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# Insulin resistance in adolescents with type I diabetes is related to a failure to suppress lipolysis

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Insulin Resistance in Adolescents with Type 1 Diabetes Is  
Related to a Failure to Suppress Lipolysis

ALLISON P. STEWART

YALE UNIVERSITY

2001

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Insulin Resistance in Adolescents with Type 1 Diabetes is Related to a  
Failure to Suppress Lipolysis

A Thesis Submitted to the  
Yale University School of Medicine  
in Partial Fulfillment of the Requirements for the  
Degree of Doctor of Medicine

by

Allison P. Stewart

2001



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## **INSULIN RESISTANCE IN ADOLESCENTS WITH TYPE 1 DIABETES IS RELATED TO A FAILURE TO SUPPRESS LIPOLYSIS**

Allison P. Stewart, Rubina A. Heptulla, Staffan Enoksson, Fran Rife, Tony Yong-Zhan Ma, Robert S. Sherwin, William V. Tamborlane, and Sonia Caprio. Section of Endocrinology, Departments of Pediatrics and Internal Medicine, Yale University, New Haven, CT.

Good metabolic control is often elusive in adolescents with type 1 diabetes (T1DM), partly due to significant insulin resistance. The failure of insulin to suppress lipolysis may be related to the insulin resistance of adolescents with poorly-controlled T1DM. To explore this question at the tissue level, we employed microdialysis to evaluate glycerol in the extracellular fluid (ECF) of fat and muscle tissues during a hyperinsulinemic glucose clamp. 10 adolescents with poorly-controlled T1DM, and 6 normal adolescents were evaluated. The T1DM group exhibited 39% less insulin-stimulated glucose metabolism than the control group. Although basal rates of carbohydrate and lipid oxidation were similar, the T1DM group had significantly less carbohydrate and more lipid oxidation during insulin infusion than the control group. Despite similar baseline plasma glycerol and free fatty acid levels, the T1DM subjects experienced less suppression of these metabolites than control subjects. Basal glycerol concentrations in adipose (T1DM:  $239.1 \pm 24.9 \mu\text{M}$ ; control:  $241.3 \pm 34.6 \mu\text{M}$ ) and muscle (T1DM:  $131.4 \pm 11.1 \mu\text{M}$ ; control:  $141.5 \pm 10.1 \mu\text{M}$ ) microdialysate were similar between groups. However, in both tissues, the T1DM subjects had significantly higher levels of ECF glycerol at the conclusion of the clamp than control subjects (muscle:  $85.3 \pm 7.5 \mu\text{M}$  for T1DM and  $51.5 \pm 7.6 \mu\text{M}$  for control,  $p = 0.01$ ; adipose:  $149.6 \pm 22.8 \mu\text{M}$  for T1DM and  $82.0 \pm 11.4 \mu\text{M}$  for control,  $p = 0.047$ ). Considering all subjects, the rate of insulin-stimulated metabolism was inversely correlated to glycerol concentration in both adipose ( $r = 0.63$ ,  $p < 0.01$ ), and muscle ( $r = 0.63$ ,  $p < 0.01$ ) microdialysate. In summary, our data is consistent with the hypothesis that there exists a failure of insulin to inhibit lipolysis in adolescents with poorly controlled T1DM which is closely related to the insulin resistance observed in this population.



## **Acknowledgements**

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## **INTRODUCTION**

### **Type 1 Diabetes Mellitus**

Type 1 diabetes mellitus (T1DM) is a chronic disease of insulin deficiency, coexisting with peripheral insulin resistance. It results from the autoimmune destruction of the insulin-producing pancreatic beta cells (1). Susceptibility to this disease is inherited, and it has been postulated that either an environmental or viral insult triggers this destruction of beta cells (1). Patients require exogenous insulin treatment for survival, yet insulin action is impaired in Type 1 diabetes, and patients are considered resistant to the action of insulin (2). T1DM has its onset primarily in childhood and adolescence. 1994 United States estimates placed the prevalence of T1DM in children under age 20 at 1.7 cases per 1,000, implying that 127,000 children and teenagers had T1DM in this country at that time (3). The incidence of T1DM on an annual basis is estimated at 18 new diagnoses per 100,000 in those younger than age 20, which translates into more than 13,000 new cases of T1DM diagnosed each year (3,4). The 0.5% risk of developing Type 1 diabetes before age 20 is greater than that of many other severe and chronic childhood illnesses, including muscular dystrophy and lupus (3).

### **Morbidity and Mortality: the DCCT**

Although the 1920s discovery of insulin was heralded as the cure for diabetes (5), this treatment introduced new problems in the diabetic population. Microvascular, macrovascular, and neurologic complications became increasingly common in patients with Type 1 diabetes as they began to survive into adulthood. In 1982, the National Institutes of Health initiated the Diabetes Control and Complications Trial (DCCT) (6) as a multi-center study testing the hypothesis that better metabolic control would lead to



fewer long-term complications of diabetes. A total of 1,441 patients with T1DM were enrolled in the study, and each was randomly assigned to receive either conventional or intensive treatment. The results of the DCCT confirmed the suspicion that intensive therapy, targeted at maintaining near-normal glycemic levels, both delayed the onset of retinopathy, neuropathy, and nephropathy, as well as slowed the progression of these complications in patients with T1DM (7). However, only 14% of subjects in the DCCT were between 13 and 17 years of age, and thus the use of intensive therapy was debated in the adolescent population. Therefore, a separate analysis was performed on the 195 adolescent subjects participating in the DCCT (8). The adolescents were also found to benefit from intensive therapy, with a decrease in risk of developing retinopathy of 53% and of retinopathy progression of 70%. Based on these results, the DCCT Research Group reiterated its recommendations for intensive treatment to prevent long-term effects of diabetes, and extended it to specifically include adolescents older than 13 years of age (8).

### **Insulin Resistance**

Insulin resistant states exist both in health as well as in disease. Insulin resistance implies insensitivity to the effects of insulin on metabolism. Patients with insulin resistance will require greater amounts of the hormone to achieve the same metabolic effect when compared to those who are insulin-sensitive. Adolescents with T1DM are at the extreme disadvantage of experiencing several insulin resistant states concurrently, which act synergistically to complicate the management of their diabetes. In a 1982 study utilizing the euglycemic, hyperinsulinemic clamp technique to measure insulin sensitivity (9), DeFronzo *et al.* set out to ascertain if insulin resistance is present in Type



1 diabetes (2). They found a severe degree of insulin resistance in their young subjects with T1DM, and demonstrated that glucose clearance was 254% better in the control subjects versus those subjects with T1DM (2). The DCCT had noted that both the intensive and conventional adolescent groups had slightly higher glycated hemoglobin than comparable adult subjects, although the differences between the treatment groups for adolescents and adults were similar ( $1.70 \pm 0.18\%$  for the adolescents versus  $1.90 \pm 0.06\%$  for the adults) (8). The DCCT documented a phenomenon already familiar to pediatric diabetes caregivers: in puberty, glycosylated hemoglobin levels typically rise as glycemic control deteriorates (10,11), despite rising insulin dosage (12). This metabolic deterioration was traditionally ascribed to the social and psychological upheaval that may occur during puberty (13). However, the possibility remained that puberty itself, in addition to the already established insulin resistance of T1DM (2), might contribute to this deterioration. To explore this theory, Amiel *et al.* employed the euglycemic clamp technique to determine the insulin sensitivity of adult, prepubertal, and pubertal subjects both with and without T1DM (14). It was possible to demonstrate that puberty is associated with a decline in insulin-stimulated glucose metabolism in both adolescents with and without diabetes, and that this decline is exaggerated in children who also have T1DM due to the additive effects of puberty and diabetes (14). Further studies utilizing glucose isotope tracer techniques, indirect calorimetry, and a sequential two-step insulin clamp (8 and 40 mU/m<sup>2</sup>·min), revealed that the insulin resistance of puberty is primarily restricted to peripheral glucose metabolism, and has little effect on hepatic glucose production, or on circulating levels of essential amino acids or free fatty acids (15). Additionally, it was postulated that this resistance is perhaps beneficial in that it



facilitates amplification of the anabolic effects of insulin (15). To determine if there existed more global effects of insulin resistance in adolescent patients with and without diabetes, Caprio *et al.* used the euglycemic insulin clamp in nondiabetic adolescents and young adults, and followed glucose, protein, and fat metabolism using leucine tracers (to follow protein metabolism), and indirect calorimetry (16). After comparing these results to those in adolescents with T1DM, they found that while the insulin resistance of normal puberty is selective for glucose metabolism, the defects in adolescents with T1DM are more profound, and affect protein and fat metabolism as well (16). Thus, the poor metabolic control of adolescents with Type 1 diabetes can now, at least partly, be explained by the synergistic insulin resistance present secondary to T1DM and puberty.

### **Mechanisms of Insulin Resistance: free fatty acids**

Almost forty years ago, based on experiments in isolated rat heart muscle and hemidiaphragms, Randle *et al.* proposed a glucose-fatty acid cycle, in which free fatty acids (FFAs) compete with glucose for oxidation, resulting in decreased glucose uptake and subsequent insulin resistance (17). The key points of this theory are that high FFAs lead to elevated acetyl-CoA and citrate levels. Acetyl CoA inhibits pyruvate dehydrogenase and thus reduces glucose oxidation. High citrate levels lead to inhibition of phosphofructokinase, and thus of glycolysis itself, eventually reducing glucose uptake as accumulation of glucose-6-phosphate inhibits hexokinase (17). Early studies found difficulty in reproducing Randle's results (18), but as the traditional emphasis for the pathogenesis of diabetes shifted from carbohydrate to lipid metabolism (19), Randle's cycle garnered interest as a possible etiology for multiple insulin resistant states and spurred on much more research.





As free fatty acid levels were known to be elevated in such diseases as non-insulin dependent diabetes mellitus, and obesity, the glucose-fatty acid cycle was of much interest to those researching in these areas. In a study of normal subjects mimicking the normal fed and diabetic states, Ferrannini *et al.* were able to confirm Randle's hypothesis that, in the well insulinized state, elevated FFA levels do effectively compete with glucose for uptake in the peripheral tissues, and that this inhibition is not overcome by hyperglycemia (20). This group further concluded that when insulin is deficient, elevated FFA levels lead to hyperglycemia through enhanced endogenous glucose production (20).

While most investigators agreed that high FFA levels inhibited carbohydrate oxidation while enhancing lipid oxidation, the effect of elevated FFAs on glucose uptake demonstrated by Ferrannini *et al.* (20) remained controversial (21,22). Further studies sought to clarify this issue, and to continue to explore the mechanism of FFA induced insulin resistance. In a 1991 study by Boden *et al.*, infusion of lipid in healthy subjects during euglycemic hyperinsulinemia was found to increase fat oxidation and decrease insulin-stimulated carbohydrate oxidation, but not to decrease glucose uptake for at least three hours (23). This decrease in glucose uptake was found to be associated with a decrease in muscle glycogen synthase, leading to the conclusion that high levels of lipid somehow interfered with glucose storage, and in this way inhibited glucose uptake (23). This time delay could explain why other investigators, using shortened experiment times, had previously failed to see the effects of elevated FFA on glucose uptake. Further studies by Boden *et al.* elucidated a dose-dependency of free fatty acid mediated



inhibition of glucose uptake (24). The mechanism for the inhibition was found to occur via two pathways. First, inhibition of carbohydrate oxidation occurred through acetyl-CoA-induced inhibition of pyruvate dehydrogenase, supporting Randle's glucose-fatty acid cycle and confirmed by other studies (25). Secondly, this decrease was observed to take place through inhibition of glycogen synthesis, both by a fat mediated interference with insulin's ability to stimulate glycogen synthase, and also perhaps by a defect in glucose transport or phosphorylation (24). A challenge to the classic Randle cycle mechanism comes from recent studies performed by Shulman and his collaborators (26, 27). In sharp contrast to the Randle cycle prediction, these investigators discovered that glucose-6-phosphate decreased in response to lipid infusion. This finding supported the theory that the inhibition of transport or phosphorylation occurred early in the pathway, and not via the metabolic bottleneck secondary to inhibition of pyruvate dehydrogenase (26) suggested by Randle (17). Subsequent investigations by Dresner *et al.* further clarified the mechanism of FFA-induced insulin resistance by measuring glycogen, glucose-6-phosphate, and intracellular glucose concentrations in healthy subjects using nuclear magnetic resonance spectroscopy during a hyperinsulinemic euglycemic clamp procedure following a five-hour infusion of lipid/heparin (28). The results of this investigation showed that the insulin resistance observed in response to lipid infusion was related to a defect in glucose transport (28). Additionally, muscle biopsy specimens obtained from these studies revealed that FFA-induced insulin resistance was temporally related to alterations in insulin-stimulated IRS-1-associated phosphatidylinositol (PI) 3-kinase activity (28). Therefore, it was concluded that elevated FFAs caused insulin resistance through inhibition of insulin-stimulated glucose transport, perhaps related to a



defect in insulin signaling through IRS-1-associated PI 3-kinase (28). Despite much research targeted at proving FFA induced insulin resistance is the mechanism for the insulin resistance of diabetes and of obesity, the exact method by which this happens remains controversial.

The glucose free fatty acid cycle also piqued interest as a possible etiology for another insulin resistant state. Elevated free fatty acids levels had likewise been noted specifically in adolescents (14), along with a failure to suppress these levels in response to insulin (16). To explore the Randle cycle as a possible etiology for the insulin resistance of puberty, Arslanian *et al.* studied prepubertal, pubertal, and adult healthy subjects using the euglycemic clamp technique, along with the use of glycerol tracers (to study rates of lipolysis), and indirect calorimetry (29). They discovered that the rate of lipid oxidation was increased during puberty, and was accompanied by a decrease in insulin-stimulated glucose metabolism. These results were felt to be consistent with the hypothesis that increased lipid oxidation, perhaps regulated by growth hormone secretion, might cause the decrease in insulin-stimulated glucose metabolism and insulin resistance appreciated during puberty (29), as suggested by Randle *et al.* (17).

Amidst the research linking elevated FFAs to the insulin resistance of diabetes, obesity, and of puberty through the Randle cycle, or a modification thereof, it has not previously been established whether the failure of insulin to inhibit lipolysis is related to the level of insulin-stimulated glucose metabolism seen in adolescents with diabetes. To examine this theory directly in target tissues of interest, we have employed the use of the





hyperinsulinemic euglycemic clamp, indirect calorimetry, and the microdialysis technique to ascertain the effect of insulin on lipolysis in adolescents with poorly controlled Type 1 diabetes mellitus. By using microdialysis, we are able to target the study of lipolysis directly to muscle as well as to adipose tissue. Recent research has revealed that both adipose and muscle make important contributions to circulating glycerol levels (30, 31), although adipose may dominate (30), making the study of both tissue sites important in the search to clarify the insulin resistance experienced by adolescents with Type 1 diabetes.

### **Microdialysis**

The microdialysis technique is an intriguing discovery that allows investigators to characterize ongoing metabolism at the tissue level, rather than being restricted to analysis of whole body metabolism by sampling from blood. Initially used in studies of the rat brain, microdialysis was first used in human research by Lonroth *et al.* in 1987 (32). This group devised a simple, although time consuming, method for *in vivo* calibration of the microdialysis probe, making this technique more accessible for use (32). Allowing direct access to the extracellular space and the microenvironment of the cell, microdialysis has several advantages. It is relatively non-traumatic, and permits the analysis of several substances at once and over long periods of time (33, 34). Given these characteristics, it is well designed to facilitate the study of local tissue metabolism in both adipose as well as muscle tissue.

A commonly used and commercially available microdialysis device is a double lumen catheter, with an inlet port leading fluid (called perfusate) into the outer lumen



towards a semipermeable dialysis membrane. This membrane sits in direct contact with the extracellular space. Low molecular weight molecules diffuse towards or away from the membrane according to their concentration gradients. The resulting fluid, called dialysate, leaves the membrane via the inner cannula to the outlet port, and is collected for analysis. The types of substances measured by the technique are determined by the permeability of the membrane, and substances that are highly lipophilic, such as FFAs, cannot be measured (33). Ideally, this arrangement mimics a blood vessel, with a microinfusion pump maintaining a low, constant flow of perfusate entering the exterior lumen, equilibrating at the membrane, and exiting as dialysate through the interior lumen (33).

Although free fatty acids are perhaps the most reliable indicator of lipolysis, there are multiple roadblocks to using them as a marker. Free fatty acids can be activated and re-esterified into new triacylglycerol (35, 36). This flux of FFAs makes them a poor candidate for tracking lipolysis. Additionally, as previously mentioned, FFAs cannot be measured with the current microdialysis techniques (33). Glycerol, on the other hand, is not only permeable to the dialysis membrane, but also is not reesterified to any significant extent (37). Although glycolysis is another potential source for glycerol, Nurjan *et al.* found that in the postabsorptive state, more than 98% of the glycerol that appears derives from lipolysis, and thus the rate of appearance of glycerol can safely be used to estimate the rates of overall lipolysis *in vivo* (35).



## **Statement of Purpose and Hypothesis**

The purpose of this study was to determine whether the failure of insulin to suppress lipolysis in adolescents with poorly controlled Type 1 diabetes mellitus is related to the insulin resistance observed in this population. Our null hypothesis was that there is no relationship between the failure of insulin to inhibit lipolysis and the insulin resistance seen in adolescents with poorly controlled Type 1 diabetes mellitus. To answer this question, we employed the euglycemic hyperinsulinemic clamp technique to determine the insulin sensitivity of adolescents with and without diabetes, while at the same time utilizing microdialysis to directly follow the appearance of glycerol (as a marker of lipolysis) in muscle and adipose tissues. Additionally, fat and carbohydrate oxidation was followed by the use of indirect calorimetry.



## **METHODS**

### **Subjects**

Sixteen subjects in total participated in the study: 10 adolescents with Type 1 diabetes mellitus, and 6 non-diabetic adolescent controls. The 10 participating subjects with diabetes attended the Pediatric Diabetes Clinic of the Yale-New Haven Medical Center. They were eligible for the study if their disease duration was greater than one year, if they had no symptoms or signs of autonomic neuropathy, if they were receiving no medications other than insulin, and if they were not suffering from any other medical illnesses. Additionally, all 10 T1DM subjects had poorly controlled diabetes, as indicated by a HbA<sub>1C</sub> greater than 9%. The non-diabetic subjects were recruited independently, and all were in good health. Prior to the study day, a detailed medical history was taken from each patient and a physical exam was performed. Adiposity was determined by calculation of body mass index (BMI) using each subject's height and weight, as well as by measurement of body fat mass assessed using dual energy x-ray absorption (DEXA) on a subsequent visit to the Yale General Clinical Research Center. Subject characteristics are summarized in Table 1.





**Table 1. Clinical Characteristics (Mean  $\pm$  S.E.M.)**

|                          | T1DM Adolescents | Normal Control Adolescents |
|--------------------------|------------------|----------------------------|
| N                        | 10               | 6                          |
| Gender (M/F)             | 7M/3F            | 3M/3F                      |
| Age (years)              | 16.2 $\pm$ 0.6   | 15.8 $\pm$ 0.9             |
| Height (m)               | 1.64 $\pm$ 0.07  | 1.62 $\pm$ 0.03            |
| Weight (kg)              | 66.8 $\pm$ 3.2   | 58.6 $\pm$ 2.5             |
| BMI (kg/m <sup>2</sup> ) | 23.2 $\pm$ 0.7   | 22.2 $\pm$ 0.8             |
| HbA <sub>1c</sub> (%)    | 10.0 $\pm$ 0.2   |                            |
| Total % Fat              | 18.5 $\pm$ 23.5  | 20.7 $\pm$ 3.6             |

The protocol was approved by the Human Investigation Committee of the Yale School of Medicine, and prior to the study, informed, written consent was obtained from all subjects and a parent of the adolescent subjects.

### **Euglycemic Hyperinsulinemic Clamp**

Subjects with diabetes were admitted to the Yale General Clinical Research Center the night prior to the study. An intravenous catheter was inserted into their left antecubital vein for infusion of insulin overnight, and later for infusion of test substances. These patients were then given continuous basal insulin infusion overnight to ensure euglycemia, based on plasma glucose measurements obtained every 30 to 60 minutes. All diabetic patients maintained their plasma glucose greater than 4.0 mmol/L during the evening prior to the study.



All subjects were available at 0700 at the Yale General Clinical Research Center, following an overnight fast of at least nine hours. Blood pressure and pulse were obtained, and patients were placed in the supine position for investigation. During the study, patients were allowed to watch movies as they rested. A retrograde cannula was inserted into a vein in the dorsum of the right hand, which was then placed inside a heated box (approximately 55° C) for sampling of arterialized venous blood (38). Infusion of small amounts of normal saline (0.9%) was used to maintain patency of this catheter between sampling. On the morning of the study, the nondiabetic subjects received an intravenous catheter in their left antecubital vein for infusion of test substances.

Blood samples were collected every ten minutes during the baseline period (forty minutes) for measurement of plasma insulin, glycerol, free fatty acid, and glucose levels. Following the baseline period, a two-step priming dose of insulin was administered (3.2 mU/kg/min followed by 1.6 mU/kg/min, each lasting for five minutes), followed by a continuous infusion of insulin at 0.8 mU/kg/min for 180 minutes. Plasma glucose levels were measured at the bedside every five minutes (Beckman glucose analyzer, Beckman Instruments, Fullerton, CA, USA), and glucose levels were kept constant (~4.6 mmol/L) by variable infusion of a 20% dextrose solution. Blood samples were taken at 10 to 20 minute intervals for analysis of plasma insulin, glycerol, and free fatty acids. Respiratory gas exchange rates were measured by a computerized open circuit indirect calorimetry (Deltatrac, Sensor Medics, Helsinki, Finland) with a ventilated hood system during the basal period, and thirty minutes prior to the end of the clamp procedure.



## Microdialysis

Two microdialysis catheters with a 30 mm membrane and a molecular weight cutoff of 20 kDa (CMA/60, CMA/microdialysis, Acton, MA) were placed: one in the gastrocnemius muscle, and one in the anterior abdominal fat tissue. One hour prior to placement of catheters, the skin was anesthetized with EMLA cream (Astra, Sodertajee, Sweden), and again at insertion with an injection of lidocaine. The muscle microdialysis probe was placed in the medial portion of the left gastrocnemius, where the muscle tapers to become the Achilles tendon. This allowed the probe to be placed parallel to the fibers of the muscle, thus preventing bending on insertion. One probe was placed in the periumbilical adipose tissue. This catheter was inserted horizontally in a medial direction from an insertion point approximately 8 cm lateral to the umbilicus (39). The input tubing of each catheter was connected to a microinfusion pump (CMA/100, CMA Medical, Stockholm, Sweden) and continuously perfused with artificial extracellular fluid (135 mmol/L NaCl, 3 mmol/L KCl, 1 mmol/L MgCl<sub>2</sub>, 1.2 mmol/L CaCl<sub>2</sub>, 300 μmol/L ascorbate, and 2 mmol/L Na phosphate buffer adjusted to pH 7.4).

After catheter insertion, a fifteen minute flush period ensued; followed by a three hour equilibration period designed to eliminate artifact secondary to the trauma of insertion of the catheters. Dialysate was collected every sixty minutes. The entire study lasted six hours (three hours of equilibration period and three hours of experimental period), during which the subjects reclined in bed.



## Substrate and Hormone Measurements

Plasma insulin was measured using a double-antibody radioimmunoassay (Linco Research, St. Louis, MO, USA). In patients with diabetes, free insulin was also determined by precipitating plasma samples at the bedside with polyethylene glycol (PEG), with all measurements being performed within one month of the study. The recovery in healthy subjects following addition of PEG was  $99 \pm 3\%$ . Total HbA<sub>1C</sub> was measured by high-performance liquid chromatography using Bio Rad (Hercules, CA, USA) equipment with a normal reference range of 0.045 - 0.059. Plasma free fatty acids were measured by the colorimetric method. Dialysate and plasma glycerol was measured in duplicate by an enzymatic fluorometric method using an automated multianalyzer (CMA/600, CMA/Microdialysis, Stockholm, Sweden).

## Calculations

During the insulin clamp study, the amount of glucose infused to maintain euglycemia provided a means of determining insulin-stimulated glucose metabolism. The glucose infusion rate was calculated at 20 minute intervals, and corrected for deviations from the target plasma glucose level, as previously described by DeFronzo *et al.* (9). Respiratory gas exchange rates were measured by a computerized open circuit indirect calorimetry (Deltatrac, Sensor Medics, Helsinki, Finland) with an ventilated hood system. Oxidation rates for carbohydrate, fat, and protein before and during the clamp procedure were calculated from the measured O<sub>2</sub> consumption, CO<sub>2</sub> production, and urinary nitrogen excretion, by assuming that each gram of urinary nitrogen is derived





from the oxidation of 6.25 grams of protein, and that each gram of substrate oxidized consumes O<sub>2</sub> and generates CO<sub>2</sub> in the following amounts: carbohydrate, 0.829 liters of O<sub>2</sub> and 0.829 liters of CO<sub>2</sub>; fat, 2.020 liters of O<sub>2</sub> and 1.428 liters of CO<sub>2</sub>; protein, 0.905 liters of O<sub>2</sub> and 0.781 liters of CO<sub>2</sub> (40).

## **Statistics**

All values are presented as mean  $\pm$  standard error of the mean (S.E.M.). Repeated measures analysis of variance (ANOVA) was performed with a single factor to compare the responses of the different groups over time. SPSS (version 10.0) was used for statistical analysis.

The thesis author performed the majority of the subject recruitment of the diabetic subjects and assisted with these microdialysis studies. Further recruitment and microdialysis studies of the control subjects were done by Dr. Rubina Heptulla and Dr. Sonia Caprio. The thesis author assisted in the compilation of the data, and was assisted by Dr. Tony Ma in the statistical analysis.



## RESULTS

**Insulin** Basal plasma insulin was not significantly different between the subjects with T1DM and the normal controls ( $13.4 \pm 1.5 \mu\text{U/ml}$  for T1DM versus  $11.8 \pm 1.3 \mu\text{U/ml}$  for controls) ( $p = 0.49$ ). During the hyperinsulinemic euglycemic clamp procedure, both groups reached similar steady-state plasma insulin levels during the last hour of the study ( $38.8 \pm 2.0 \mu\text{U/ml}$  for T1DM versus  $41.7 \pm 2.8 \mu\text{U/ml}$  for controls) ( $p = 0.41$ ).

**Glucose** Basal plasma glucose levels were significantly different between groups ( $119.0 \pm 5.9 \text{ mg}$  for T1DM versus  $91.8 \pm 2.5 \text{ mg}$  for controls) ( $p < 0.05$ ), but became and remained similar as the clamp study proceeded ( $96.6 \pm 2.6 \text{ mg}$  for T1DM versus  $87.5 \pm 3.7 \text{ mg}$  for controls, at 30 minutes from initiation of clamp) ( $p > 0.05$ ), and care was given to maintain plasma glucose at  $\sim 89 \text{ mg}$  ( $5\text{mM}$ ).

### Glucose Metabolism

The amount of glucose infused to maintain euglycemia during the clamp procedure serves as an index of insulin-stimulated glucose metabolism. During the clamp procedure, this “M” value was 39% lower in subjects with poorly controlled diabetes ( $120.0 \pm 15.8 \text{ mg/m}^2 \cdot \text{min}$ ) when compared with normal controls ( $196.8 \pm 39.8 \text{ mg/m}^2 \cdot \text{min}$ ) ( $p = 0.051$  assuming a nonparametric distribution, Figure 1).



Basal carbohydrate oxidation rates were similar in the poorly controlled T1DM group ( $32.8 \pm 3.9\%$  of total energy expenditure (EE)) when compared to the normal control group ( $44.3 \pm 5.1\%$  of total EE) ( $p = 0.09$ ). However, during insulin infusion, while the control group increased their carbohydrate oxidation significantly by 43% (from a basal value of  $44.3 \pm 5.1\%$  of total EE to a clamp value of  $63.6 \pm 5.0\%$  of total EE) ( $p = 0.003$ , Figure 2A), the T1DM group also increased their carbohydrate oxidation, but not significantly (from basal value of  $32.8 \pm 3.9\%$  of total EE to a clamp value of  $44.1 \pm 6.1\%$  of total EE) ( $p > 0.05$ , Figure 2A). As a result, rates of carbohydrate oxidation during the clamp were significantly lower in the T1DM group ( $44.1 \pm 6.1\%$  of total EE) when compared to the control group ( $63.6 \pm 5.0\%$  of total EE) ( $p < 0.05$ , Figure 2A).

### **Fat Metabolism**

There was not a significant difference in basal rates of fat oxidation between the T1DM group ( $53.8 \pm 4.2\%$  of total EE) and the normal control group ( $40.2 \pm 5.1\%$  of total EE) ( $p = 0.06$ , Figure 2B). However, during the infusion of insulin, the control group significantly suppressed their rate of fat oxidation by 44% (from a basal value of  $40.2 \pm 5.1\%$  of total EE to a clamp value of  $22.5 \pm 4.7\%$  of total EE) ( $p = 0.002$ , Figure 2B), whereas in the T1DM group, fat oxidation changed minimally (from a basal rate of  $53.8 \pm 4.2\%$  of total EE to a clamp value of  $42.8 \pm 6.3\%$  of total EE) ( $p = 0.06$ , Figure 2B). Therefore, rates of fat oxidation during the infusion of insulin were significantly higher in the T1DM group ( $42.8 \pm 6.3\%$  of total EE) when compared to the control group ( $22.5 \pm 4.7\%$  of total EE) ( $p < 0.05$ , Figure 2B).



Basal plasma free fatty acid levels were similar between groups ( $500.8 \pm 73.3 \mu\text{M}$  for the T1DM group versus  $663.2 \pm 129.1 \mu\text{M}$  for the control group) ( $p = 0.26$ ). During the infusion of insulin, the T1DM group showed significantly less percent suppression of plasma FFA from baseline in the second hour ( $62.9 \pm 6.2\%$  for T1DM versus  $84.4 \pm 5.8\%$  for control) ( $p = 0.035$ , Figure 3) and in the third hour ( $49.5 \pm 8.1\%$  for T1DM versus  $85.1 \pm 4.1\%$  for control) ( $p = 0.006$ , Figure 3) when compared to the control group.

Basal plasma glycerol levels were similar between groups, as shown in Figure 4. During insulin infusion, plasma glycerol remained near baseline in the T1DM group, while the control group experienced a decrease in plasma glycerol. The T1DM group had a significantly higher plasma glycerol at the end of the clamp procedure when compared to the control group ( $p < 0.05$ ).

### **Glycerol Concentrations in Adipose Microdialysate**

Basal values of glycerol present in adipose microdialysate were similar in both the T1DM group ( $239.1 \pm 24.9 \mu\text{M}$ ) and the control group ( $241.3 \pm 34.6 \mu\text{M}$ ) ( $p = 0.96$ , Figure 5A). However, during the second and third hours of the clamp procedure, glycerol concentration in adipose microdialysate were significantly higher in the T1DM group compared to the control group ( $149.6 \pm 18.5 \mu\text{M}$  for T1DM versus  $89.2 \pm 7.3 \mu\text{M}$  for controls during the second hour,  $p = 0.03$ , and  $149.6 \pm 22.8 \mu\text{M}$  for T1DM versus  $82.0 \pm 11.4 \mu\text{M}$  for controls during the third hour,  $p = .047$ , Figure 5A). This change is





similarly reflected in Figure 5B, which shows a significantly lower percent suppression of glycerol from baseline experienced in the T1DM group during the second ( $36.6 \pm 3.7\%$  for T1DM versus  $59.7 \pm 5.5\%$  for controls) ( $p = 0.003$ ) and third hours ( $38.0 \pm 4.8\%$  for T1DM versus  $63.2 \pm 6.5\%$  for controls) ( $p = 0.007$ ) of the clamp procedure when compared to the control group.

### **Glycerol Concentrations in Muscle Microdialysate**

Basal muscle glycerol concentrations in microdialysate were similar in the T1DM group ( $131.4 \pm 11.1 \mu\text{M}$ ) and the normal control group ( $141.5 \pm 10.1 \mu\text{M}$ ) ( $p = 0.55$ , Figure 6A). By the third hour of the clamp procedure, the T1DM group had a significantly higher muscle glycerol concentration in microdialysate when compared to the control group ( $85.3 \pm 7.5 \mu\text{M}$  for the T1DM group versus  $51.5 \pm 7.6 \mu\text{M}$  for the control group) ( $p = 0.01$ , Figure 6A). This failure to suppress glycerol production in muscle is also reflected in Figure 6B, where the percent suppression of glycerol from baseline in muscle microdialysate is significantly lower in the T1DM group at hour two ( $29.8 \pm 4.2\%$  for T1DM versus  $47.7 \pm 3.36\%$  for control) ( $p = 0.01$ ) as well as at hour three ( $33.6 \pm 3.9\%$  for T1DM versus  $63.5 \pm 4.8\%$  for controls) ( $p < 0.001$ ), when compared to control subjects.

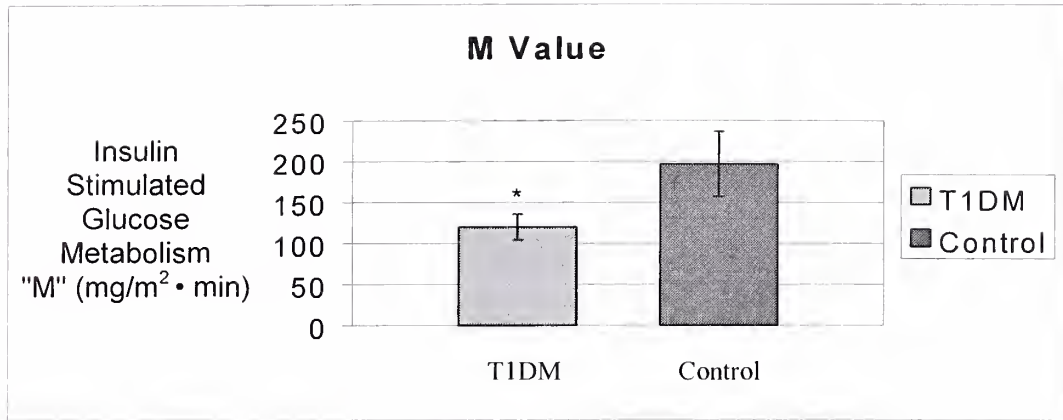
### **Insulin-stimulated Glucose Metabolism and Dialysate Glycerol Concentration**

An inverse relationship is observed between all subjects' insulin-stimulated glucose metabolism, represented by their M values, and the concentration of glycerol in the extracellular fluid of adipose and muscle tissues at the end of the clamp period (after

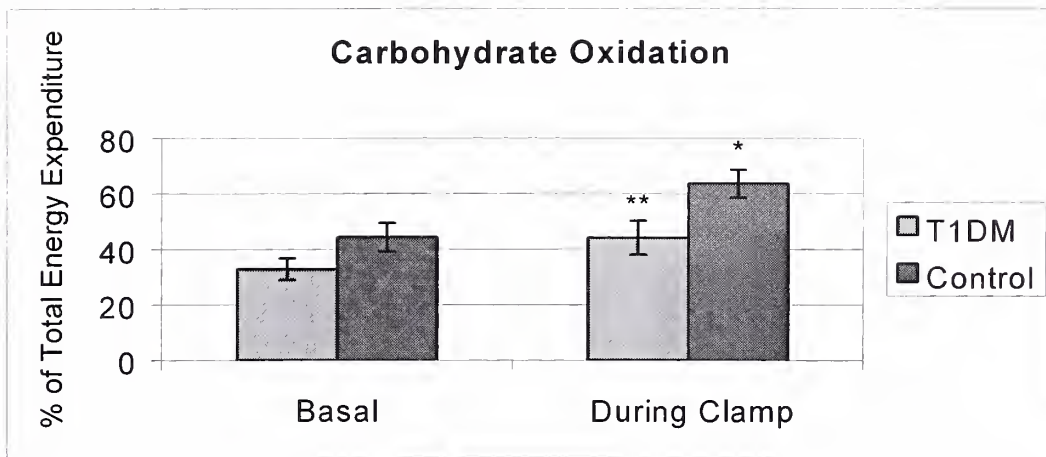


three hours of euglycemic hyperinsulinemia). This relationship is demonstrated in Figures 7A and 7B. In adipose tissues, this relationship is defined by an  $r = 0.63$  ( $p < 0.05$ , Figure 7A), and in muscle tissue, an  $r = 0.63$  is observed ( $p < 0.05$ , Figure 7B). This correlation persists even when the subjects are separated into individual groups and analyzed independently (data not shown).

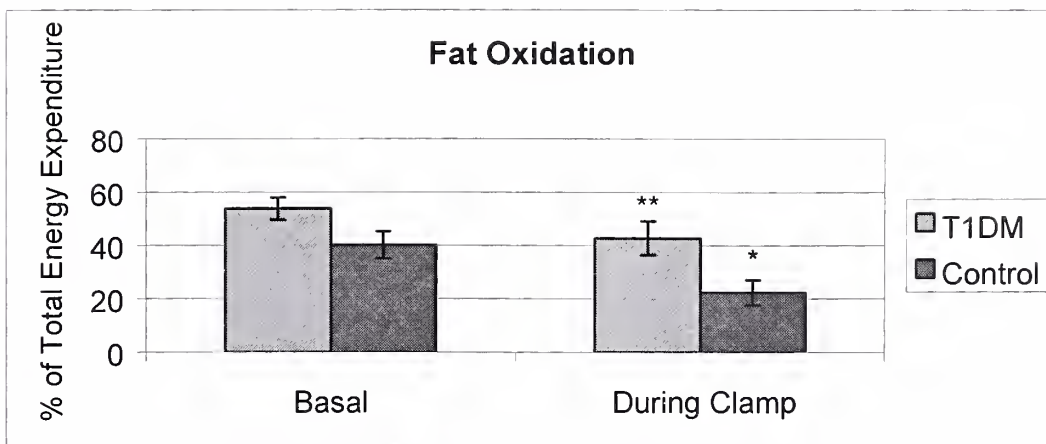




**Figure 1.** Reduced insulin stimulated glucose metabolism in subjects with Type 1 diabetes versus normal controls as an indication of insulin resistance. The single asterisk denotes  $p = 0.05$  for adolescents with diabetes versus the control group.

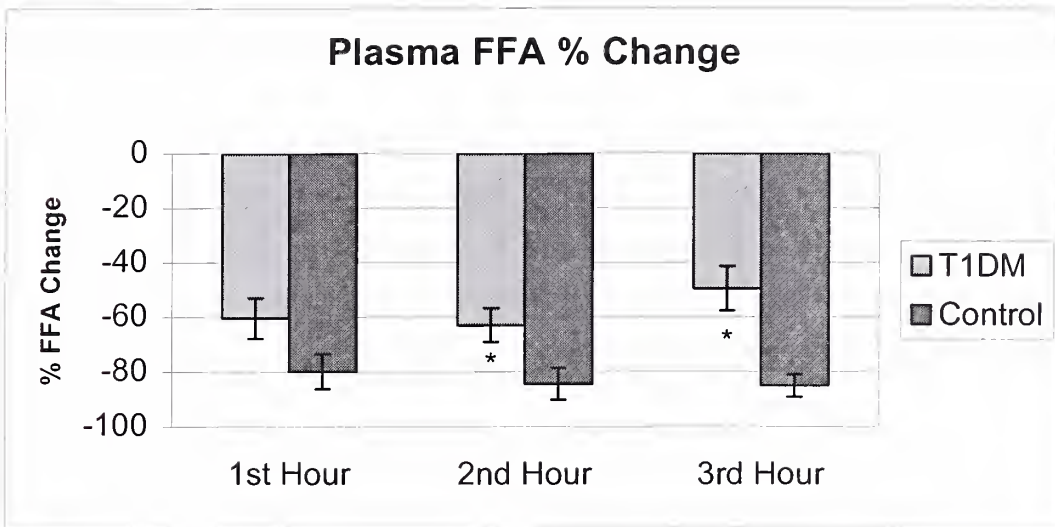


**Figure 2A.** Rates of oxidation of carbohydrate from calorimetry analysis before and during the glucose clamp procedure for T1DM and control groups. The single asterisk denotes  $p = 0.003$  for the control group during the clamp versus this group's basal value. The double asterisk denotes  $p < 0.05$  for the T1DM group when compared to the control group during the clamp procedure.

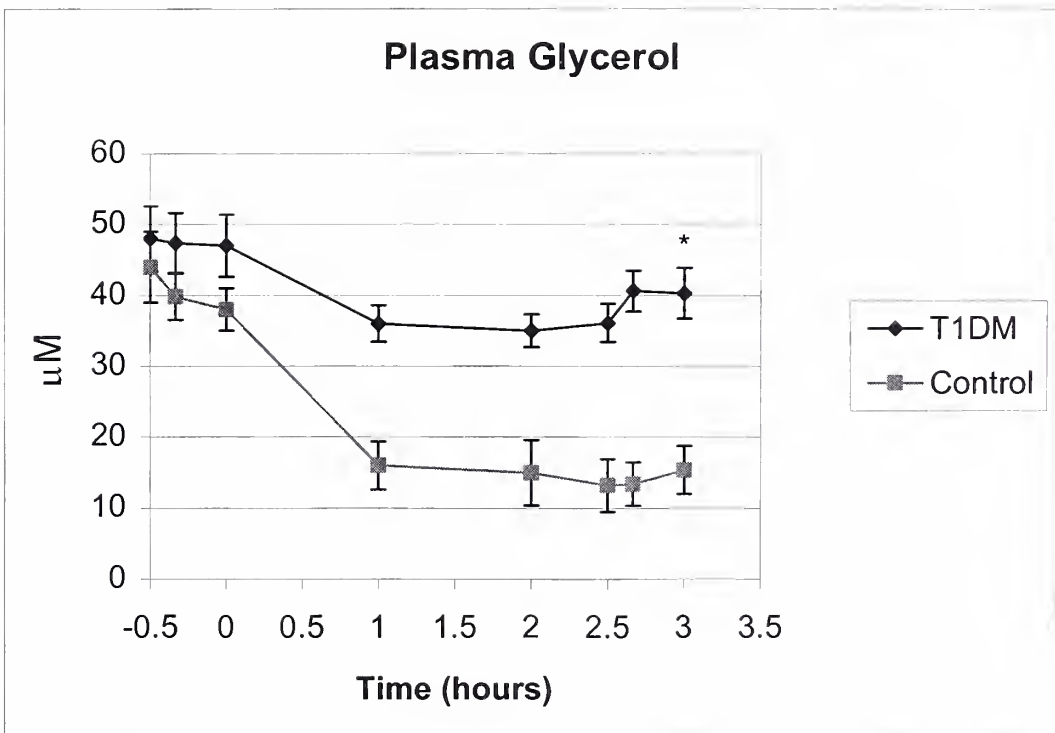


**Figure 2B.** Rates of oxidation of fat from calorimetry analysis before and during the glucose clamp procedure for T1DM and control groups. The single asterisk denotes  $p = 0.002$  for the control group during the clamp versus this group's basal value. The double asterisk denotes  $p < 0.05$  for the T1DM group when compared to the control group during the clamp procedure.





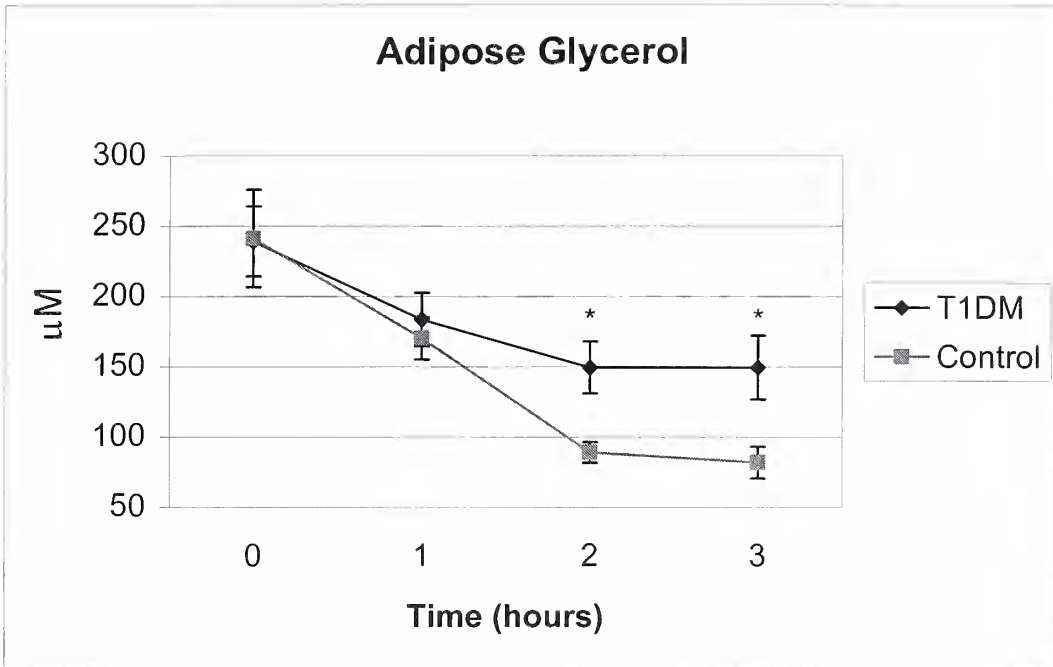
**Figure 3.** Percent change from baseline in plasma free fatty acids for the T1DM and control groups during the three-hour course of the glucose clamp procedure. The negative percents imply a net suppression of free fatty acid concentration in plasma. The single asterisk denotes  $p < 0.05$  for the T1DM group versus the control group.



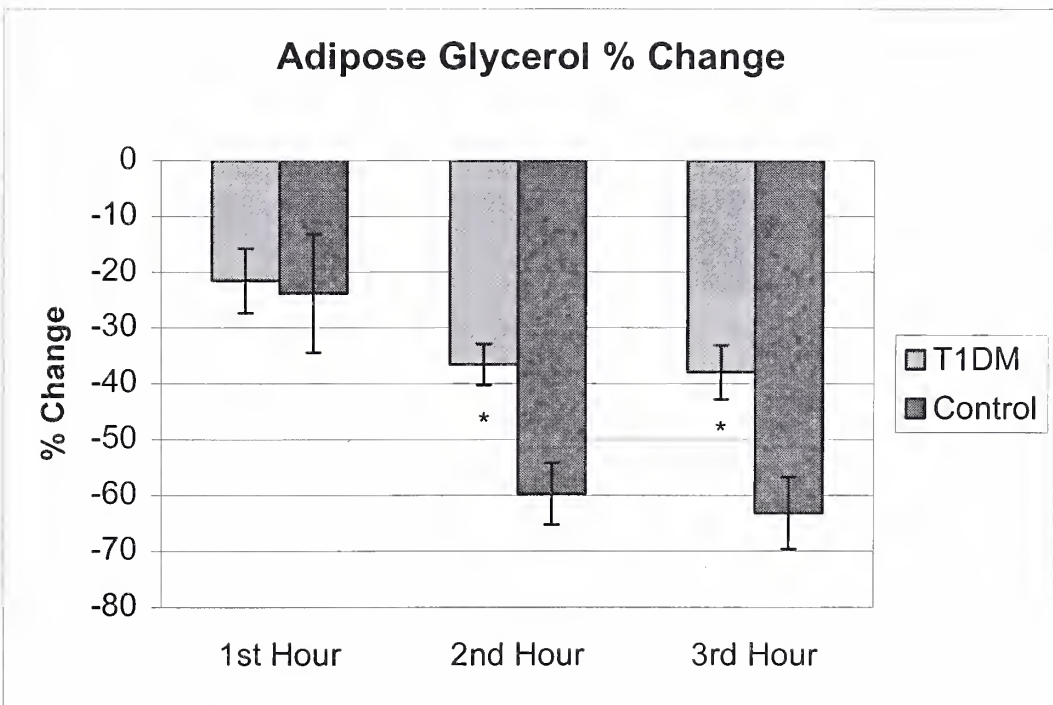
**Figure 4.** Concentration of glycerol in plasma during the course of the glucose clamp for T1DM and control groups. The single asterisk denotes  $p < 0.05$  for the T1DM group versus the control group.





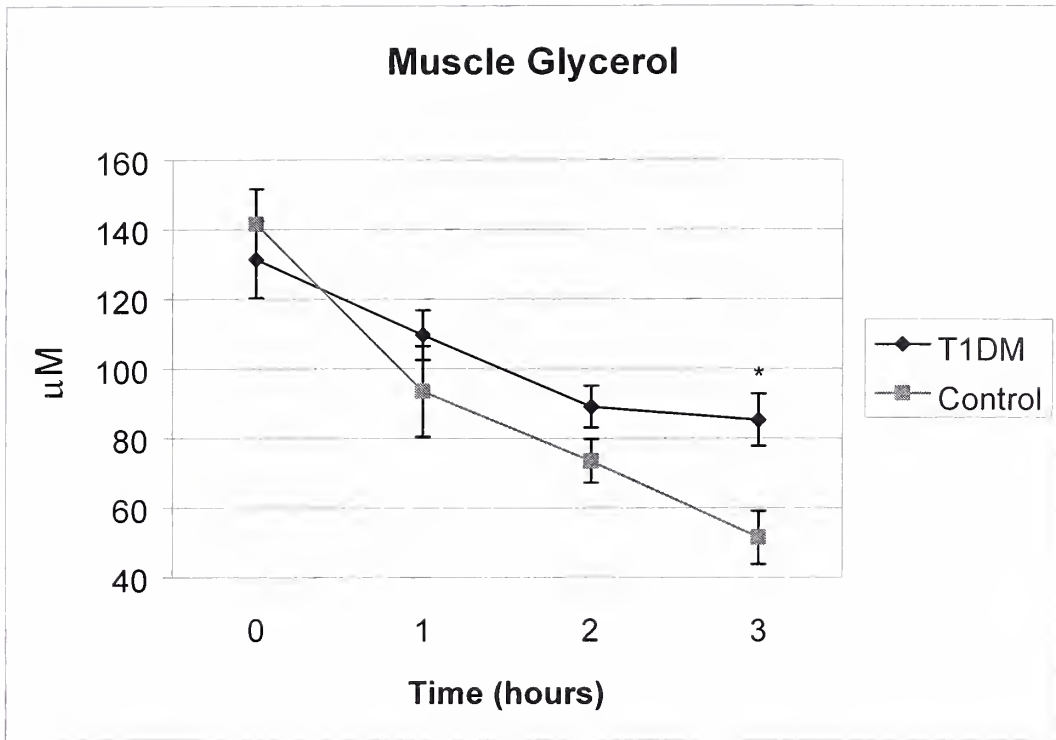


**Figure 5A.** Absolute concentration of glycerol in microdialysate from adipose tissue during the three-hour course of the glucose clamp procedure. The single asterisk denotes  $p < 0.05$  for the T1DM group versus the control group.

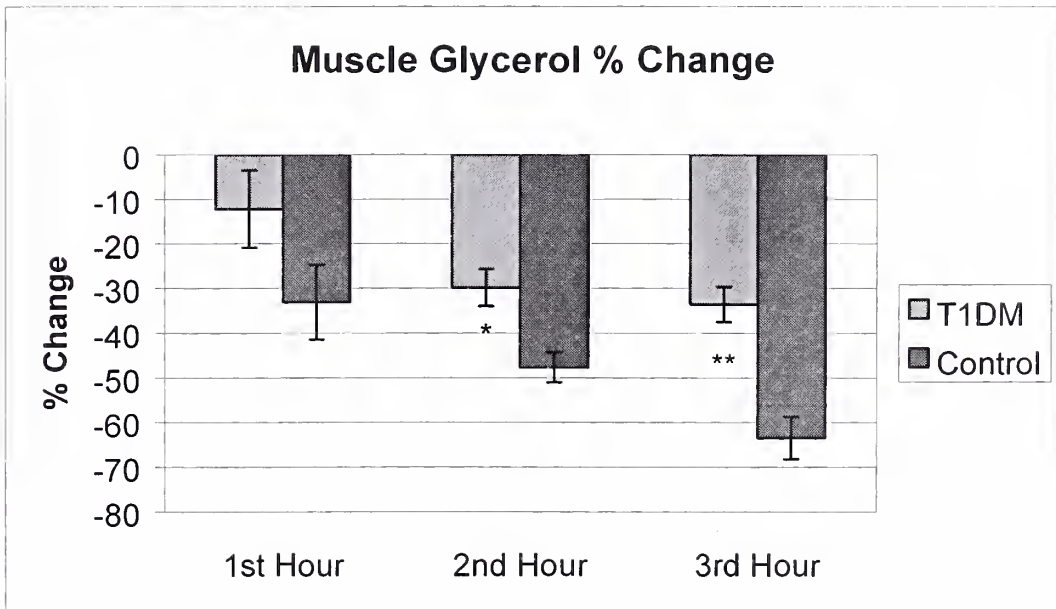


**Figure 5B.** Percent change from baseline in glycerol concentration from adipose microdialysate during the glucose clamp procedure. The negative percents imply a net suppression of glycerol concentration in adipose tissue extracellular fluid. The single asterisk denotes  $p < 0.01$  for the T1DM group versus the control group.



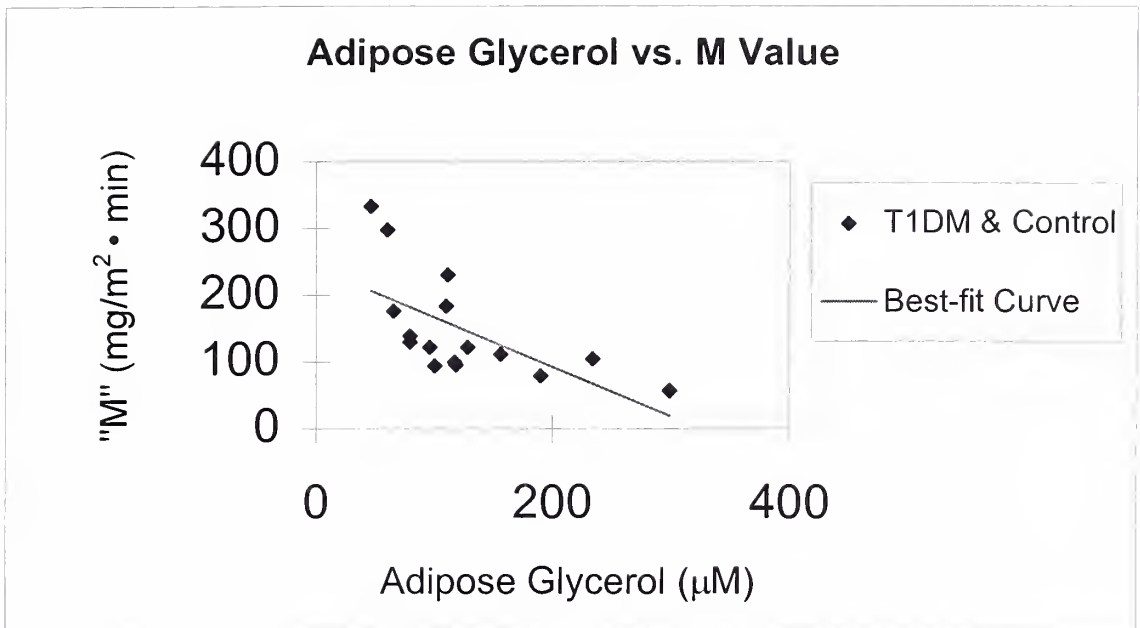


**Figure 6A.** Absolute concentration of glycerol in microdialysate from muscle tissue during the three-hour course of the glucose clamp procedure. The single asterisk denotes  $p = 0.01$  for the T1DM group versus the control group.

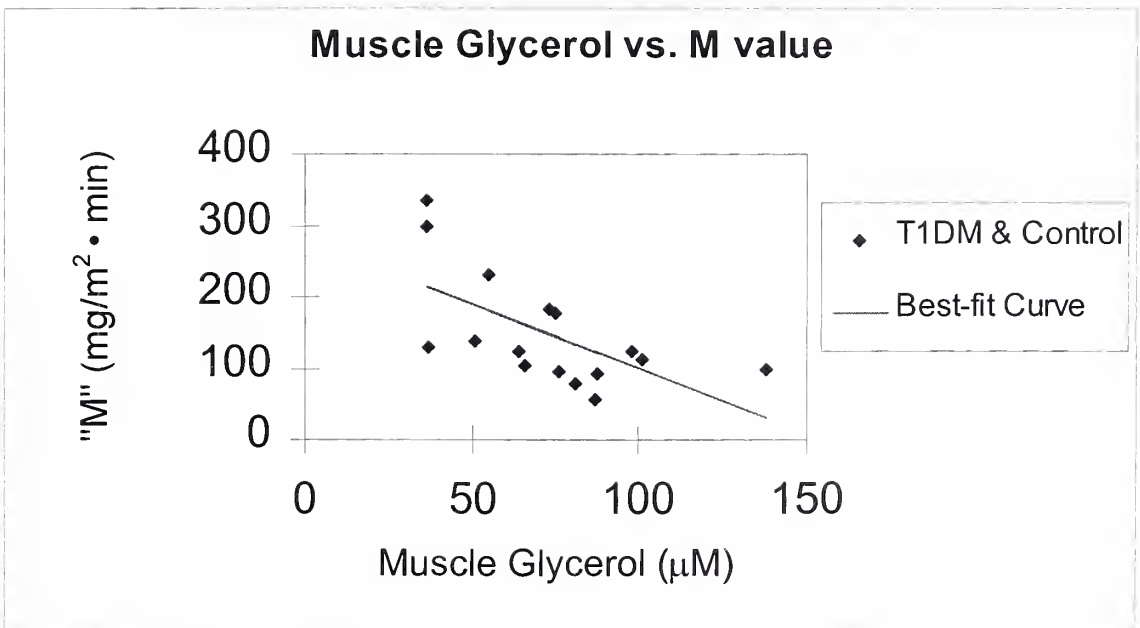


**Figure 6B.** Percent change from baseline in glycerol concentration from muscle microdialysate during the glucose clamp procedure. The negative percents imply a net suppression of glycerol concentration in muscle tissue extracellular fluid. The single asterisk denotes  $p = 0.01$  for the T1DM group versus the control group. The double asterisk denotes  $p < 0.001$  for the T1DM group versus the control group.





**Figure 7A.** Absolute concentration of glycerol in adipose microdialysate at the end of the three-hour glucose clamp procedure versus M value. The inverse correlation observed is defined by  $r = 0.63$ , and  $p < 0.01$ .



**Figure 7B.** Absolute concentration of glycerol in muscle microdialysate at the end of the three-hour glucose clamp procedure versus M value. The inverse correlation observed is defined by  $r = 0.63$ , and  $p < 0.01$ .



## DISCUSSION

In designing the present investigation, we set out to explore a potential mechanism by which a defect in insulin action might lead to the substantial insulin resistance encountered in adolescents with Type 1 diabetes. To accomplish this task, we used the technique of microdialysis, the glucose clamp procedure, and indirect calorimetry to evaluate insulin's action on lipolysis in adolescents with poorly controlled Type 1 diabetes, and compared the observed response to that of normal adolescents. By utilizing the microdialysis technique to monitor glycerol concentration in the extracellular fluid, we were permitted to target the study of lipolysis directly to tissues of interest, specifically adipose and muscle, with minimal invasiveness. In our study, we have noted a specific failure of insulin to suppress lipolysis in the T1DM group as compared to the normal controls which is correlated with their degree of insulin resistance.

In our adolescent subjects with Type 1 diabetes, we found a significant derangement of insulin-stimulated glucose metabolism when compared to the control group of adolescents. Insulin-stimulated glucose metabolism in total was reduced, indicated by the smaller glucose infusion necessary to maintain euglycemia during the clamp procedure (and reflected by a lower "M" value) in the T1DM group when compared to the control group. This reduction can be partly explained by decreased utilization of carbohydrate fuel in the T1DM group versus the control group during insulin infusion. Although basal rates of carbohydrate metabolism were similar between the two groups, the response to infusion of insulin was not. While the control group





significantly increased their rate of carbohydrate oxidation in response to insulin infusion, the T1DM group also increased, but not significantly. Overall, the T1DM group experienced significantly less carbohydrate oxidation during the insulin infusion than did the control group. In general, T1DM group was less able to react appropriately to the infusion of insulin by utilizing available carbohydrates, as reflected by lower “M” values and reduced rates of carbohydrate oxidation, when compared to the normal control adolescent group.

Profound difficulties with the management of fat metabolism in response to insulin infusion were observed in our adolescents with diabetes when contrasted to the control group. Although basal lipid oxidation rates were similar in both groups, the control group appropriately suppressed fat oxidation in response to insulin infusion, while the T1DM group suppressed much less. This translated into a significant difference in suppression of lipid oxidation between the two groups during the clamp procedure, with persistent utilization of lipid fuel by the T1DM group. These findings were echoed at the tissue level. Using the microdialysis technique in both muscle and adipose tissues, we discovered that adolescents with Type 1 diabetes failed to suppress the appearance of glycerol in the extracellular fluid compartments of both of these tissue sites. A significant difference between the experimental and control groups in glycerol concentration in the microdialysate samples from adipose tissue was observed after as little as two hours of insulin infusion. Since glycerol can appropriately be used as a marker of lipolysis (35-37), our findings imply a failure of insulin to inhibit lipolysis in adipose and muscle tissues in the T1DM group when compared to the control group.



This failure of suppression was also recognized at the whole body level, as the T1DM group was found to have higher concentrations of free fatty acids and glycerol in plasma during insulin infusion than did their normal counterparts. These results suggest that the adolescents with diabetes failed to react appropriately to the infusion of insulin by slowing lipid metabolism via reduced oxidation of fat and decreased lipolysis.

The effects on glucose and fat metabolism seen in the group of adolescents with Type 1 diabetes in this study illustrate a generalized insulin resistance. The reduction in insulin sensitivity we observed is not wholly unexpected, as adolescents typically have poorer metabolic control (10,11) and require higher insulin dosage (12) than their adult counterparts. This insulin insensitivity can be partly explained and substantiated by the results of two previous studies. The insulin resistance of Type 1 diabetes has been previously described by DeFronzo *et al.* (2), and is undoubtedly a factor contributing to the lack of insulin sensitivity that we have observed. In addition to this is the insulin resistance related to puberty, first described by Amiel *et al.* in 1986 (15). Our experimental group of subjects is at the intersection of these two insulin resistant states, and thus their reduced insulin sensitivity was anticipated.

The clarification of the mechanism of the insulin insensitivity seen in adolescents with Type 1 diabetes, and demonstrated in this study, was the ultimate goal of this investigation. In general, the mechanism by which insulin resistance occurs is controversial. Since Randle first proposed his glucose-fatty acid cycle in 1963 (17), free



fatty acids have been the focus for much of the research in this area. Many have tried to elucidate a mechanism that would link insulin resistant states to elevated circulating FFAs, and have succeeded to varying degrees (20-29, 41). Elevated FFAs have been noted in adolescents (14), accompanied by a failure to suppress lipolysis in response to insulin (16). These observations prompted investigation into the mechanism of the insulin resistance of puberty (29). Arslanian *et al.* found elevated rates of lipid oxidation and decreased insulin-stimulated glucose metabolism in pubertal subjects when compared with prepubertal and adult subjects. Based on these findings, they concluded that the insulin resistance of puberty is related to insulin's impaired suppression of lipid oxidation (29). More recently, Shulman has proposed a mechanism, based on his (28) and others' investigations, to account for the insulin resistance associated with increased FFAs (41). He theorizes that the accumulation of free fatty acid metabolites prevents the appropriate action of the insulin receptor, leading to failure to activate IRS-1-associated phosphatidylinositol 3-kinase and resulting in decreased activation of glucose transport (41, 28). The present study was undertaken to investigate whether the failure of insulin to suppress lipolysis is an important influence on the insulin resistance seen in adolescents with T1DM. Additionally, we sought to clarify the role of adipose and muscle tissues in this failure of inhibition, given recent research that has unmasked the importance of these sites in the regulation of fat metabolism and insulin resistance (30,31). In the current investigation, we have observed an inverse correlation between the absolute concentration of glycerol in adipose and muscle microdialysate during insulin infusion and insulin-stimulated glucose metabolism ("M" value). Although causation cannot be concluded from the correlation we have noted, our data does suggest



that the insulin resistance seen in adolescents with Type 1 diabetes may in part be due to the failure of insulin to inhibit lipolysis, and that this failure occurs at the tissue level. It is conceivable that the unchecked lipolysis demonstrated in this study induces insulin resistance through FFA-induced inhibition of IRS-1-associated PI 3-kinase, as suggested by Shulman and his collaborators (28, 41). Our study underscores the importance of examining the effects of insulin on metabolic fuels other than glucose, since, as we have demonstrated, there exists an extensive network of interrelations between the metabolism of many insulin-sensitive fuels.

In summary, we have observed that the insulin resistance that normally develops during puberty is exaggerated in the presence of poorly controlled Type 1 diabetes, and that a failure of insulin to suppress lipolysis may partly explain this phenomenon. In addition to documenting this deficiency at a total body level, by pioneering the use of the microdialysis technique in this population of patients, we were able to directly target our study of lipolysis to the muscle and adipose tissues believed to be crucial to the regulation of lipolysis. Although causation cannot be inferred from the correlation we have demonstrated, our data is consistent with the hypothesis that a failure of insulin to inhibit lipolysis in adolescents with T1DM leads to elevated FFAs, which compete with glucose for utilization, ultimately causing the marked insulin resistance seen in these patients.





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