approached the percentage values observed in vehicle-treated *+/db* animals (57.2±7% vs. 54±8%, $p > 0.05$). These data suggest that T_3 stimulates GLUT4 transporter translocation to the plasma membrane of myocytes in *db/db* mice. The significant increase in the percentage of GLUT4 transporters

associated with the plasma membrane observed in myocytes from *db/db* mice is important since this may facilitate increased glucose uptake in skeletal muscle.

T_3 treatment regulated the induction and vesicular translocation mechanisms in adipose tissue of *db/db* animals. T_3 did not induce GLUT4 expression in *+/db* mice since total GLUT4 expression decreased with treatment but did induce GLUT4 expression in *db/db* mice. Thus, the decrease in cytoplasmic GLUT4 that was observed within adipocytes of *+/db* animals may be attributed to either decreased induction of the GLUT4 glucose transporter or decreased plasma membrane internalization. In vehicle-treated *+/db* animals, the cytoplasmic GLUT4 quantity decreased with T_3 whereas in *db/db* mice it increased. The decreased cytoplasmic GLUT4 transporter quantity observed in vehicle-treated *db/db* animals may reflect a problem with the induction process. Vehicle treatment in *db/db* mice represents "hypothyroid" concentrations and treatment with T_3 restores the animal to functionally euthyroid T_3 levels. Thus, with T_3 treatment, GLUT4 transporter levels approximate those of normal animals. The plasma membrane associated transporter quantity in normal mice was unaltered with T_3 doses. Although translocation of the cytoplasmic GLUT4 towards the plasma membrane was observed in *db/db* animals with thyroid hormone treatment, a decrease in membrane-associated GLUT4 transporter quantity with a concomitant increase in the cytoplasmic pool was observed at the supraphysiological doses which may represent either attenuated T_3 responsiveness or accelerated internalization of GLUT4 transporters.

Alterations in skeletal muscle GLUT4 expression have been reported in NIDDM subjects. However, the current study reports induction of GLUT4 transporters by T_3 in

myocytes of *+/db* animals as well as both GLUT4 induction and restoration of vesicular translocation in myocytes of *db/db* animals. A decrease in the cytoplasmic pool with a concomitant increase in the GLUT4 transporter quantity associated with the plasma membrane was also observed. This suggests that T_3 regulates GLUT4 transporter translocation from the cytoplasm to the plasma membrane.

Core and Radiant Temperature Studies

As a means of assessing metabolic expenditures and thermal responses to T_3, the core body and radiant temperatures of *+/db* and *db/db* mice were measured. An increase in these temperatures was observed in both phenotypes relative to their vehicle-treated counterparts. Figure 9 shows dose-dependent increases, except at 100 ng T_3/g BW dose, in the core body

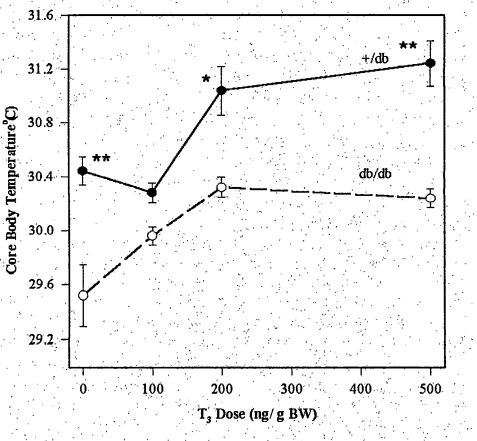


Figure 9. Core body temperatures of normal (*+/db*) and diabetic (*db/db*) mice treated with T_3 (0, 100, 200, and 500 ng/g BW). Comparisons are between phenotypes at the given dose (Values are ± SEM, n=5).

temperatures of *+/db* mice on day 10. These data confirm that T_3 treatment increases core body temperatures in normal mice and represent the normal thermogenic response.

The core body temperatures of vehicle-treated *db/db* mice were less than those of normal animals (29.52±0.23°C vs. 30.44±0.10°C, respectively; $p \leq 0.01$). Thyroid hormone treatment induced dose-dependent increases in core body temperatures of vehicle-treated *db/db* animals with a maximal response being reached at 200 ng T_3/g BW. The 500 ng T_3/g BW dose caused no further change. These data show that the core body temperatures of vehicle-treated *db/db* mice are less than *+/db* mice and suggest that T_3 treatment increases core body temperatures in *db/db* mice to levels of *+/db* animals. Core body temperatures of *db/db* mice were, however, lower than similarly treated *+/db* mice at all doses suggesting T_3 hyporesponsiveness and/or reduced thermogenic capacity.

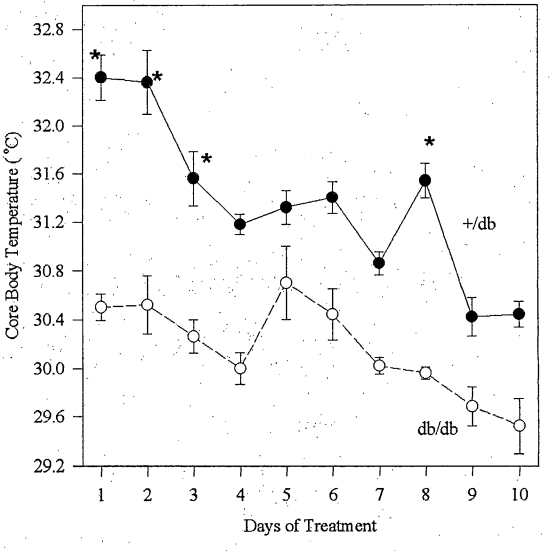


Figure 10. Daily core body temperatures of vehicle-treated normal (*+/db*) and diabetic (*db/db*) mice. Comparisons are between phenotypes at the given day (Values are ± SEM, n=5).

Figure 10 shows that the core body temperatures of vehicle-treated *+/db* mice gradually declined throughout the study resulting in day 10 values less than those observed on day 1 (32.4±0.19°C vs. 30.44±0.10°C, respectively; $p \leq 0.05$) while vehicle-treated *db/db* animals showed a similar but not statistically distinguishable decrease (30.5±0.11°C vs. 29.5±0.23°C, respectively, $p > 0.05$). A temperature decrease observed in *+/db* animals was unexpected and cannot be explained using available data.

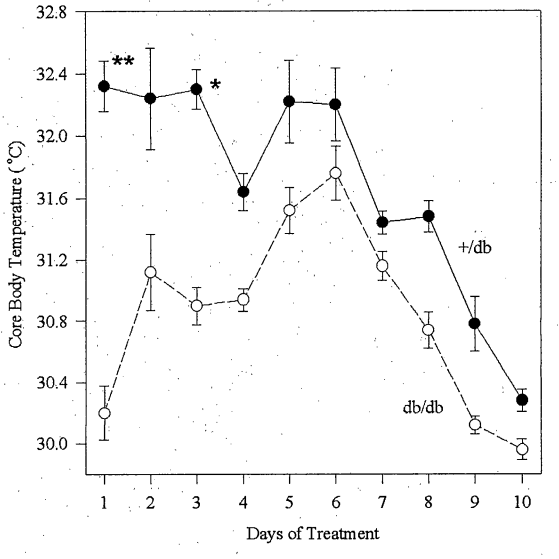


Figure 11. Daily core body temperatures of normal (*+/db*) and diabetic (*db/db*) mice treated with 100 ng T_3/g BW. Comparisons are between phenotypes at the given day (Values are ± SEM, n=5).

The core body temperatures of *+/db* mice treated with 100 ng T_3/g BW remained unchanged through day 6, but decreased on days 7 through day 10 (Figure 11). In *db/db* animals receiving the replacement dose, core body temperatures increased and a maximal response was observed on day 6. In a pattern similar to *+/db mice*, the temperatures in *db/db* animals decreased on days 7 through day 10. The fluctuations in temperature may

be attributed to induced stress resulting from handling and methods in obtaining colonic temperature readings, however this was not tested.

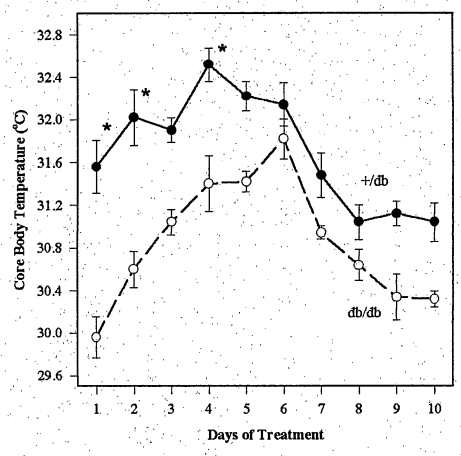


Figure 12. Daily core body temperatures of normal (*+/db*) and diabetic (*db/db*) mice treated with 200 ng T_3/g BW. Comparisons are between phenotypes at the given day (Values are ± SEM, n=5).

Daily core body temperatures were recorded to assess the chronology of thermogenic responses. Figure 12 shows the responses of *+/db* and *db/db* mice treated with 200 ng T_3/g BW and these represent the maximal metabolic responses recorded for all treatment groups. The maximal response was observed on day 4 (32.52±0.16°C) after which core body temperatures continued to decrease until day 8 where they remained constant until day 10 (31.04±0.18°C). In *db/db* mice, the baseline core body temperatures were considerably less than those of normal mice, but T_3 treatment caused daily increases in core body temperatures with the maximal response being observed on day 6. Temperatures then decreased until day 9 where they remained constant through day 10. Similar chronological

responses to T_3-induced thermogenesis were observed in both *+/db* and *db/db* mice. However, the *db/db* animals consistently exhibited lower temperatures.

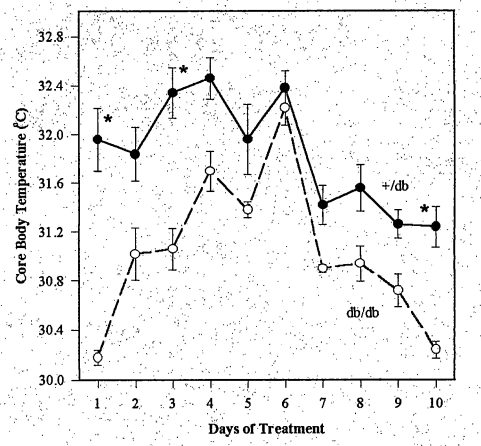


Figure 13. Daily core body temperatures of normal (*+/db*) and diabetic (*db/db*) mice treated with 500 ng T_3/g BW. Comparisons are between phenotypes at the given day (Values are ± SEM, n=5).

The core body temperatures from *+/db* and *db/db* mice treated with 500 ng T_3/g BW were also recorded on days 1 through 10 (Figure 13). The temperature changes in these animals paralleled those observed in mice treated with 200 ng T_3/g BW. The core body temperatures represent the internal regulated temperatures while the radiant temperatures represent the heat that is dissipated. As core body temperatures increased in response to T_3, a maximal threshold was achieved on day 6 and a concomitant decrease in core body temperatures occurred on days 7-10. Excess heat is dissipated on days 7 through 10.

In order to account for the amount of heat being radiated, the radiant temperatures of *+/db* and *db/db* mice were recorded on days 7-10. Figure 14 shows that the radiant

temperatures of *+/db* mice increased in response to T_3 with a maximal response occurring at 200 ng T_3/g BW and subsequently decreasing at the supraphysiological dose of 500 ng T_3/g BW. There was an increase in radiant heat as the body core temperatures decreased. As core body temperatures of *+/db* and *db/db* mice decreased on day 7 of treatment, the radiant temperatures of these animals increased at all T_3 doses.

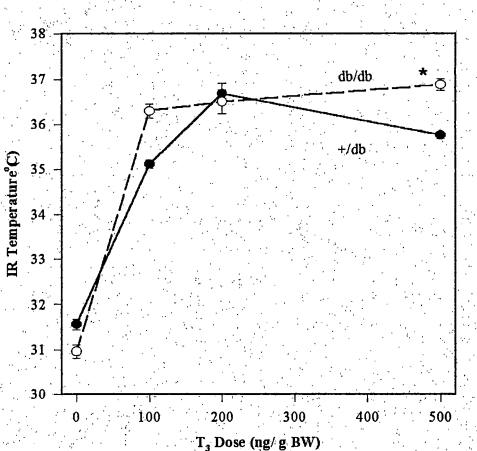


Figure 14. Radiant temperatures of normal (*+/db*) and diabetic (*db/db*) mice treated with T_3 (0, 100, 200, and 500 ng/g BW). Comparisons are between phenotypes at the given dose (Values are ± SEM, n=5).

The radiant temperatures of *db/db* mice increased in response to T_3 at all doses (Figure 14). The baseline radiant temperature of vehicle-treated *db/db* mice was lower than *+/db* animals (30.96±0.14°C vs. 31.56±0.12°C, $p \leq 0.05$). The maximal response occurred at 500 ng T_3/g BW at which the radiant temperature was also greater than *+/db* mice. These data show that T_3 induces thermogenesis in both *+/db* and *db/db* animals. The increase in radiant temperature as the core body temperatures decreased occurred in order to dissipate the

excess heat to thermoregulate the core temperature. This pattern was also observed in *+/db* mice.

The radiant temperatures from *+/db* mice treated with vehicle and 100, 200, and 500 ng T_3/g BW were recorded on days 7 through 10. An increase in radiant temperature was observed at the 100 ng T_3/g BW dose in *+/db* and *db/db* animals on days 7 and 8 (data not shown), a pattern similar to the group of mice treated with 200 ng T_3/g BW. However, radiant temperatures of *+/db* mice decreased on days 9-10 and were not different than temperatures recorded on day 7 whereas radiant temperatures in *db/db* mice increased through day 10.

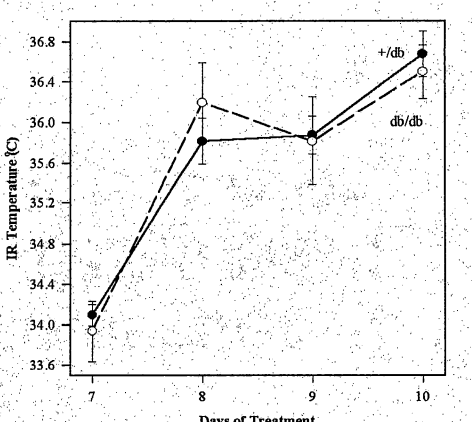


Figure 15. Radiant temperatures of normal (*+/db*) and diabetic (*db/db*) mice treated with 200 ng T_3/g BW. Comparisons are between phenotypes at the given day (Values are ± SEM, n=5).

Radiant temperatures from the 200 (Figure 15) and 500 ng T_3/g BW (Figure 16) doses are shown since maximal thermic responses were observed at these doses. Figure 15 shows

that the radiant temperature in *+/db* mice on day 7 was 34.1±0.10°C and continued to increase until a maximal response was observed on day 10 (36.68±0.22°C). In *db/db* mice, radiant temperatures increased with T_3 treatment and the maximal response was observed on day 10 (36.5±0.27°C) where the radiant temperature approached that of *+/db* animals. These observations suggest that, with T_3 treatment, radiant temperatures of *db/db* mice approach normal and imply that *db/db* animals are utilizing as much energy as *+/db* animals to drive thermogenesis.

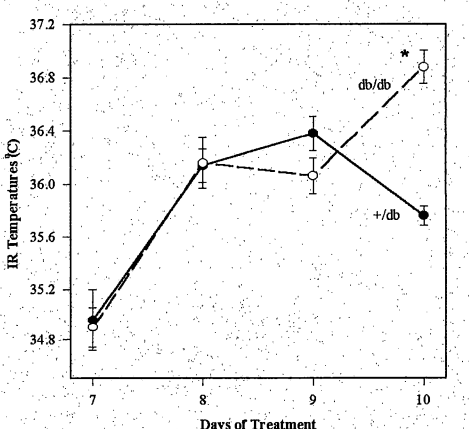


Figure 16. Radiant temperatures of normal (*+/db*) and diabetic (*db/db*) mice treated with 500 ng T_3/g BW. Comparisons are between phenotypes at the given day (Values are ± SEM, n=5).

The radiant temperatures of *+/db* mice treated with 500 ng T_3/g BW were also recorded on days 7 through 10 (Figure 16) and the temperatures followed a pattern similar to that observed in mice treated with 200 ng T_3/g BW. The day 7 radiant temperatures of normal animals were 34.96±0.24°C with the maximal response observed on day 9 and a

decrease on day 10. In *db/db* mice, the radiant temperature on day 7 was 34.94±0.16°C and the maximal response observed on day 10 was greater than that of *+/db* animals (36.88±0.13°C vs. 35.76±0.08°C, $p \leq 0.05$). These observations suggest that T_3 treatment increases the radiant temperatures of *db/db* mice beyond those of normal animals implying that greater energy is being utilized and that *db/db* animals are dissipating more heat than *+/db* mice. The concomitant decrease in core body temperature observed in *+/db* and *db/db* animals is likely due to thermoregulation in order to maintain a low core temperature.

Serum Glucose Determination

Serum glucose concentrations were evaluated in *+/db* and *db/db* animals in order to determine if thyroid hormone treatment influenced serum glucose levels. The baseline

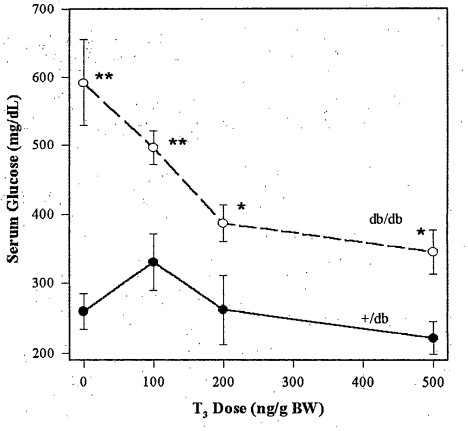


Figure 17. Serum glucose concentrations of normal (*+/db*) and diabetic (*db/db*) mice treated with T_3 (0, 100, 200, and 500 ng/g BW). Comparisons are between phenotypes at the given dose (Values are ± SEM, n=5).

glucose levels in vehicle-treated *+/db* mice were 260.2±25.3 mg glucose/dL and these remained relatively constant with T_3 treatment (Figure 17). In *db/db* mice, the baseline glucose concentration was 592.0±63.0 mg glucose/dL and this is considered severely hyperglycemic. Serum glucose levels decreased with T_3 treatment in a dose-dependent fashion. At 500 ng T_3/g BW, serum glucose levels decreased approximately 42% when compared to vehicle-treated mice (592.0±63.0 vs. 344.5±31.4 mg glucose/dL, respectively; $p \leq 0.01$) which indicates that T_3 treatment improved the glycemic status in *db/db* animals.

Respiratory Quotient (RQ) Determination

The respiratory quotient, RQ, (ml CO_2 / ml O_2) was monitored daily in *+/db* and *db/db* mice over a nine day period in order to determine the effect T_3 treatment might have on

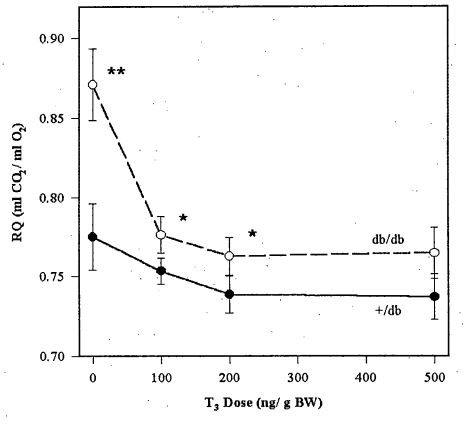


Figure 18. Respiratory quotients (RQ) of normal (*+/db*) and diabetic (*db/db*) mice treated with T_3 (0, 100, 200, and 500 ng/g BW). Comparisons are between phenotypes at the given dose (Values are ± SEM, n=5).

metabolic substrate utilization. The RQ of *+/db* mice treated with 0 ng T_3/g BW was 0.78±0.03 (Figure 18) which suggests that a combination of proteins, lipids and carbohydrates were potentially being used as the metabolic substrate. With T_3 treatment, the RQ value decreased to 0.74±0.01 which suggests a shift toward more fat or protein catabolism. In vehicle-treated *db/db* mice, the RQ was 0.87±0.05 (Figure 18). This indicates that substantially more carbohydrates were being used as a metabolic substrate than is typically used by *+/db* animals. With increasing doses of thyroid hormone, the RQ decreased significantly. Diabetic mice treated with 100 ng T_3/g BW had a respiratory quotient that was less than vehicle-treated animals (0.78±0.02 vs. 0.87±0.05, $p \leq 0.05$) and suggests that the primary substrate being utilized had shifted from carbohydrates to fats or proteins. The RQ values of *db/db* mice treated with either 200 or 500 ng T_3/g BW approached those of vehicle-treated *+/db* animals (0.76±0.03 vs. 0.74±0.03, $p > 0.05$) and indicates that the *db/db* mice were undergoing fat catabolism. This also suggests that the diabetic mice have undergone normalization in substrate utilization.

CHAPTER FOUR: DISCUSSION

The insulin-regulatable GLUT4 transporter is expressed in skeletal muscle, adipose tissue, and cardiac muscle (Pessin and Bell 1992; Mueckler 1994; Stephens and Pilch 1994) and regulates glucose uptake in those target tissues. The present study investigated whether thyroid hormone treatment could restore the insulin effects on GLUT4 glucose transporter induction and/or translocation in the insulin-resistant *db/db* mouse. This effect was assessed by examining the total GLUT4 quantity as an indicator of the induction process and the cellular GLUT4 distributions which represent translocation within the cell. These phenomena were evaluated in lean C57BL/KsJ *+/db* mice and obese-diabetic C57BL/KsJ *db/db* mice, which are insulin-resistant (Hummel *et al.* 1966), T_3-resistant, and functionally hypothyroid (Fehn *et al.* 1988).

Matthaei *et al.* (1995) showed that the total GLUT4 glucose transporter quantity in adipocytes of male Sprague-Dawley rats decreased in hypothyroid animals, but increased in hyperthyroid rats. These results agree with data presented in this study since the functionally hypothyroid and T_3-resistant *db/db* mice are brought to functionally euthyroid status using hyperthyroid doses of T_3. The total GLUT4 quantity was assessed in the adipocytes of *+/db* and *db/db* mice in order to determine if administration of T_3 stimulated GLUT4 induction. Total GLUT4 transporter quantity in *+/db* mice remained unchanged with increasing doses of T_3 which suggests that induction the GLUT4 transporter is not likely under direct regulation at normal physiologic concentrations of T_3. However, attenuation was observed at the maximal dose of T_3. In *db/db* mice, which are T_3-resistant

and functionally hypothyroid, the total GLUT4 quantity was 50% that of vehicle-treated *+/db* animals and this increased 2-fold with T_3 treatment and approached levels equivalent to those of vehicle-treated *+/db* animals (Figure 1). The findings in this study are consistent with results showing that T_3 increases total GLUT4 protein expression (Casla *et al.* 1990). This suggests that T_3 stimulates GLUT4 induction and normalizes GLUT4 quantities in *db/db* mice. A reduction in the degradation rate to account for increased GLUT4 cannot, however, be discounted.

Zorzano *et al.* (1996) showed in STZ-induced diabetic (insulin-deficient) Wistar rats that the quantity of GLUT4 decreased in the cytoplasm of adipocytes and increased in the plasma membrane with increased insulin concentrations. This author suggests that insulin promotes redistribution of the glucose transporter to the plasma membrane. Although these results explain the established effects of insulin, the current study addresses thyroid hormone effects and the potential restoration of insulin action in a model for NIDDM. In the current study, T_3 mimics the effects Zorzano *et al.* (1996) reported for insulin action. Zorzano's results are in agreement with the results observed in the *+/db* mice from this study since these animals remain insulin-responsive. Cytoplasmic and membrane-bound GLUT4 in adipocytes of *+/db* mice remained constant with T_3 treatment and only the cytoplasmic portion decreased with supraphysiological doses (Figures 2 and 3). This represents normal expression, GLUT4 transporter recycling, and regulation in a normal endocrine environment. However, the decreased cytoplasmic GLUT4 at the 500 ng T_3/g BW dose suggests increased degradation of membrane components or attenuation of GLUT4 synthesis. Such attenuation is a blunted response typically observed within systems that

have been pushed to physiological extremes.

Previous studies on adipocytes from insulin-deficient diabetic rats (Kahn *et al.* 1989; Berger *et al.* 1989) and in studies using 3T3-L1 adipocytes cultured *in vitro* without insulin (Calderhead *et al.* 1990), showed that cytoplasmic GLUT4 quantities were less than those of normal rats and insulin-treated cells, respectively. Decreased expression of the GLUT4 transporter has also been documented in NIDDM patients as well as in diabetic animal models (Koranyi *et al.* 1990; Kahn 1996). The decrease in cytoplasmic GLUT4 number reduces their availability to be mobilized to the plasma membrane and could explain the lower plasma membrane content. It has also been reported that insulin-stimulated GLUT4 translocation is impaired in adipocytes from NIDDM individuals (Ciaraldi *et al.* 1991). However, the mechanism by which translocation is regulated has not yet been explained and has recently received much attention as being the potential site of regulatory defects (Kahn 1996). The current study showed that cytoplasmic GLUT4 in vehicle-treated *db/db* mice was 50% less than vehicle-treated *+/db* mice. This quantity decreased in response to T_3 treatment but increased 2-fold with the 500 ng T_3/g BW dose and therefore approached levels of vehicle-treated *+/db* mice (Figure 2). As cytoplasmic GLUT4 is disproportionately depleted, a concomitant increase in the quantity of membrane-bound GLUT4 is observed which approaches that of vehicle-treated *+/db* mice (Figures 2, 3). This suggests that T_3 facilitates GLUT4 translocation from the cytoplasm to the plasma membrane and normalizes GLUT4 within adipose tissue of functionally "hypothyroid" animals.

The proportion of GLUT4 glucose transporters associated with the plasma membrane was calculated in *+/db* and *db/db* mice to determine if thyroid hormone administration plays

a role in the trafficking of the GLUT4 transporter, specifically in the translocation process. This type of analysis has not been reported previously, thus there are no studies with which to compare the current data. The percent of the GLUT4 transporter population associated with the plasma membrane of adipocytes in *+/db* mice did not change with T_3 treatment (Figure 4). Thyroid hormone either did not alter the GLUT4 transporter turnover or it equally affected the rates of internalization and association of GLUT4 transporters with the plasma membrane. Although the absolute amount of membrane-bound GLUT4 in *db/db* mice was less than in *+/db* mice, the percent of GLUT4 glucose transporters associated with the plasma membrane was similar to that of *+/db* mice (Figure 4). This shows that the membrane-bound GLUT4 quantity in relation to the total GLUT4 in *db/db* mice is proportional to that of *+/db* animals, thus, the percentage is scaled down proportionately in *db/db* mice. In *db/db* animals treated with T_3, the percent of GLUT4 associated with the plasma membrane increased suggesting that T_3 may regulate GLUT4 translocation from the cytoplasm to the plasma membrane. Since the final proportions approached those of *+/db* mice, it appears that T_3 normalizes the GLUT4 quantity associated with the plasma membranes of *db/db* mice. Thyroid hormone does not induce the synthesis of additional GLUT4 in *+/db* mice nor is the cellular distribution affected. As a result of overcoming T_3 resistance in *db/db* mice, T_3 induces GLUT4 synthesis resulting in increased cellular GLUT4 content. The cytoplasmic population consequently is redistributed towards the plasma membrane suggesting that T_3 restores the translocation process.

GLUT4 transporter expression and subcellular distribution were assessed in myocytes because skeletal muscle is a metabolically active tissue which utilizes approximately 85%

of total glucose available. Skeletal muscle is the major site for glucose disposal and is the major site of insulin resistance within NIDDM subjects (Brozinick *et al.* 1996). The total GLUT4 quantity was assessed in the myocytes of *+/db* and *db/db* mice to determine if thyroid hormone administration stimulated GLUT4 induction. Total GLUT4 quantity in *+/db* mice increased with T_3 treatment at physiological doses but to a lesser degree with the supraphysiological dose. This shows that T_3 facilitates GLUT4 transporter induction. In vehicle-treated *db/db* mice, the total GLUT4 transporter quantity was similar to that of vehicle-treated *+/db* animals and total GLUT4 quantity also increased with T_3 treatment showing that T_3 induces the GLUT4 transporter.

The current study proposes a model whereby T_3 either overcomes thyroid hormone resistance and restores the insulin response in target tissues of *db/db* mice or T_3 has a direct effect in GLUT4 transporter regulation. If the insulin response is restored with T_3 treatment, the results from the current study then contradict published reports which show that GLUT4 transporter expression within skeletal muscle remains unchanged in response to insulin treatments (Kahn 1996; Hainhault *et al.* 1991; Pedersen *et al.* 1990). A possible reason for this discrepancy is that all animal and human subjects were fasted at least 8 hours prior to testing and fasting causes a decrease in thyroid hormone levels thereby rendering the animals hypothyroid. Weinstein *et al.* (1991) showed that T_3 could induce GLUT4 expression in skeletal muscle of hypothyroid non-diabetic animals that were restored to euthyroid status. Since the animals were fasted prior to tissue collection, the GLUT4 values and thyroid levels are not accurate representations of animals in a non-fasting state. At the supraphysiological dose, total GLUT4 in myocytes of *db/db* mice approached GLUT4 levels

found in vehicle-treated *+/db* animals (Figure 5). This suggests that total GLUT4 is normalized in *db/db* mice that have overcome thyroid hormone resistance. Although total GLUT4 quantities in vehicle-treated *+/db* and *db/db* mice were not different (Figure 5), the subcellular distributions were different in the two phenotypes (Figures 6, 7). However, T_3 treatment allows for "normal" GLUT4 expression and a more appropriate distribution of subcellular GLUT4 quantities. These data show that T_3 stimulates GLUT4 induction and likely plays a role in either the transcriptional or translational processing of the GLUT4 glucose transporter.

The quantity of cytoplasmic GLUT4 in myocytes of *+/db* mice increased in response to thyroid hormone treatment and these results are consistent with the published literature (Casla et al. 1990; Weinstein *et al.* 1991; Weinstein *et al.* 1994). Induction of GLUT4 has been established above, but the increased cytoplasmic quantity can also reflect increased internalization of plasma membrane components (Figure 6). This response contrasts with observations made in adipocytes of *+/db* animals and suggests tissue-specific responses to T_3. The membrane-bound GLUT4 in *+/db* mice decreased with T_3. This suggests attenuation phenomena or increased GLUT4 transporter internalization as was seen with the adipocytes of *+/db* mice. Based on the observations within myocytes, T_3 appears to regulate GLUT4 transporter induction and vesicular translocation at a basal level whereby T_3 exhibits its permissive action under normal physiological conditions.

Several studies have shown that cellular GLUT4 expression levels (either protein or mRNA) are unchanged in the skeletal muscle of diabetic subjects (Kahn 1996; Hainhault *et al.* 1991; Pedersen *et al.* 1990) including the quadriceps of 5 week-old *db/db* mice (Koranyi

et al. 1991). In each of these studies, only the plasma membrane-associated content of GLUT4 glucose transporters has been shown to be altered in skeletal muscle. However, results from the current study show that the cytoplasmic GLUT4 in *db/db* mice is greater than the cytoplasmic GLUT4 content found within *+/db* mice and these quantities decrease in response to T_3. This indicates that the pool of cytoplasmic GLUT4 in myocytes of 8-10 week old *db/db* mice is regulated by T_3. These results are contrary to reports of unalterable GLUT4 expression in quadriceps of 5 week old *db/db* mice. This discrepancy can be attributed to the age differences of the mice. Fehn *et al.* (1988) reported that the onset of a defect in T_3 responsiveness occurs after 6 weeks of age thus Koranyi *et al.* (1990) missed the onset period and obtained results from animals not yet displaying the T_3-resistance. Also, a concomitant disproportionate depletion in cytoplasmic GLUT4 quantities was observed while membrane-bound GLUT4 increased in *db/db* mice (Figures 6, 7). This was similar to the pattern observed in adipocytes and indicates that T_3 may regulate GLUT4 translocation from the cytoplasm towards the plasma membrane. This observation is extremely important since skeletal muscle requires the GLUT4 transporter for the majority of glucose uptake. Although T_3 regulation of GLUT4 translocation has not been reported in the literature, the results reported here are consistent with the depletion of a cytoplasmic vesicular pool in response to a hormonal signal (Weinstein *et al.* 1994). Therefore, T_3 is herein proposed as a regulator of GLUT4 translocation process.

At the supraphysiological dose of T_3 (500 ng/g BW), the membrane bound GLUT4 transporter quantity in *db/db* mice decreases, although it still remains greater in comparison to the vehicle-treated counterpart. As the membrane-bound quantity decreases

(approaching values similar to the baseline expression levels of +/*db* vehicle-treated mice), the quantity of cytoplasmic GLUT4-containing vesicles increases also approaching values similar to +/*db* vehicle-treated mice. This suggests a normalization phenomenon within the skeletal muscle in response to thyroid hormone treatment.

The current study establishes that T_3 increases GLUT4 transporter induction because an increase is seen in the total cellular quantity of GLUT4 transporters. These data agree with the findings of Casla *et al.* (1990) and Weinstein *et al.* (1991). More recently, Torrance *et al.* (1997) showed that T_3 regulates GLUT4 expression at the transcriptional level in one muscle fiber type and increased both membrane-bound GLUT4 quantities and GLUT4 mRNA. However, Torrance *et al.* (1997) failed to recognize that T_3 regulates the redistribution of the GLUT4 transporter from the cytoplasm to the plasma membrane. This redistribution of GLUT4 transporters towards the plasma membrane is important since it may be the pathway for increased cellular glucose uptake.

In normal mice, the percent of the GLUT4 transporter associated with the plasma membrane of myocytes decreased with thyroid hormone treatment and shows that T_3 altered GLUT4 transporter turnover (Figure 8). Perhaps overloading the system with an excess number of GLUT4 transporters surpasses the quantity of docking complexes available within the cell leading to accumulation in the cytoplasm. Alternatively, internalization of membrane components may be accelerated as part of an increased recycling rate phenomena. The percent of GLUT4 glucose transporters associated with the plasma membrane of vehicle-treated *db/db* mice was less than that of vehicle-treated +/*db* mice (Figure 8). This quantity increased with thyroid hormone treatment and indicates that T_3 regulates GLUT4

vesicular translocation from the cytoplasm towards the plasma membrane. This is extremely important since the increased percent of GLUT4 transporters associated with the plasma membrane indicates translocation and docking and suggests a greater probability that GLUT4 transporters have fused with plasma membrane. Increased fusion of GLUT4 transporters with the plasma membrane of myocytes would facilitate increased glucose uptake which would provide the substrate for increased thermogenic activity in the skeletal muscle. Although these data show that T_3 regulates the translocation mechanism from the cytoplasm to the plasma membrane, one cannot say that the GLUT4 transporters within the vesicle membrane are incorporated into the plasma membrane. Thus, the vesicular fusion mechanism warrants further investigation.

The current study establishes that T_3 plays a role in GLUT4 transporter regulation. Thyroid hormone induced the GLUT4 transporter in both adipocytes and myocytes, but to varying degrees. This shows the tissue specific responses to T_3 in relation to GLUT4. Also, T_3 promoted the translocation of cytoplasmic GLUT4 towards the plasma membrane in both myocytes and adipocytes and thus contributed to the increased plasma membrane GLUT4 content. This is particularly important in relation to NIDDM since this increased quantity may facilitate increased glucose uptake within these cells. The increased glucose uptake would thus provide the cells, particularly myocytes with a substrate that can be utilized for metabolism and thermogenesis.

The core body temperatures in *+/db* and *db/db* animals were measured to determine the effect of T_3 treatment on thermogenesis. Core body temperatures of *+/db* mice were greater than *db/db* animals which is what is expected since diabetic animals exhibit impaired

thermogenesis (Hummel *et al.* 1966). The temperatures of both *+/db* and *db/db* mice increased with thyroid hormone treatment demonstrating thermogenic responses in both phenotypes. These results are similar to those reported by Oh and Kaplan (1994) for the phenotypically-related *obese* (*ob/ob*) mouse model. Obese *ob/ob* mice are hypothermic making them similar to *db/db* animals (Oh and Kaplan 1994; Hummel *et al.* 1966). However, *ob/ob* mice are hypothyroid, meaning that T_3 levels are decreased, yet are still responsive to T_3. In contrast, *db/db* mice are hyperthyroid but exhibit hyporesponsiveness to the action of thyroid hormone, which is indicative of T_3-resistance (Fehn *et al.* 1983, 1988). Thyroid hormone treatment elicits thermogenic responses in both *ob/ob* mice and in *db/db* mice although *db/db* animals require supraphysiological doses to be effective. Thus, T_3 increases the core body temperatures in both animal models. When the core body temperatures were monitored throughout the ten day study, animals of both phenotypes receiving T_3 doses of 200 and 500 ng/g BW, exhibited declining temperatures on days 7 through 10. This, however, does not reflect a decrease in thermogenesis because lower core body temperatures were accompanied by higher radiant temperatures. A reasonable explanation for this observation is that the animals maintain their core body temperatures by dissipating excess heat produced in response to T_3 treatment as radiant heat. This phenomenon represents normal T_3-induced thermoregulation and accounts for the changes in temperature observed within these animals.

Radiant temperatures have not previously been reported for such studies, therefore data from this study cannot be compared with published reports since this is a novel approach to account for thermogenic activity. It does, however, account more accurately

for increases in metabolism associated with T_3 treatment. Radiant temperatures in *+/db* and *db/db* mice both increase with thyroid hormone treatment. However, the increase in radiant temperature was greatest in *db/db* animals. The increase in radiant temperatures might be potentially explained by increased thermogenic capacity in response to increased GLUT4 expression in the plasma membranes of myocytes. This increase in GLUT4 transporters would account for increased glucose uptake by myocytes which would then utilize the additional substrate in subsequent heat production (thermogenesis). Thyroid hormone also has been shown to stimulate membrane permeability to ions as well as induce Na^+/K^+ pumps in skeletal muscle, both of which are believed to play a role in the thermogenic response (Voldstedlund *et al.* 1995). The increase in thermogenic activity may also be associated with higher cytoplasmic glucose concentrations due to T_3-induced GLUT4 vesicular fusion (integration) with the plasma membrane of myocytes. Thus, T_3-induced GLUT4 expression in the plasma membrane of myocytes may support thermogenic activity.

Hyperglycemia is characteristic of NIDDM individuals since glucose uptake is not adequately handled due to decreased insertion of the insulin-regulatable GLUT4 transporter into cell membranes. In the current study, serum glucose levels were examined to determine if thyroid hormone treatment improved the glycemic status of *db/db* mice. Serum glucose levels of normal animals remained unchanged with T_3 treatment which suggests that T_3 does not affect the normal glucose handling mechanism in insulin-responsive animals (Figure 17). However, serum glucose concentrations in vehicle-treated *db/db* mice were approximately 50% greater than in *+/db* animals, as could be expected for animals displaying insulin resistance. Thyroid hormone decreased glucose concentrations about 40% which suggests

increased glucose utilization or restoration of the insulin response. Although still considered hyperglycemic, the glycemic status of *db/db* animals was improved significantly with thyroid hormone treatment. This suggests that tissues were able to take up glucose, however which tissue specifically does this remains to be determined and warrants further study.

One approach to this problem might be to evaluate substrate utilization which accompanies the redistribution of GLUT4. The respiratory quotient (RQ) is an indicator of which substrate is being utilized as the fuel source within the organism. In this study, the respiratory quotient was determined to see if thyroid hormone treatment causes a shift in the metabolic substrate utilized by *+/db* and *db/db* animals. The RQ values of +/db mice decreased in response to thyroid hormone treatment to levels suggestive of a combination of carbohydrates, proteins, and fats as substrate sources (Figure 18). However, the RQ in *db/db* mice shifts from 0.87± 0.05, which indicates carbohydrate as the predominant substrate source to 0.76±0.03 which indicates increased utilization of fats, proteins, or both as the metabolic substrates in response to thyroid hormone treatment. Evidence from a previous study wherein the adipocyte volumes of *db/db* mice decreased in response to T_3 suggests that fats were the primary source of energy (Estrada unpublished results). However, the decrease in serum glucose levels with T_3 treatment in *db/db* mice from the current study suggests that there is substantial uptake but not utilization as a metabolic substrate. Informal examination of livers in *db/db* mice suggests increased deposition of fats which may be attributed to increased hepatic glucose uptake leading to enhanced lipogenesis (data not shown). Although the glucose transporters in hepatocytes are not insulin-regulatable, the lipogenic enzymes still remain inducible by T_3 and may account for the

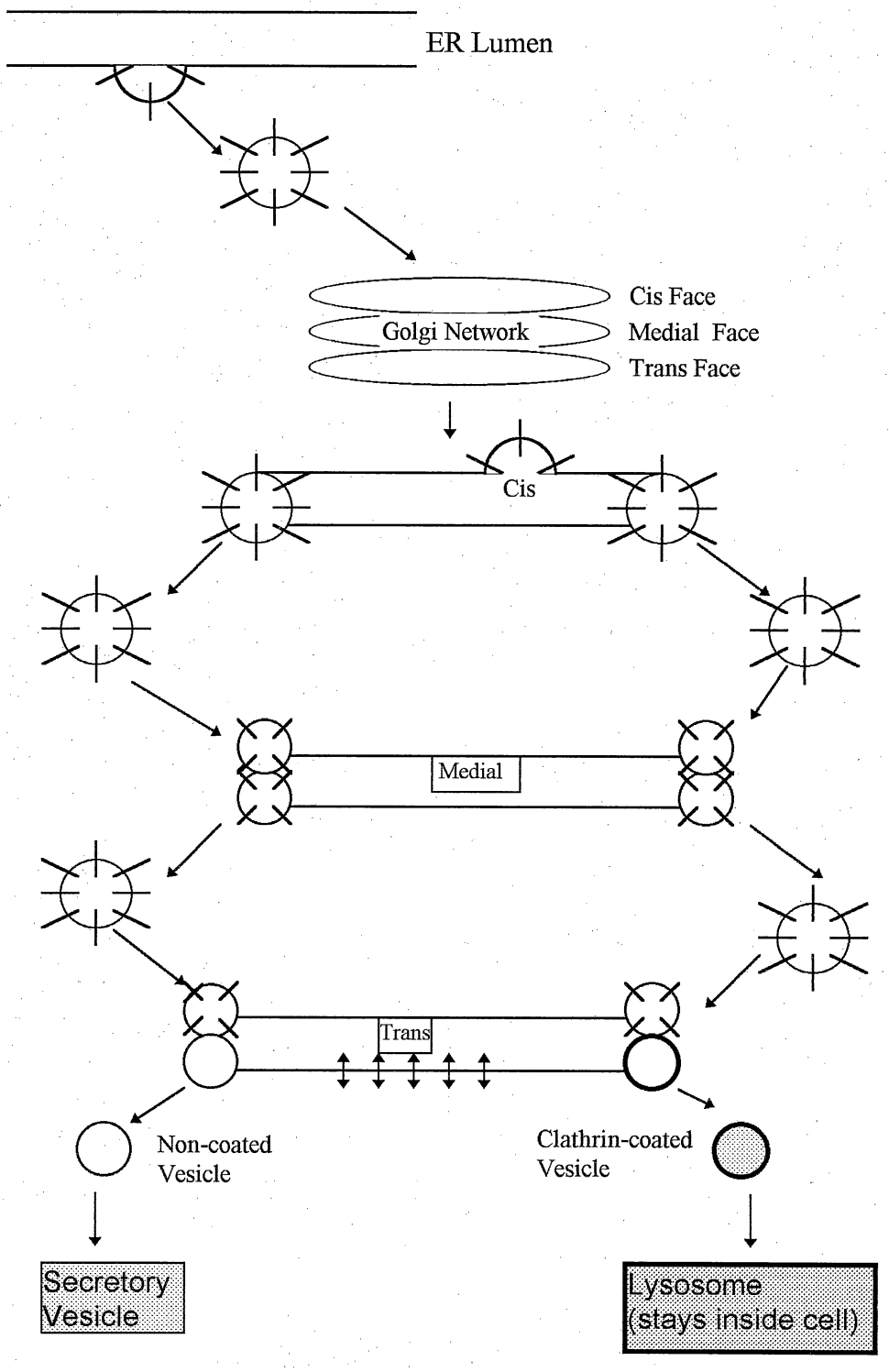
increased disposal of glucose from the serum. However, the RQ values in the *db/db* mice treated with supraphysiological doses of thyroid hormone approached those of vehicle-treated *+/db* mice and suggest, once again, that normalization has taken place.

The RQ values indicating a shift to fat catabolism do not support the observation of increased membrane-associated GLUT4 transporters in myocytes facilitating increased cellular glucose uptake and utilization. An explanation for increased GLUT4 associated with the plasma membrane in the absence of glucose utilization may be that the GLUT4 transporter-containing vesicles dock with the plasma membrane and are isolated with plasma membrane fractions. The docked vesicles, however, may not be fused and therefore the GLUT4 transporters are not integrated into the plasma membrane. Thus, glucose uptake by myocytes cannot be facilitated since the GLUT4 transporter is not inserted correctly. However, the energy by which thermogenesis is fueled is probably provided by fatty acids, an idea which is supported by an RQ shift indicating fat catabolism, in conjunction with an increase in radiant temperatures. The mechanism by which the vesicles containing the GLUT4 glucose transporters fuse with the plasma membrane warrants further investigation to see if that step within the trafficking mechanism is defective within NIDDM subjects.

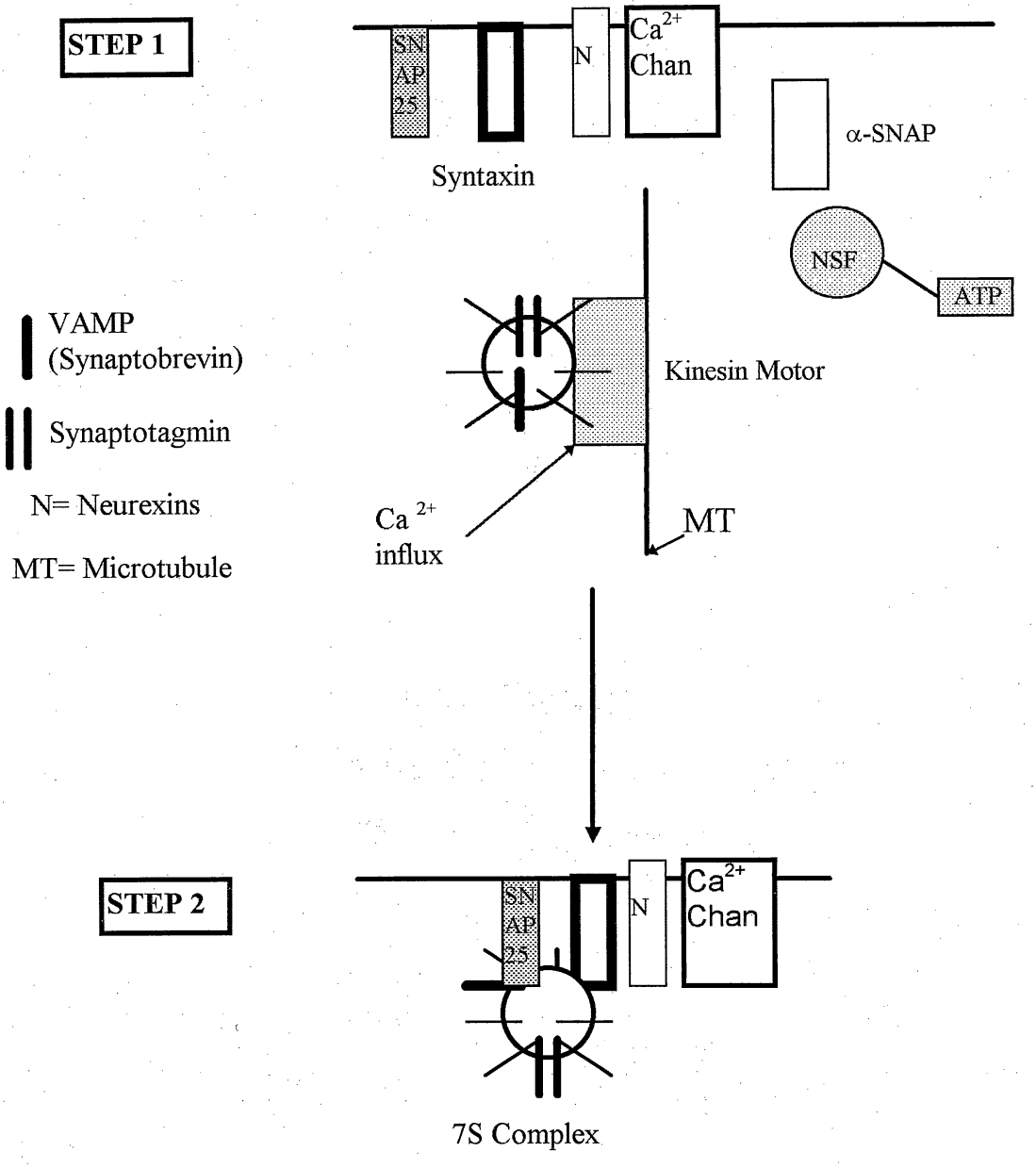
This study has demonstrated reduced total GLUT4 expression in adipocytes and decreased GLUT4 transporters associated with the plasma membranes of myocytes of *db/db* mice but an increases in these quantities in response to exogenous T_3. The current study also demonstrated that T_3 treatment induces GLUT4 glucose transporter redistribution from the cytoplasm to the plasma membrane of adipocytes and myocytes in *db/db* mice. Observations from adipocytes are consistent with studies by Zorzano *et al.* (1996), Kahn

et al. (1989), and Berger *et al.* (1989). Gibbs *et al.* (1995) alluded to a trafficking problem in GLUT4 transgenic *db/db* mice in which GLUT4 overexpression could compensate for a trafficking defect. This is consistent with the proportional fusion from the cytoplasm to the plasma membrane demonstrated in this study, but T_3 corrects the GLUT4 distribution in *db/db* mice. The transgenic *db/db* mice showed a marked decrease in hyperglycemia due to increased glucose transport. However, Gibbs *et al.* (1995) did not evaluate the cellular GLUT4 distribution in all tissues of transgenic mice and the potential consequences of increased glucose disposal by tissues that typically do not express the GLUT4 transporter. More recently, Torrance *et al.* (1997) showed that T_3 regulates GLUT4 transporter expression at the transcriptional level in hypothyroid rats made hyperthyroid. Although not pointed out by the authors, T_3 was shown to regulate GLUT4 transporter translocation because distribution differences in GLUT4 transporters are observed in response to T_3 administration. The results of the current study indicate that GLUT4 transporter translocation from the cytoplasm to the plasma membrane is regulated by T_3 in *db/db* mice and is the first report of such regulation. The increased thermogenic response observed in this study parallels increased numbers of GLUT4 transporters in the plasma membrane of myocytes but the RQ values do not support carbohydrate utilization as the metabolic substrate for the thermogenic response. However, decreased serum glucose levels in *db/db* mice in response to T_3 treatment indicates that glucose is taken up but is not utilized as the metabolic substrate for thermogenesis. The liver may take up the glucose via GLUT2 transporters which are not insulin-regulated. Absorbed glucose may then be converted to fat and stored away. If this is the case, this may imply that the GLUT4 glucose transporters

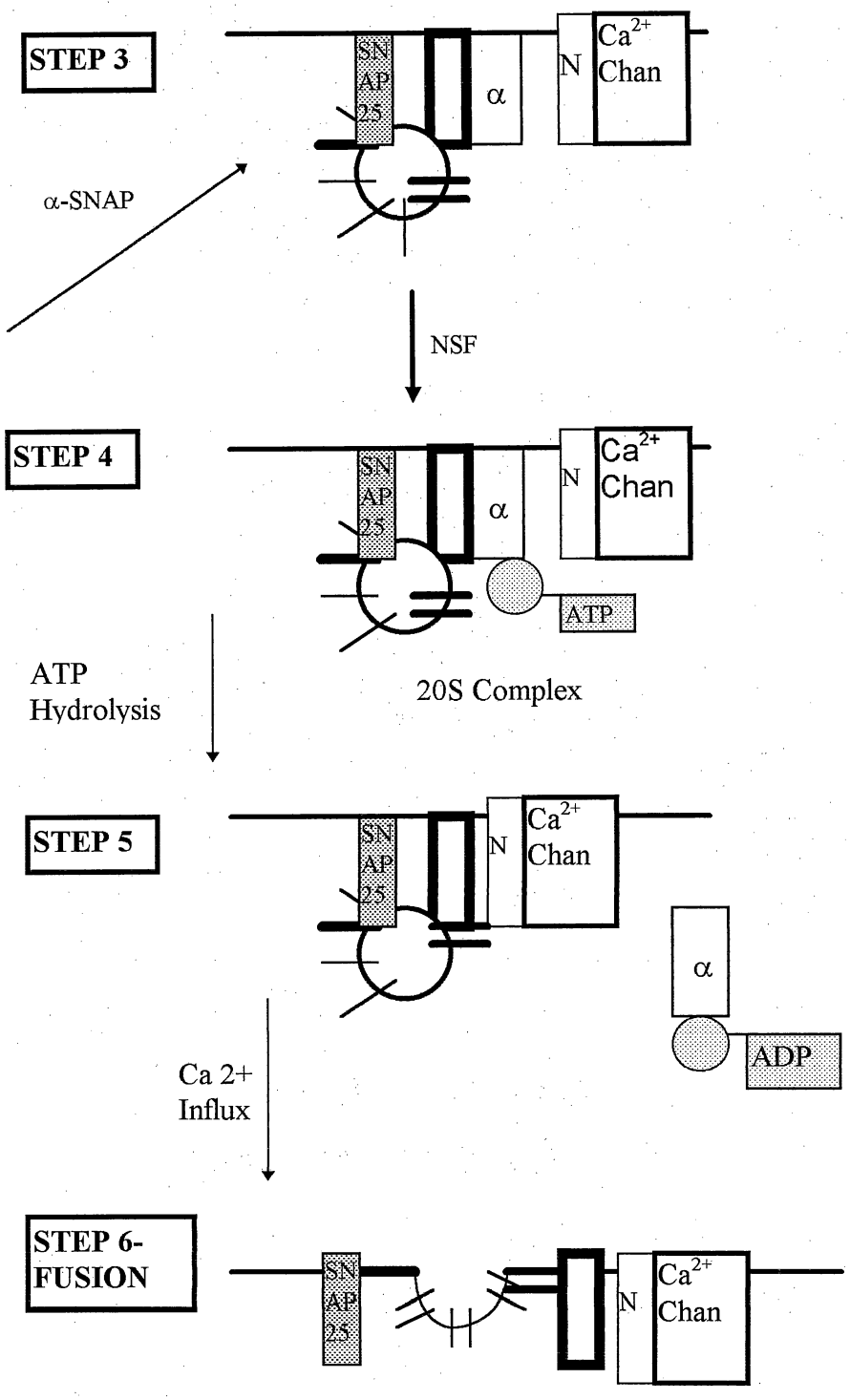
associated with the plasma membrane may not be integrated and are unable to facilitate glucose uptake. The energy to drive the observed metabolic responses is likely supplied by fats. This study suggests a model wherein high doses of T_3 overcome thyroid hormone resistance in the *db/db* mouse and restore GLUT4 transporter translocation.



Appendix 1. Vesicular Trafficking .



Appendix 2. Vesicular translocation, docking, and fusion.



Appendix 2. (Cont.) Vesicular translocation, docking, and fusion.

REFERENCES

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

Beck, K. editor. 1995. Principles and Practice of Endocrinology and Metabolism 2nd ed., Philadelphia, J.B. Lippincott Company, p. 1155-1163.

Berger, J., C. Biswas, P. Vicario, H. Strout, R. Saperstein, and P. Pilch. 1989. Decreased expression of the insulin-responsive glucose transporter in diabetes and fasting. *Nature* 30: 70-72.

Bjorntorp, P. 1987. Fat cell distribution and metabolism. *Annals of the New York Academy of Sciences* 499: 66-72.

Bradford, M. 1976. A rapid sensitive model for quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry* 72: 248-254.

Boissoneault, G., M. Hornshuh, J. Simons, D. Romsos, and G. Leveille. 1978. Oxygen consumption and body fat content of young lean and obese (*ob/ob*) mice. *Proceedings of the Society for Experimental Biology and Medicine* 157: 402-406.

Bray, G. and D. York. 1979. Hypothalamic and genetic obesity in experimental animals: An autonomic and endocrine hypothesis. *Physiological Reviews* 59: 719-808.

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

Burcelin, R. 1993. Changes in uncoupling protein and GLUT 4 glucose transporter expressions in interscapular brown adipose tissue of diabetic rats: Relative roles of hyperglycemia and hypoinsulinemia. *Biochemical Journal* 291: 109-113.

Calderhead, D., K. Kitagawa, L. Tanner, G. Holman, and G. Lienhard. 1990. Insulin regulation of the two glucose transporters in 3T3-L1 adipocytes. *The Journal of Biological Chemistry* 265: 13800-13808.

Casla, A., A. Rovira, J. Wells, and G. L. Dohm. 1990. Increased glucose transporter (GLUT4) protein expression in hyperthyroidism. *Biochemical and Biophysical Research Communications* 171: 1862-1868.

Ciaraldi, T., J. Molina, and J. Olefsky. 1991. Insulin action kinetics in adipocytes from obese and non-insulin dependent diabetes mellitus subjects: Identification of multiple cellular defects in glucose transport. *Journal of Clinical Endocrinology and Metabolism* 72: 876-882.

Clark, C. 1995. 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.

Chua, S., W. Chung, X. Zhang, S. Liu, L. Tartaglia, and R. Liebel. 1996. Phenotypes of mouse *diabetes* and rat *fatty* due to mutations in OB (Leptin) receptor. *Science* 269: 540-543.

Considine, R., E. Considine, C. Williams, T. Hyde, and J. Caro. 1996. The hypothalamic receptor in humans: Identification of incidental sequence polymorphisms and absence of the *db/db* mouse and *fa/fa* rat mutations. *Diabetes* 19: 992-994.

DeFronzo, R., R. Bonadonna, and E. Ferrannini. 1992. Pathogenesis of NIDDM. *Diabetes Care* 15: 318-368.

Fabres-Machado, U. and M. Saito. 1995. The effect of adipose cell size on the measurement of GLUT4 in white adipose tissue of obese mice. *Brazilian Journal of Medical and Biological Research* 28: 369-376.

Fehn, R., S. Roulette, S. Lee, and R. Chiasson. 1988. Thyroid suppression reversal in the *db* mouse by reducing peripheral 5' monodeiodinase activity. 72nd Annual Meeting of the Federation of American Societies for Experimental Biology, #7525.

Fehn, R. and R. Chiasson. 1983. Pituitary-thyroid function in the *db/db* mouse: A model for non-insulin dependent diabetes mellitus. American Society of Zoologists Western Regional Conference on Comparative Endocrinology.

Gautier, M., N. Garreau, L. Madeddu, and L. Sperling. 1994. Evidence for defects in membrane traffic in Paramecium secretory mutants unable to produce functional storage granules. *The Journal of Cell Biology* 124: 893-902.

Glass, C. and J. Holloway. 1990. Regulation of gene expression by the thyroid hormone receptor. *Biochemica et Biophysica* 1032: 157-176.

Guerre-Millo, M. 1996. Glucose transporters in obesity. *Proceedings of the Nutrition Society* 55: 237-244.

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

Hadley, M. 1992. Endocrinology, New Jersey, Prentice Hall, p.292-6, 303, 344-346, and 352-353.

Haffner, S., M. Stern, H. Miettinen, M. Wei, and R. Gingerich. 1996. Leptin concentrations in diabetic and non-diabetic Mexican-Americans. *Diabetes* 45: 822-824.

Hainhault, I., M. Guerre-Millo, C. Guichard, and M. Lavau. 1991. Differential regulation of adipose tissue glucose transporters in genetic obesity (Fatty fat). *Journal of Clinical Investigation* 87: 1127-1131.

Halaas, J., K. Gajiwala, M. Maffei, S. Cohen, B. Chait, D. Rabinowitz, R. Lallone, S. Burley, and J. Friedman. 1995. Weight-reducing effects of the plasma protein encoded by the *obese* gene. *Science* 269: 543-546.

Hansen, P., E. Gulve, B. Marshall, J. Gao, J. Pessin, J. Holloszy, and M. Mueckler. 1995. Skeletal muscle glucose transport and metabolism are enhanced in transgenic mice overexpressing the GLUT4 glucose transporter. *The Journal of Biological Chemistry* 270: 1679-1684.

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

Hummel, K., M. Dick, and K. Coleman. 1966. Diabetes, a new mutation in the mouse. *Science* 153:1227-1228.

Jequier, E. 1987. Energy utilization in human obesity. *Annals of the New York Academy of Sciences* 499: 73-83.

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

Kahn, B., M. Charron, H. Lodish, S. Cushman, and J. Flier. 1989. Differential regulation of two glucose transporters in adipose cells from diabetic and insulin-treated diabetic rats. *Journal of Clinical Investigations* 84: 404-411.

Kahn, C. R. and G. Weir editors. Diabetes Mellitus 13th ed., Philadelphia, Lea and Febiger, 1994, p. 193-7, 320-1, 586-595.

Kalousek, A. K. 1986. The effect of intraperitoneally administered thyroxine, triiodothyronine, and iopanoic acid on the in vivo and in vitro oxygen consumption rates of normal C57BL/KsJ db/m mice. M.S. Thesis, California State University, San Bernardino, San Bernardino.

Koranyi, L., D. James, M. Mueckler, and M. A. Permutt. 1991. Glucose transporter levels in spontaneously obese (*db/db*) insulin resistant mice. *Journal of Clinical Investigation* 85: 962-967.

Leonard, J. 1997. Direct thyroid hormone action on the cytoskeleton of nerve cells. Experimental Biology Annual Meetings.

Livingstone, C., F. Thomson, M. Arbuckle, I. Campbell, T. Jess, S. Kane, C. Moyes, L. Porter, J. Rice, M. Seatter, and G. Gould. 1996. Hormonal regulation of the insulin-responsive glucose transporter, GLUT4: Some recent advances. *Proceedings of the Nutrition Society* 55: 179-90.

Livingstone, C. and G. Gould. 1995. Insulin resistance in diabetes mellitus. *Biochemical Society Transactions* 40: 37-39.

Mason, R., R. Gunst, and J. Hess. Statistical Design and Analysis of Experiments, New York, John Wiley & Sons, 1989, p. 301 and 347.

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

Morris, A. and R. Frizzell. 1994. Vesicle targeting and ion secretion in epithelial cells. *Annual Reviews of Physiology* 56: 371-397.

Mueckler, M. 1994. Facilitative glucose transporters. *European Journal of Biochemistry* 219: 713-725.

Oh, S. and M. Kaplan. 1994. 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 Biology and Medicine* 207: 260-267.

Olson, A. and J. Pessin. 1995. Transcriptional regulation of the human *GLUT4* gene promoter in diabetic transgenic mice. *The Journal of Biological Chemistry* 270: 23491-23495.

Oppenheimer, J., H. Scwartz, and K. Strait. 1994. Thyroid hormone action 1994: The plot thickens. *European Journal of Endocrinology* 130: 16-24.

Pedersen, O., J. Bak, P. Anderson, S. Lund, D. Moller, J. Flier, and B. Kahn. 1990. Evidence against altered expression of GLUT1 or GLUT4 in skeletal muscle of patients with obesity or NIDDM. *Diabetes* 39: 865-870.

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

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

Scheller, R. 1995. Membrane trafficking in the presynaptic nerve terminal. *Neuron* 14: 893-897.

Sinha, M. C. Maldonado, C. Buchanan, W. Pories, C. Carter-Su, P. Pilch, and J Caro. 1991. Adipose tissue glucose transporters in NIDDM. *Diabetes* 40: 472-477.

Stephens, J. and P. Pilch. 1995. The metabolic regulation and vesicular transport of GLUT4, the major insulin-responsive glucose transporter. *Endocrine Reviews* 16: 529-546.

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

Voldstedlund, M., J. Jensen, A. Handberg, and J. Vinten. 1995. Quantity of Na/K ATPase and glucose transporters in the plasma membrane of rat adipocytes is reduced by in vivo triiodothyronine. *European Journal of Endocrinology* 133: 626-634.

Weinstein, S., J. Watts, R. Haber. 1991. Thyroid hormone increases muscle/fat glucose transporter gene expression in rat skeletal muscle. *Endocrinology* 129: 455-464.

Weinstein, S., E. O'Boyle, R. Haber. 1994. Thyroid hormone increases basal and insulin-stimulated glucose transport in skeletal muscle: The role of GLUT4 transporter expression. *Diabetes* 43: 1185-1189.

Zorzano, A., P. Munoz, M. Cramps, C. Mora, X. Testar, and M. Palacin. 1996. Insulin-induced redistribution of GLUT4 glucose carriers in the muscle fiber: In search of GLUT4 trafficking pathways. *Diabetes* 45(Suppl. 1): S70-S81.