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EFFECT OF TRIIODOTHYRONINE AND INSULIN ON GLUCOSE METABOLISM IN TISSUE EXPLANTS AND ISOLATED ADIPOCYTES FROM LEAN AND OBESE ZUCKER RATS

Iowa State University PH.D. 1985

Effect of triiodothyronine and insulin on glucose metabolism in tissue explants and isolated adipocytes from lean and obese Zucker rats

by

James Wilbur Bailey

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TABLE OF CONTENTS

INTRODUCTION

Thryoid hormones modulate energy metabolism and play a role in growth and development. Until recently it was believed that thyroid hormones had their major effect by binding to nuclear receptors. Plasma membrane receptors for thyroid hormones have been found which affect plasma membrane sugar transport (134) and a direct action of thyroid hormone on the mitochondria has been reported (144,145). This dissertation is designed to examine the role of thyroid hormone administration on glucose utilization by adipocytes.

A number of disease states are associated with altered thyroid hormone metabolism and action. Decreased plasma concentrations of thyroid hormones are found in a high percentage of diabetic (127) and obese patients (17,46). The genetically obese Zucker rat (fa/fa) is an excellent animal model in which to study the effects of thyroid hormone. The adult obese Zucker exhibits hyperphagia (14), fat cell hyperplasia and hypertrophy (50,86), hyperinsulinemia (14), hypertriglyceridemia (11), and a defective themogenic capacity (84,123). Some of the endocrine disorders concomitant with the hyperinsulinemia are decreased serum levels of thyroid hormones (17,46), growth hormone (97) and prolactin (97).

Thyroid hormones are known to affect adipose tissue metabolism (39,49,57). Fat cells grown in culture possess high-affinity binding sites for thyroid hormone (1,57). These cells have enhanced de novo fatty acid synthesis with chronic exposure to physiological levels of

thyroid hormone. A striking response of hepatic lipogenic enzymes to both thyroid hormones and the administration of simple carbohydrates is reported (96,111).

Correze et al. (29) and van Inwegen et al. (153) showed that the fat cells from thyroidectomized rats lose their lipolytic capacity and these same cells exhibit increased lipogenesis. Therefore, thyroid hormones might modulate a critical regulatory step or steps in lipogenesis and lipolysis. In contrast to this finding, other investigators (4,34,62) reported that thyroidectomy has no effect on the stimulation of CO_2 formation from glucose. The age of the animals, type of animal model and isolation procedure varied between investigators which may explain the different results.

Recent research shows a relationship between insulin and thyroid hormone. Experimentally induced hypothyroid animals become hyperinsulinemic, and hyperthyroid animals exhibit lower circulating insulin levels (92). Administration of insulin to dogs increases thyroid secretions. This effect is secondary to insulin-induced hypoglycemia (9).

It is evident from the above discussion that the action of thyroid hormone by itself and in concert with other hormones plays an important role in intermediary metabolism. The following literature review will examine these actions and interactions further.

Explanation of dissertation format

This dissertation is written in the alternate format. It contains an abstract, introduction, literature review, two papers to be submitted for publication, and a final discussion.

I performed all of the experimental work in the Food and Nutrition department at Iowa State University under the direction of Dr. Murray Kaplan. Elaine Herink and Gina Matkin-Baker assisted me with the glucose utilization and transport studies.

LITERATURE REVIEW

Thyroid hormone and growth and development

Thyroid hormones have profound effects on metabolism and growth and development. At the cellular level, a great number of thyroid hormone-dependent (and sometimes tissue specific) biochemical responses are observed in mammals (161). The underlying molecular basis for the changes is, as yet, poorly understood. Postnatal growth is clearly thyroid hormone dependent. The literature is replete with reports of marked retardation of growth in all mammalian species deprived of thyroid hormones. The developmental effects of thyroid hormones are perhaps most dramatically exemplified in amphibian metamorphosis. Many effects of thyroid hormones on mammalian development are believed to be mediated via growth factors, including somatomedins, erythropoetin, nerve growth factor and epidermal growth factor (51). Administration of growth hormone to neonatally thyroidectomized or hypophysectomized-thyroidectomized rats stimulates a significant gain in growth (131). Thyroid hormone administration causes only a minimal stimulation of growth in the absence of growth hormone. Animals given both hormones grow at a much greater rate than those given maximal doses of either one alone (151). Thyroid hormones also play an important role in the development of enzymes and metabolic pathways. Succinic dehydrogenase, a mitochondrial enzyme, normally begins to increase to adult levels at the tenth day of life. In neonates made hypothyroid at birth, reduced activity is observed between the tenth and fifteenth day (55).

Replacement therapy begun at the tenth day leads to normalization of enzyme activity. If treatment is delayed until day fifteen, the hormone has little effect (69). This critical period of hormone-dependent biochemical maturation extends to a wide variety of enzymes.

Reduced levels of circulating thyroid hormones results in the release of thyroid hormone stimulating hormone from the pituitary gland, which in turn accelerates the many reactions leading to increased hormone synthesis. The sequence of events resulting in the secretion of T_4 and T_3 into the circulation are: 1) concentration of iodide ion by the thyroid gland; 2) iodination of the tyrosine residues of thyroglobulin; 3) coupling of iodotyrosine residues; and 4) proteolytic digestion of thyroglobulin.

Thyroglobulin, with its iodinated tyrosine residues, is stored in the lumen of the thyroid follicles. Droplets of thyroglobulin are fused with cellular lysosomes where digestion with proteolytic enzymes occurs. Free mono- and di-iodotyrosines are almost completely deiodinated within the cell and most of the iodide ion is conserved and reconcentrated into the thyroid gland. Free T_4 and T_3 are resistant to intracellular deiodinase and are released unchanged into the circulation. The hormones are poorly soluble in water at the physiological pH of 7.4 but they are solubilized and transported in the blood by their structurally-specific high-affinity associations with proteins.

Thyroid hormone mode of action

Thyroid hormones are generally considered as gene activating hormones. Tata and Widnell (150) suggested in the early 1960s that nuclear RNA synthesis was stimulated as a primary response to thyroid hormone. Oppenheimer and co-workers (110) provided the first evidence for the existence of nuclear receptors for thyroid hormones, however, none were found in other cellular components. Similarly, Samuels and Tsai (125) confirmed the presence of high affinity, limited capacity T_3 and T_4 binding sites on the nuclei of GH_1 cells. Buergi and Abbuehl (18) in 1984 were the first investigators to describe T_3 nuclear binding in isolated nuclei from rat epididymal fat pad adipocytes. Anselmet et al. (1) have recently shown that cells from the preadipocyte cell lines, ob 17 and HGFu, possess a small number of high affinity T_3 receptor sites. A strong indication that the binding sites were receptors was provided by the excellent correlation of the binding affinities of numerous thyroid hormone analogs to the biological activities of the compounds. Recent data support the binding and physiological importance of thyroid hormones in other cellular fractions. The failure of these investigators to demonstrate binding sites other than the nucleus by in vivo techniques could be due to: 1) a large amount of the total hormone being bound nonspecifically by the various cellular fractions when administered in vivo, making it difficult to detect specific binding; 2) a redistribution of the label during cell fractionation; or 3) the generation of thyroid hormone binding sites during the preparation of the cellular fractions to be used for the in vitro binding studies.

The cells of a preadipose clonal line (ob 17) isolated from epididymal fat pad of ob/ob mice were found to possess high-affinity binding sites for T_3 (1,57). The investigators found enhanced de novo fatty acid synthesis with chronic exposure to physiological levels of T_3. This was supported by increased activity of fatty acid synthetase. The expression of the lipogenic enzymes was greatly enhanced by insulin and a two fold increase was seen with physiological concentrations of triiodothyronine. The increase in the lipogenic enzyme activity seen with triiodothyronine is due to pretranslational (mRNA transcription or processing) effect of T_3 (58). Sterling et al. (144,145) reported that intravenous injection of T_3 in physiological doses in the rat results in the direct stimulation of mitochondrial oxidative phosphorylation.

Recent data support the physiological relevance of thyroid hormone on extranuclear events. Muller and Seitz (106) have hypothesized that the thyrometabolic state of a single cell is the result of the simultaneous action of T_3 at the nucleus, mitochondria and plasma membrane. They believe the interaction on T_3 with the plasma membrane and inner mitochondrial membrane represent the target of the primary or initiating action of the hormone, whereas the nucleus is responsible for the late sustained effects of T_3. In the rat thymocyte plasma membrane T_3 binding sites with Kd values of 0.95 and 25 nM have been detected (134). Other investigators found saturable binding sites for T_3 in the plasma membranes of hepatocytes and fibroblasts (56,99,114). Two hypotheses have been presented for the physiological function of these

membrane binding sites (82). The first is that the binding of thyroid hormones to the membrane binding sites influences the cellular levels of amino acids and/or sugars. The second hypothesis is that the sites play a role in the cellular uptake of thyroid hormones either by serving as carriers of the thyroid hormones from the outside to the inside of the cell or as transporters of thyroid hormones across the cell membrane.

The prevailing view regarding the uptake of triiodothyronine and thyroxine by tissues is that cellular uptake is largely dependent upon the concentration of free hormones in the blood and extracellular fluids (81,120). Although it is assumed that thyroid hormones enter the cell by passive diffusion through the plasma membrane because of their lipophilic characteristics, evidence exists that the uptake may be at least partly carrier mediated (112). Cheng et al. (24) found that in fibroblasts, the appearance of intracellular tetramethylrhodamine-labeled T_3 (rho-T_3) was preceded by its binding to cell surface binding proteins and their clustering in coated pits. Inside the cell, the T_3 was concentrated in vesicles that were morphologically different from lysosomes and mitochondria. Within 1 hour, these vesicles were found clustered near the nucleus. The clustering of the binding protein on the cell surface was inhibited by methylamine, dansylcadaverine or bacitracin compounds known to inhibit the clustering of membrane proteins. In GH_3 cells, it also appears that clustering of T_3-membrane binding protein complexes is important for the cellular uptake of T_3 because the uptake was blocked by monodansylcadaverine (77). Similar conclusions were drawn in the study of Halpern and Hinkle (68).

Starvation and dietary manipulations change the thyroid status. During total starvation the serum thyroxine concentration remains unchanged while the serum triiodothyronine level drops about 50 percent; at the same time the level of the biologically inactive reverse T_3 (rT_3) increases about 50 percent, resulting in a low T_3-high rT_3 state (102,115,157). The low T_3-high rT_3 state is found to be associated with a number of diseases, including, diabetes (127) and obesity (17,46), and is classified as the 'low T_3 syndrome'. The change in thyroid hormone status is due to a lowering of the peripheral conversion of T_4 to T_3 and of the slow metabolic degradation of rT_3 while the conversion of T_4 to rT_3 is enhanced (47,149). Another mechanism for the decreased effect of thyroid hormones could be a decreased number of nuclear T_3 receptor sites (5,129). The serum concentrations of the specific thyroid hormone binding proteins thyroxine-binding globulin (TBG) and thyroxine-binding prealbumin (TBPA) are lower during energy restriction (21,104,147). These adaptive mechanisms may act to limit protein catabolism.

Overfeeding increases the production rate of T_3 without altering production or clearance of T_4, resulting in increased serum T_3 concentration and unchanged T_4 concentration. The increase in serum T_3 levels during overfeeding appears to be the result of greater peripheral conversion of T_4 to T_3 rather than increased thyroidal secretion of thyroid hormone, since T_4 production is unchanged by overfeeding (35,159). A study done by Krotkiewski et al. (88) showed that changes in T_3 and rT_3 after exercise are in the same general direction as after starvation or carbohydrate restriction.

Hypothyroidism is clearly associated with some forms of obesity in man and obese animal models, and it in fact may precede the onset of hyperinsulinemia, insulin resistance, hyperphagia and obesity in some subgroups (162). It is reported that in obese female rats, the thyroid uptake of radioiodine is significantly lower than in lean ones. There is a direct correlation between the degree of obesity and decreased T_3 concentration in the serum (15,17,46). Sheppard and Ramsden (137) monitored the thyroid hormone concentration in moderately obese patients during and after dietary controls and found little improvement. It appears that obesity is frequently accompanied by a decrease of thyroid metabolism similar to hypothyroidism. Other investigators however, find no correlation between thyroid hormone levels and obesity. Glennon and Brech (59) measured protein binding iodine (PBI) in obese and lean patients and could find no differenes. Serum concentrations of free T_3 and total T_3 were measured in 25 grossly obese patients matched in sex and age to non-obese volunteers and no differenes were found (148).

Recent evidence empasizes a strong relationship among carbohydrate, lipid and thyroid hormone metabolism. There is a striking response of hepatic lipogenic enzymes to both thyroid hormones and administration of simple sugars (96,111). The peripheral conversion of thyroxine to triiodothyronine appears to be determined in part by the availability of carbohydrate (19,36). Several investigators document that hypothyroid patients have relatively low fasting blood glucose concentrations (44,100) and low basal insulin concentration (103). Elevated levels of

fasting blood glucose in the hyperthyroid state are associated with elevated basal plasma insulin concentrations (158). A recent study by Dimitriadis et al. (43) showed that injections of humans with levels of T_3 to mimic short-term thyrotoxicosis resulted in increased postabsorptive plasma glucose, insulin and C-peptide concentrations. Despite the hyperinsulinemia, glucose tolerance was not improved. This implies that elevated short-term levels of T_3 induce insulin resistance.

Thyroid hormones are known to affect lipid metabolism in the liver. T_3 promotes an increase of both biosynthesis (41,89,95,96) and oxidation (41) of fatty acids. The effect of thyroid hormones on adipose tissue was first studied by Rich et al. (119). They found that hyperthyroidism increased plasma free fatty acids. Subsequently, other workers (2,20,29,38,62,64,80,122) demonstrated that the epinephrine-stimulated lipolysis was influenced by the thyroid state of the animal: enhanced in hyperthyroidism, markedly reduced or abolished in hypothyroidism. Correze et al. (32) and Omri et al. (109) report that thyroid hormones exert two types of effects on the lipogenic pathway of the fat cells: one is independent of cyclic AMP and the other, through a 'permissive effect' on lipolytic hormones, is under nucleotide control. It is well established that some lipolytic and lipogenic enzymes can be regulated through a mechanism of phosphorylation-dephosphorylation by a cyclic AMP-dependent kinase. This was shown for hormone-sensitive lipase (54) and acetyl CoA carboxylase (71,118). Omri et al. (109) very recently showed that thyroid hormones control the ^{32}P labeling of proteins of the

cytosol and plasma membrane fractions of rat fat cells and, at least in some cases, the lipolytic and lipogenic pathways.

Hypercholesterolemia or hypocholesterolemia have been reported to be associated with hypothyroidism or hyperthyroidism, respectively (28). Considerable variablility has been reported in plasma concentrations of triglycerides, which may be increased, decreased or normal in hyperthyroidism (107,152). In hypothyroidism, the plasma triglyceride level is usually increased but may be normal (90,107). Alterations in thyroid status can change lipoprotein concentration and/or distribution (160). These alterations may be a consequence of or the reason for the altered lipid metabolism.

Most reports agree that insulin plays a decisive role as the stimulus for glucose utilization and conversion to lipid in adipose tissue. Adipocytes obtained from euthyroid animals display marked increases in glucose uptake and lipid synthesis when insulin is added to the incubation medium (34). It is interesting to note the conflicting results on the effects of insulin on glucose metabolism in hypothyroid animals. Some investigators (29,153,155) show that adipocytes obtained from hypothyroid animals have increased rates of glucose uptake and lipid formation from glucose. These cells also were reported to be insulin sensitive. In contrast, other investigators (34,128,136) show that hypothyroid adipocytes do not respond to insulin by increasing lipogenesis. These studies show that in the presence of insulin, the euthyroid adipocyte has a greater rate of glucose uptake, CO_2 production

and lipid synthesis compared to the hypothyroid adipocyte in vitro. The reasons for these discrepancies are unclear, however, the age and sex of the animals, type of animal model, isolation procedure and mode of presentation of the data varied among investigators, which may help explain the different results.

Studies with hypophysectomized rats (128) have suggested a possible mechanism for the insulin insensitivity seen by some investigators in hypothyroid adipose tissue. Thyroid hormone administration to hypophysectomized rats increased both the basal uptake and metabolism of glucose and the rate of lipid formation in isolated adipocytes. However, thyroid hormone was incapable of restoring the insulin induced stimulation of glucose uptake and metabolism. Growth hormone administration did restore the responsiveness of adipocytes to insulin. Since thyroid hormone deprivation is known to lead to growth hormone deficiency in rats (73,103), it is possible that the lack of adipose sensitivity to insulin in thyroidectomized rats is due to the lack of growth hormone secretion.

The thyroid hormone-insulin interaction has largely been studied in thyroidectomized rats, or in rats injected with thyroid hormones, and the results are contradictory. The insulin receptor binding, insulin insensitivity and insulin responsiveness are found to be unchanged, increased or decreased by thyroid hormones (30,31,34,40,72,74). Triiodothyronine inhibits insulin secretion (91,98). Studies performed by Cech and Amatruda (23) indicate decreased sensitivity of T_3 treated

cells to insulin. They found a decreased number of insulin receptors with the same binding characteristics as those of the control cells. In contrast, Arner et al. (3) observed a large increase in insulin binding in isolated human adipocytes from hypothyroid patients and a marked decrease in the hyperthyroid groups. They found these results to be due to alterations in insulin receptor number. In hypothyroidism, the action of insulin was almost completely inhibited despite increased insulin receptor binding. This suggests a postreceptor inhibition of insulin action on glucose utilization in adipose tissue by hypothyroidism. Insulin stimulated glucose oxidation was significantly increased in hyperthyroidism and decreased in hypothyroidism. A synergy between insulin and T_3 in the developmemt of some liver lipogenic enzymes is reported (63). Mariash and Oppenheimer (94) recently suggested that insulin could act by accelerating the intracellular metabolism of glucose and thus the production of a factor which could interact with the nuclear T_3 signal.

Glucose transport and utilization

The interest in adipose tissue was aroused when it became clear in the 1940s and 1950s that it was not only a storage site but actually possessed a very high rate of metabolic turnover. For example, D-glucose is rapidly metabolized in adipose tissue, such as rat epididymal fat pads, and this process is markedly enhanced by insulin. Glucose metabolism is vital for the adipocyte. Fatty acids could not be esterified and triglycerides could not be stored in the absence of

production of ∝-glycerolphosphate. The overall rate of glucose concentration in an isolated cell such as the rat adipocyte depends on the sequential function of both glucose transport and intracellular glucose metabolism. An understanding of these two processes is important not only because their interaction determines the overall metabolic rate, but also because each may have varying relative responses to hormonal treatment or may be affected differently by abnormal conditions, including obesity.

Crofford and Renold (33) showed that glucose is mainly transferred across the cell membrane of adipose tissue by carrier-mediated (facilitated) diffusion. At low glucose concentrations, the rate of transmembrane glucose transport limits the rate of glucose metabolism and the transport step is therefore a major point of regulation. The plasma membranes of adipocytes are equipped with special structures referred to as carriers which greatly facilitate the transfer of hexoses from the extracellular fluid to the cytosol. Several hormones, most notably insulin, influence the permeability of the plasma membrane. The properties of the transport system also are modulated when cells metabolize glucose at a high rate. Insulin causes a manyfold increase in Vmax without influencing Km significantly (108,156). Insulin can increase maximal transport capacity by either increasing the number of activated transport units or the mobility of already functioning transport units. Recent work (79,138) strongly suggests that the insulin-induced increase in Vmax is brought about by a translocation of

transporters from a site in which they are nonoperative (intracellular pool) to the plasma membrane. In several insulin-resistant states, including the streptozotocin-induced diabetic rat (87), the high fat-fed rat (76), the obese male rat (75) and guinea pig adipose tissue (79), decreased glucose transport in response to insulin correlated with a decreased pool of intracellular glucose transporters, suggesting that glucose transport in these animals is limited by the quantity of glucose transporters available for translocation to the plasma membrane. Hissin et al. (75) found that regardless of the basal or insulin-stimulated state, a roughly 12 fold increase in cell size was associated with less than a threefold increase in the total number of glucose transport systems per cell. Their results suggest that large adipocytes appear to be locked in an insulin-stimulated-like state which does not reverse in the absence of insulin, perhaps as a consequence of the chronic hyperinsulinemia associated with the aging rat model of obesity. The alterations in glucose transport and metabolism and their regulation by insulin that accompany cellular enlargement very well may reflect the consequence of normal growth processes that ultimately prevent the cell from expanding its triglyceride stores beyond some maximal volume (50).

The radiolabeled glucose analog, 2-deoxy-D-glucose (2-DG), is taken up by cells with the D-glucose transport system and provides a good index of the rate of glucose transport (108). 2-DG is phosphorylated by hexokinase after it is transported into cells. The incorporation of 2-DG into cells is the result of both transport and phosphorylation, and

the measurement of the incorporation would be a valid measure of 2-DG transported only under conditions where all the 2-DG transported is phosphorylated as rapidly as it enters the cell. Such conditions have been established for 2-DG (108).

3-O-Methyl-D-glucose (3-OMG) is another glucose analog widely used to study glucose transport. However, unlike 2-DG, this compound cannot be "trapped" by phosphorylation, and efflux is significant at very early time points. The time course of uptake of this analog is rapidly curvilinear, due to rapid filling of the relatively small intracellular water space of adipocytes. There is a report (121) that 3-OMG has a slightly reduced specificity for the D-glucose transport system. Another potential problem associated with the use of 3-OMG is that under certain conditions decomposition of ^{3}H 3-O-methyl-D-glucose can form radioactive impurities which can seriously interfere with proper measurements of transport rates.

Goldrick (61) found that an increase in the glucose concentration in the incubation medium enhanced triglyceride synthesis from glucose in short incubations. Horn and Goodner (78) found a significant difference in insulin sensitivity among different fat depots in the rat. Omental fat was relatively insensitive to insulin stimulation whereas epididymal and subcutaneous fat were insulin sensitive. It is becoming increasingly apparent that adipose tissue is heterogeneous with respect to hormone binding and sensitivity, morphology, and physiologic regulation of storage and mobilization of triglyceride. Some

investigators have reported sex differences in glucose transport in rat and human adipocytes (52,67). These reports indicate that adipocytes from females are more sensitive and responsive to insulin.

In the rat thymocyte, T_3 enhances the uptake of the glucose analog 2-DG (133). The relative effectiveness of thyroid hormone analogs in promoting glucose uptake was very similar to their relative binding to the plasma membrane. This effect was first seen at 1 nM T_3 and increased linearly up to 10 μM. The lower limit of this range is bordering on physiological levels. A more physiologically significant effect of T_3 is on 2-DG uptake in cultured chick embryo heart cells (135). The T_3 concentration that produced half-maximal uptake of 2-DG, 0.87 nM, is well within physiological levels. During the first six hours of incubation of the chick embryo heart cells with T_3, the hormone's stimulatory effect was not inhibited by actinomycin D, puromycin or cyclohexamide (132). Therefore, RNA and protein synthesis are not required for the T_3 response. T_3 does not have to enter the cell to produce this effect, since uptake was also promoted by T_3 coupled to red blood cells (42).

Tissue explants

There are limited methodological possibilities to investigate morphologic changes of adipose tissue exposed to variations in the biochemical environment for more than a few hours. The application of tissue culture offers such a possibility. Using terminology as proposed by the Tissue Culture Association (126), tissue culture is the maintenance or growth of tissues in vitro, to allow preservation or differentiation of the appropriate cellular architecture and/or function. Explant culture is the in vitro growth or maintenance of undissociated tissue or organs taken directly from the animal. Cell culture is the growth or maintenance of cells no longer organized into tissues. A primary culture is a culture initiated with tisses or cells from an intact animal. A primary cell culture is usually initiated by mechanically, chemically or enzymatically dispersing a tissue into individual cells. The cells, or selected subpopulations, are placed in a culture flask containing nutritive media and allowed to attach and grow. Explants are simply suspended or are held in place in the flask by a coverslip or something similar.

Explants of human adipose tissue have been maintained in vitro for 30 weeks with minor morphological changes from that of freshly excised tissue (140). These tissue explants were found to be sensitive to insulin and the incorporation of glucose into lipids as well as the release of glycerol was normal for at least one week (40). Using this technique, these same investigators showed that the long term effect of

a hormone may differ from that found in short term incubations. The effect of long term exposure to insulin enhanced the lipolytic rate while acute effects are antilipolytic (142). Long term exposure of explants to insulin stimulated the basal rate of glucose incorporation in the subsequent short term incubations used to study glucose utilization (143). When insulin was added in the short term incubations these explants did not further respond to the hormone, while explants cultured without insulin responded to additional insulin. Thus, it appears that prolonged exposure to insulin leads to a diminished acute effect of the hormone on glucose metabolism. Prolonged exposure to insulin influences the cellular responsiveness to the hormone. This finding is relevant in that large fat cells from subjects with obesity and hyperinsulinemia exhibit a diminished responsiveness to insulin in vitro (124) as well as in vivo (116).

Bernstein (8) found glucose utilization was present but diminished in rat epididymal tissue explants. He also found that insulin responsiveness in some metabolic pathways was absent. The discrepancies between Smith and his collaborators and Bernstein may be that human adipose tissue begins with lower glucose utilization relative to the rat (66). For example, the rate of 3-O-methyl-L-glucose transport into human adipocytes is much slower than in rat adipocytes (25).

Coiro et al. (27) utilized adipose tissue explants and isolated cells to measure the effect of hypophysectomy on glucose transport and glucose utilization. Their data indicate that isolated cells metabolize

glucose more rapidly than cells in tissue explants, regardless of prior treatment of the rats. Other investigators have made similar observations (60,66). An obvious explanation for these results is that availability of substrate to the cells embedded in tissue may be limited by long tortuous pathways for diffusion from the incubation medium to the cell surface. The fact that the discrepancy between the rates of glucose oxidation in tissue and isolated cells decreased as the concentration of glucose in the medium increased is consistent with this concept. That is, as the diffusion gradient between the incubation medium and the fluid in the immediate pericellular space becomes greater, the delivery of glucose to the cells becomes less limiting, and glucose oxidation more nearly approximates the rates achieved by isolated cells. Another possible explanation for the differences seen between isolated cells and tissue explants is that adipose tissue explants contain a variety of cell types, including endothelial cells, fibroblasts, mast cells and other connective tissue elements. In contrast, a rather pure population of fat cells can be separated from other cells via centrifugation in the isolated cell suspensions. The adipocyte cell population may be varied in the cell isolation procedure because this procedure selects for the cells with larger fat droplets, and hence lower density, because these cells are more likely to be driven into the fat cake that accumulates at the top of the centrifuge tube during the various washings.

Zucker rat

To date there are approximately 50 different types of animal model obesities. The models have a number of different etiologies: neural, endocrine, pharmacological, nutritional, seasonal, genetic, viral and idiopathic. The many differences among the various animal obesities indicate that no single type can serve as a general model of human obesity. Although obese animals cannot serve as exact models of the human condition, they have provided important insights into the causes, consequences and cures of human adiposity. The largest class of models includes the genetic obesities. Genetically obese animals have been divided according to mode of inheritance into the single gene dominant strains, single gene recessive strains and the polygenic inbred or hybrid strains (130). Of these, the single gene recessive models, in particular, the obese mouse (ob/ob), diabetic mouse (db/db) and fatty or Zucker rat (fa/fa) have been the most extensively studied. In these cases, the obesity arises as a result of classical Mendelian genetics. The single gene mutants have several advantages, although it can be argued that from an inheritance point of view, the polygenic strains are more realistic models of human obesity. In such cases, obesity arises as a result of having an extreme array of genes that determines factors such as growth rate, appetite, metabolic rate, physical activity and social reactivity. There is probably no single genetic lesion. The mode of inheritance is difficult to disentangle and the environmental influences are generally strong. So far it has not been possible to detect the primary lesion in any of the mutants.

The genetically obese (fa/fa) Zucker rat is an excellent rodent model for early onset hypercellular-hypertrophic human obesity. These animals appeared as a spontaneous mutant in the laboratories of Zucker and Zucker (14) from a Sherman and Merek Stock M cross. The phenotypic expression of this trait is not visually manifest at birth. The obese rats cannot be recognized for the first 2 to 3 weeks of life. However, it has been shown by Kaplan (84) that gross energy metabolism is already impaired by this time. Planche et al. (113) showed that 7 day old Zucker rat pups showed a reduction in oxygen consumption, respiratory CO_2 production, in vivo oxidation of palmitate, and core temperature. The adult obese Zucker rat has the following characteristics; hyperphagia, fat cell hyperplasia, hyperinsulinemia, hypertriglyceridemia, glucose intolerence, insulin resistance, decreased oxygen consumption, defective thermogenic capacity and possible peripheral hypothyroidism. Obese Zucker rats that have been pair-fed throughout life with lean rats fed ad libitum have heavier fat depots than lean rats and a greater percentange body fatness than obese rats fed ad libitum (26). Therefore, in this model, overeating itself is not necessary for the development or maintenance of obesity. Recent studies show that increased physical activity alone is not sufficient to produce a large loss of body weight or body fatness in the genetically obese rat (6,48). However, a slight improvement in glucose tolerance and insulin sensitivity was achieved.

In a study performed by Stolz and Martin (146), obese female Zucker rats were made streptozotocin diabetic and supplemented with insulin to equal that of normal lean Zucker rats. They found that the obese continued to partition more energy toward carcass lipid deposition. This occurred in spite of similar total body weight gain and food intake per body weight to that of the lean animals. The hyperinsulinemia seen in obesity is not essential for the lipid deposition observed in the obese Zucker rat. Smith and Kaplan (139) reported increased hepatic lipogenesis at 4 weeks of age in obese rats. Peak rates of lipogenesis occur at 6 weeks in both lean and obese rats. The fatty acid formation from radiolabeled glucose by obese adipocytes is manyfold higher than the lean adipocytes at this time (139). Pair feeding the obese rat to its lean littermate does not normalize hepatic lipogenesis in the obese rat (7,97), but does normalize adipose lipogenesis (7).

The obese rat does not convert dietary protein into lean body mass as efficiently as the lean rat (164). Radcliffe and Webster (117) propose that the increased intake of calories seen in the obese rat is a response to a protein-metabolism deficit. However, when Castonguay et al. (22) allowed obese rats to compose their own diets from three macronutrient sources, they selected a diet higher in fat but lower in protein than their lean littermates. If hyperphagia is a response to increased need for protein, the obese animal should eat more of the protein source. Therefore, an altered protein drive probably does not contribute to the hyperphagia that is observed when obese rats are fed a complete diet.

The obese Zucker rat has a lower maintenance requirement than its lean littermates (105). During fasting, obese rats have been found to have the same rate of energy expenditure per unit of metabolic body weight (kilograms$^{0.75}$) as lean rats (45). Dunn et al. (45) state that since the fasting metabolic rates are similar in both genotypes, the reduced basal metabolic rate is not the primary cause of obesity. Additional evidence which supports this conclusion is work by Kaplan (85), who showed obese and lean rats have identical oxygen consumption rates expressed on a body protein basis.

Elevated lipoprotein lipase (LPL) enzyme activity and fat cell hypertrophy is clearly present by the second postnatal week (10). Cleary et al. (26) interpreted this to mean that the early increased LPL activity directs or preferentially pulls the increased uptake of dietary derived triglycerides into adipocytes of obese rats, secondarily depriving lean body tissues. According to this hypothesis, a defect in nonshivering thermogenesis would result from substrate deprivation in brown adipose tissue because this tissue is thought to be the dominant tissue site for generation of extra heat (53). The triglyceride content of intrascapular brown adipose tissue is higher in the 7 day old obese pups than in their lean littermates, whereas lipoprotein lipase activity in this tissue is not increased in the obese pups (11). Our data (unpublished) show no decrease in brown adipose tissue oxygen consumption of obese rats. These observations suggest decreased substrate utilization rather than substrate deprivation. The elevated

LPL activity in white adipose tissue develops even when hyperphagia is prevented (26). The obese rat has hypertriglyceridemia by 2 weeks of age (11). Therefore, the obese rat has sufficient lipid prior to hyperphagia for muscle and other tissues to utilize. The LPL activity is similar in the muscle of lean and obese Zucker rats (101). These results suggest that the elevated LPL activity in adipose tissue does not appear to be the primary lesion in the obese animal.

In addition to the hypotheses discussed so far, there are many others that have been presented to explain the biochemical abnormalities seen in obese animal models (13). Some of these hypotheses include: 1) impaired oxidation of acetate, 2) abnormal response to growth hormone, 3) increased concentrations of glycerol kinase in adipose tissue, 4) increased cellularity of adipose tissue, 5) triglyceride storage diseases due to a decrease in lipolysis or to enhanced lipogenesis or esterification, 6) increased insulin secretion, 7) decreased insulin receptors, 8) reduced sodium pump activity, 9) reduced levels of brain catecholamines, 10) decreased concentration of cholecystokinin in the brain, 11) decreased response to thyroid hormone, 12) increased concentrations of endorphin in the pituitary and circulation and 13) decreased thermogenesis.

The obese rat has more detectable body fat than the lean rat at 2 weeks of age (11). Adipose tissue hypoplasia has been observed in young obese Zucker rats (83). Concomitant with adipocyte hypoplasia is hypothyroidism (12). The concentration of thyrotropin, uptake of

radioactive iodine and circulating concentrations of triiodothyronine are diminished, indicating that the fatty rat is hypothyroid. Levacher et al. (93) studied the effect of thyroxine treatment on adipose tissue cellularity in this strain. T_4 treatment to obese weanlings increased their adipocyte number up to the level of lean untreated rats (93). There was a slight reduction in the size of the adipocytes. They found that the lack of thyroid hormone does not alter adipocyte number permanently. The body growth of obese Zucker rats was reduced by T_4 treatment (93). However, the genetically obese rat remains obese. Genetically obese Zucker rats remain obese even with food restriction (16,26), adrenalectomy (163), thyroid hormone replacement (93), exercise (37), serum insulin levels normalized (146), or injection of a lipogenic inhibitor (65).

In the experiments reported here, the tissue samples and isolation procedures were carried out at the same time of day, keeping all experimental conditions identical on any given day. However, as has been noted by other investigators (61,154), there is a great deal of variability in the basal glucose utilization response of isolated adipocytes. A seven-fold difference in plasma insulin levels, determined at various times over a 24-month period, in one monkey has been reported (70). Antecedent diet, duration of overnight fast and conditions of sampling were all rigorously controlled. This exemplifies the importance of expressing the data as a relative change compared to the basal state for each time an experiment is carried out. Most of the

data presented in this thesis will be as a percent change from the basal response.

PAPER I. EFFECT OF TRIIODOTHYRONINE TREATMENT ON GLUCOSE METABOLISM IN ISOLATED ADIPOCYTES FROM LEAN AND OBESE ZUCKER RATS

ABSTRACT

The effect of triiodothyronine (T_3) and insulin treatment on glucose metabolism in isolated adipocytes of 6 week old lean and obese Zucker rats was examined. Insulin had a strong stimulatory effect on glucose metabolism. Insulin stimulated and non-stimulated glucose utilization was greater in the obese than the lean rats. The effect of insulin and phenotype on glucose utilization was pathway specific as fatty acid, CO_2 and glycerol-glyceride formation were stimulated by different magnitudes. Pretreatment of isolated adipocytes with pharmacological concentrations of T_3 for 1/2 hour prior to measurement of glucose metabolism decreased fatty acid synthesis more in the adipocytes from lean than obese rats. The presence of insulin was required in the T_3 pretreated cells to observe any effect of T_3. The effect of a 2 hour pretreatment of adipocytes with T_3 was dependent upon the phenotype of the cells, insulin status and the specific pathway of glucose utilization. The effect of T_3 in the presence of insulin in both lean and obese adipocytes was to decrease lipogenesis. In the absence of insulin, the 2 hour pretreatment effect of T_3 in tissue from a euthyroid animal was to increase lipogenesis at physiological concentrations and decrease lipogenesis at pharmacological concentrations. A pharmacological dose of T_3 was required to increase lipogenesis in the absence of insulin in hypothyroid tissue. This is suggestive that obese tissue is resistant to thyroid hormones. However, in the presence of insulin, physiological levels of T_3 decreased fatty

acid synthesis in both genotypes. Glucose transport was unaltered by T_3 pretreatment of the adipocytes. This indicates that the action of T_3 on glucose metabolism is due to a postreceptor effect. These results suggest that thyroid hormones might modulate a critical regulatory step(s) common to lipogenesis and lipolysis.

KEY WORDS

Adipose tissue, triiodothyronine, insulin, obesity, Zucker rat, glucose metabolism

INTRODUCTION

The genetically obese Zucker rat is an example of a single gene recessive model of obesity. The obesity is early onset (6), and energy metabolism is impaired in the very young (34,49). The adult obese Zucker rat exhibits hyperphagia (7), hyperinsulinemia (6), fat cell hyperplasia and hypertrophy (35), hypertriglyceridemia (5), defective thermic capacity (34,54) and decreased serum levels of thyroid hormones (20). Hypothyroidism is clearly associated with some forms of obesity in man (8) and obese animal models (20), and may in fact precede the onset of hyperinsulinemia, hyperphagia and obesity (69).

Thyroid hormones are known to affect adipose tissue metabolism (11,17,21) mostly by augmentation of catecholamine induced lipolysis. The major effect of thyroid hormones is believed to be by binding to nuclear receptors (46,55,65), followed by stimulation in mRNA and protein synthesis. Buergi and Abbuehl (9) describe 3,3',5-triiodo-L-thyronine (T_3) nuclear binding in isolated nuclei from rat epididymal fat pad adipocytes. Adipocytes grown in culture possess high-affinity binding sites for thyroid hormones, which upon stimulation with physiological levels of thyroid hormone exhibit enhanced fatty acid synthesis (24).

A strong relationship exists between carbohydrate and lipid metabolism and thyroid hormone. Upon administration of simple sugars, a striking response of hepatic lipogenic enzymes and thyroid hormones is observed (41,47). Thyroid hormones are reported to increase both the

hepatic biosynthesis (18,37,40,41) and oxidation (18) of fatty acids. One of the best documented effects of thyroid hormones on adipose tissue is that of the influence of the thyroid hormone status of an animal on the epinephrine-stimulated lipolysis; enhanced in hyperthyroidism, markedly reduced or abolished in hypothyroidism (16,27,32,53). Correze et al. (12) and van Inwegen et al. (66) showed that fat cells from thyroidectomized rats lose their lipolytic capacity. These same cells had an increase in lipogenesis. Omri et al. (45) very recently showed that thyroid hormones control the ^{32}P labelling of proteins involved in both the lipogenic and lipolytic pathways of rat fat cells. In contrast to these findings, other investigators (4,15,27) have reported that thyroidectomy has no effect on the stimulation of CO_2 formation from glucose. The age and type of the animals used and the isolation procedure varied among investigators, which may help explain these differences. Also in vivo treatment with thyroid hormones may result in perturbations resultant from indirect effects of T_3 or T_4. Recent studies show thyroid hormones have direct stimulatory effects on mitochondrial oxidative phosphorylation (63,64) and on plasma membrane sugar transport (58,59). Research on the direct acute action on thyroid hormone on adipose tissue metabolism has provided contradictory results. Further investigation of the effects of thyroid hormone on adipose tissue metabolism is important to elucidate the role of this hormone in disease states such as obesity and diabetes. Previous work in this laboratory (3,51) demonstrated that thyroid hormones have an immediate

action on adipocyte metabolism. The purpose of this study is to investigate the direct effects of T_3 on glucose transport and metabolism in isolated adipocytes from the Zucker rat.

MATERIALS AND METHODS

Animals

Six week old male lean (?/+) and obese (fa/fa) Zucker rats were obtained from the breeding colony at Iowa State University. Animals were housed in suspended wire cages and allowed free access to water and a commercial lab diet (Simonsen Mill Inc., Quimby, Iowa). The rats were subjected to a 12 hour light-dark cycle and maintained at 24 °C and 50% relative humidity.

Materials

Bovine serum albumin (fraction V, RIA grade), porcine insulin, 2-deoxy-Dglucose and 3,3'-5-triiodo-L-thyronine (sodium salt) were obtained from Sigma Chemical Co., (St. Louis, MO). Collagenase (type IV) was obtained from Cooper Biochemical (Freehold, NJ). Osmium tetroxide (OsO_4) was obtained from Electron Microscopy Sciences (Fort Washington, PA). Nylon filtration screen was obtained from Tetko (Elmsford, NY). Silicone oil (d=0.943) and 2,4,6-trimethylpyridine were obtained from Aldrich (Milwaukee, WI). Omnifluor scintillant was obtained from New England Nuclear (Boston, MA). D-(U-^{14}C) glucose, 2-deoxy-D-(1-^{3}H) glucose and inulin-(^{14}C) carboxylic acid were obtained from Amersham (Arlington Heights, IL). Petroleum ether (bp 38 - 54.5 °C), scintiverse II scintillation solution and all other chemicals were obtained from Fisher Scientific Co. (Springfield, NJ).

Cell isolation protocol

Following an overnight fast, animals were decapitated and the epididymal fat pads were removed. The fat pads from 3 obese or 4 lean rats were pooled and the individual adipocytes were isolated by the method of Rodbell (52). The fat pads were rinsed in Krebs Ringer bicarbonate (KRB) buffer, containing 3% bovine serum albumin (BSA), pH 7.4. The tissue pieces were then minced with scissors into 2-5 mg pieces. The tissue pieces were placed into 50 ml polypropylene beakers containing the following collagenase digestion mixture: 30 mg BSA and 1 mg collagenase/ml KRB, pH 7.4. Two ml of the digestion mixture was used per gm of tissue. The beakers were placed in a gyrotory metabolic shaker at 37 °C and gassed for the 1 hour incubation with 95% CO_2-5% O_2. The digestion mixture was filtered through a 250 μm nylon screen with two KRB rinses. The crude cell suspension then was centrifuged (50 X g for 1 minute) and washed 2 times and resuspended in KRB containing 3% BSA, pH 7.4. The number of isolated cells and their viability was determined microscopically using a cell counting chamber. Exclusion of trypan blue (0.6%) was used as an indication of cell viability. In all cases, the cell viability was found to be greater than 93%. The volume of the cell suspension was adjusted so that it contained approximately 5 X 10^5 cells/ml. An aliquot of the cell suspension was fixed in 2,4,6-trimethylpyridine buffer containing 2% osmium tetroxide according to the method of Hirsch and Gallian (30). The fixed adipocytes were filtered consecutively through 250 μm mesh and 25 μm mesh nylon screens.

Adipocytes were then rinsed and taken up in a known volume of 0.9% NaCl-0.004% Triton X-100 for counting in a Coulter Counter Particle Size Analyzer.

Glucose utilization

Approximately 5 X 10^5 adipocytes were added to 25 ml polycarbonate flasks that contained various levels of 3,3'-5-triiodothyronine (T_3), glucose and insulin. The flasks were incubated for either 30 minutes or 2 hours at 37 °C in a metabolic shaker and gassed with 95% O_2-5% CO_2. Measurement of glucose utilization was initiated by the addition of 5 mM D-glucose and 0.5 μCi/ml D-(U-^{14}C) glucose. The incubation flasks were capped with rubber stoppers containing suspended plastic wells with 3 cm^2 filter paper strips. After 60 minutes, 0.1 ml of 25% KOH was injected onto the paper strips in each flask, followed immediately by addition of 0.5 ml of 2N H_2SO_4 into the media to stop the reactions. The released CO_2 was trapped on the paper strips during an additional 1 hour incubation with shaking.

The filter paper strips were placed in scintillation vials and air dried for 2 hours. Ten ml of toluene based scintillation fluid (4 g Omnifluor, 240 ml methanol and 760 ml toluene/l) were added to the vials, and radioactivity was determined by liquid scintillation spectrometry. The radiolabeled lipids were extracted using the method of Dole and Meinertz (19) with the following modifications. Five ml of an extraction mixture (20% petroleum ether: 80% 2-propanol) were added to the contents of the flasks and shaken at 25 °C for 2 hours. The

contents of the flasks were then quantitatively transferred to screwcapped glass tubes with two 5 ml rinses. Nine ml of petroleum ether and 9 ml of water were added and the tubes were mixed. After the phases separated, the upper organic phase was removed and placed in new tubes. Nine ml of water were added and the tubes were mixed and centrifuged at 500 X g at ambient temperature. The organic phase was transferred to new tubes and evaporated at 60 °C. The extracted lipids were saponified by the addition of 6 ml of ethanolic KOH (5 g KOH/100 ml of 95% ethanol) and heated at 80-85 °C for 45 minutes. Six ml of water were added and the non-saponified lipids were extracted three times with 5 ml petroleum ether. Two ml of 12.4 N HCl were added to the tubes. The fatty acids were quantitatively extracted 3 times with 5 ml petroleum ether and collected into scintillation vials and air dried. Ten ml of scintillation fluid were added to the vials and radioactivity was determined as stated above. Radioactivity in the glycerol-glyceride in the remaining aqueous fraction was determined by placing a 2 ml sample of the aqueous phase into 15 ml of scintiverse II scintillation solution and measurement of the remaining volume of the aqueous phase.

2-Deoxyglucose transport

Approximately 5 X 10^4 adipocytes were added in duplicate to 12 mm X 75 mm polystyrene tubes that contained various levels of T_3 and insulin in KRB. The tubes were placed in a metabolic shaker at 37 °C for 30 minutes or 2 hours and gassed with 95% O_2-5% CO_2. Measurement of 2-deoxyglucose uptake was initiated by the addition of 2-deoxy-D-(1-^{3}H)

glucose (2-DG) and inulin-(^{14}C) carboxylic acid (inulin). Each incubation tube contained 0.1 mM 2-DG, 2.0 μCi/ml ^{3}H 2-DG, 0.5 μCi/ml ^{14}C inulin and 10 mg/ml BSA in a final volume of 0.3 ml. The 2-DG uptake experiments were carried out for 1-15 minutes. Seconds prior to stopping the reaction, 0.2 ml of the reaction mixture was layered on 0.7 ml of silicone oil in microcentrifugation tubes. The reaction was stopped by separating the adipocytes from the media according to the method of Gammeltoft and Gliemann (22). This was accomplished by centrifuging the tubes at 10,000 X g for 15 seconds. The centrifugation tubes were placed in a -80 °C freezer. The tubes were cut midway through the oil layer. The top half of the centrifugation tube was placed into a scintillation vial. Fifteen ml of scintiverse II were added to each vial and radioactivity was determined. The ^{14}C-inulin is used to determine the amount of extracellular space in this system (26) and the amount of 2-deoxyglucose that was in the extracellular space.

Statistics

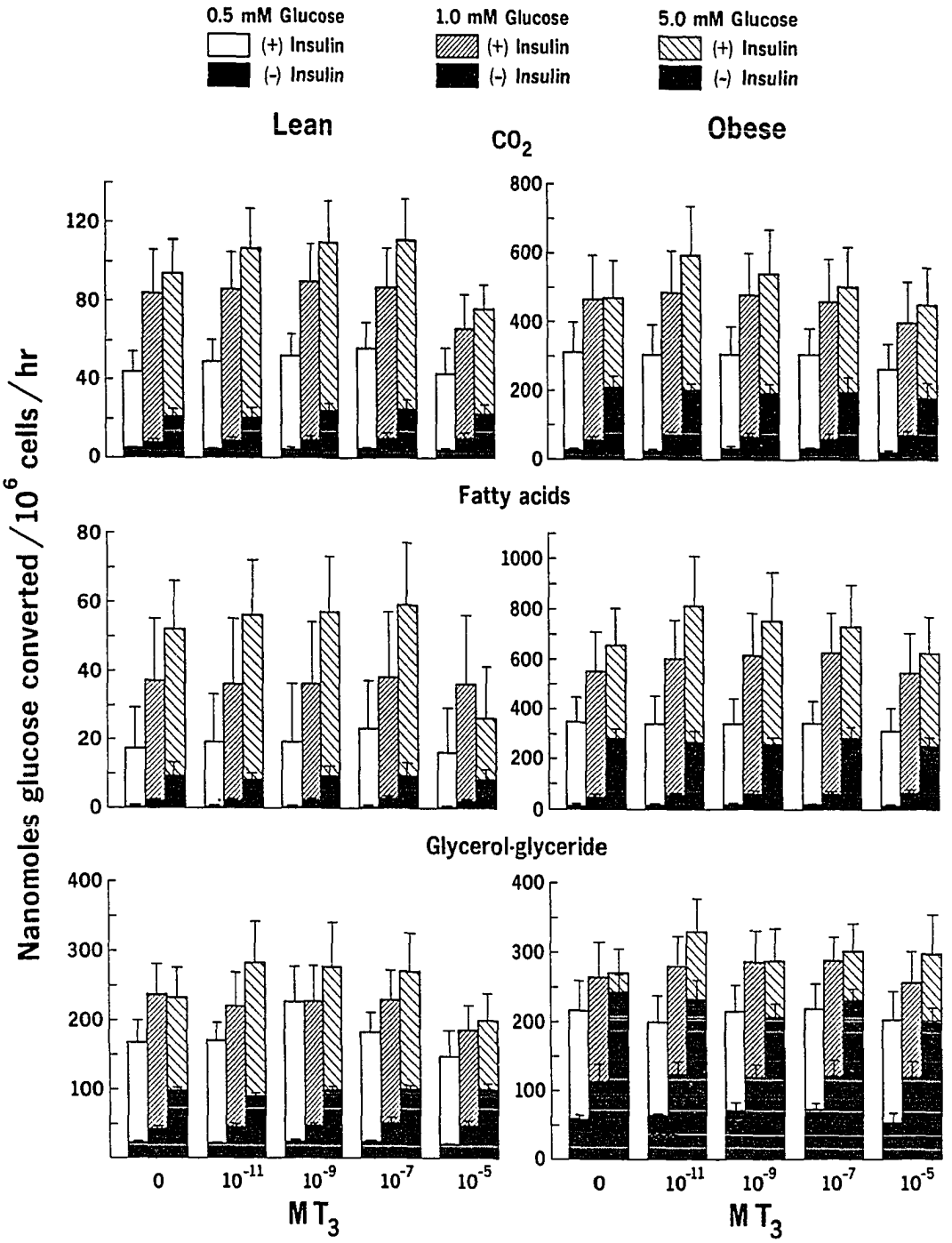
All statistical analysis were done using the Statistical Analysis System (SAS) at the Computer Center at Iowa State University. 2-deoxyglucose experiments and the main effects of the glucose utilization experiments including genotypes, hormone levels, and substrate levels were analyzed by analysis of variance (ANOVA). The t test (62), using the pooled mean squared error term, was used to measure differences between the levels of T_3 used in the glucose utilization experiments. A probability of 0.05 or less was taken to be statistically significant.

RESULTS

Adipocytes were incubated with 0, 10^{-11}, 10^{-9}, 10^{-7} or 10^{-5} M T_3 for either 30 minutes or 2 hours prior to addition of radiolabeled glucose. Figure 1 shows the effect of glucose, insulin and T_3 on glucose utilization. The concentration of glucose in the incubation media had a very dramatic effect on the amount of ^{14}C glucose utilized by the adipocytes. As the glucose concentration in the media was increased from 0.5 to 1.0 to 5.0 mM, the amount of glucose converted to CO_2, fatty acids and glycerol-glyceride increased. In no case was there a glucose X T_3 interaction, indicating that the effect of glucose concentration on glucose utilization was a parallel increase. Therefore, all subsequent glucose utilization studies were done with 5.0 mM glucose which closely mimics physiological levels of glucose. As has been noted by other investigators (15,25,29), the presence of insulin has a large stimulating effect on glucose utilization (Fig. 1). The insulin stimulated increase in glucose utilization was between 4 and 20 fold in lean and obese adipocytes. The fat cells isolated from obese rats had much higher levels of CO_2, fatty acids and glycerol-glyceride produced than the lean rats (Fig. 1). This genetic difference was most pronounced in the amount of fatty acid produced, approximately a 12 fold increase. CO_2 and glycerol-glyceride syntheses were increased approximately 8 and 2 fold, respectively, in the obese over the leans.

A large variability was evident in the basal glucose utilization response in these experiments due to the daily variability between

Figure 1. Effect of glucose, insulin and triiodothyronine on glucose utilization by adipocytes from lean and obese rats. Each value is the mean ± SEM of 5 observations. Adipocytes were pre-incubated for 30 minutes with or without T_3 at 37 °C before the addition of U-[^{14}C] glucose, which initiated the 1 hour incubation period. Insulin, when present during the 1 hour incubation, was at 1000 μU/ml. Incubations and extractions of metabolic products were performed as described in "Materials and Methods".

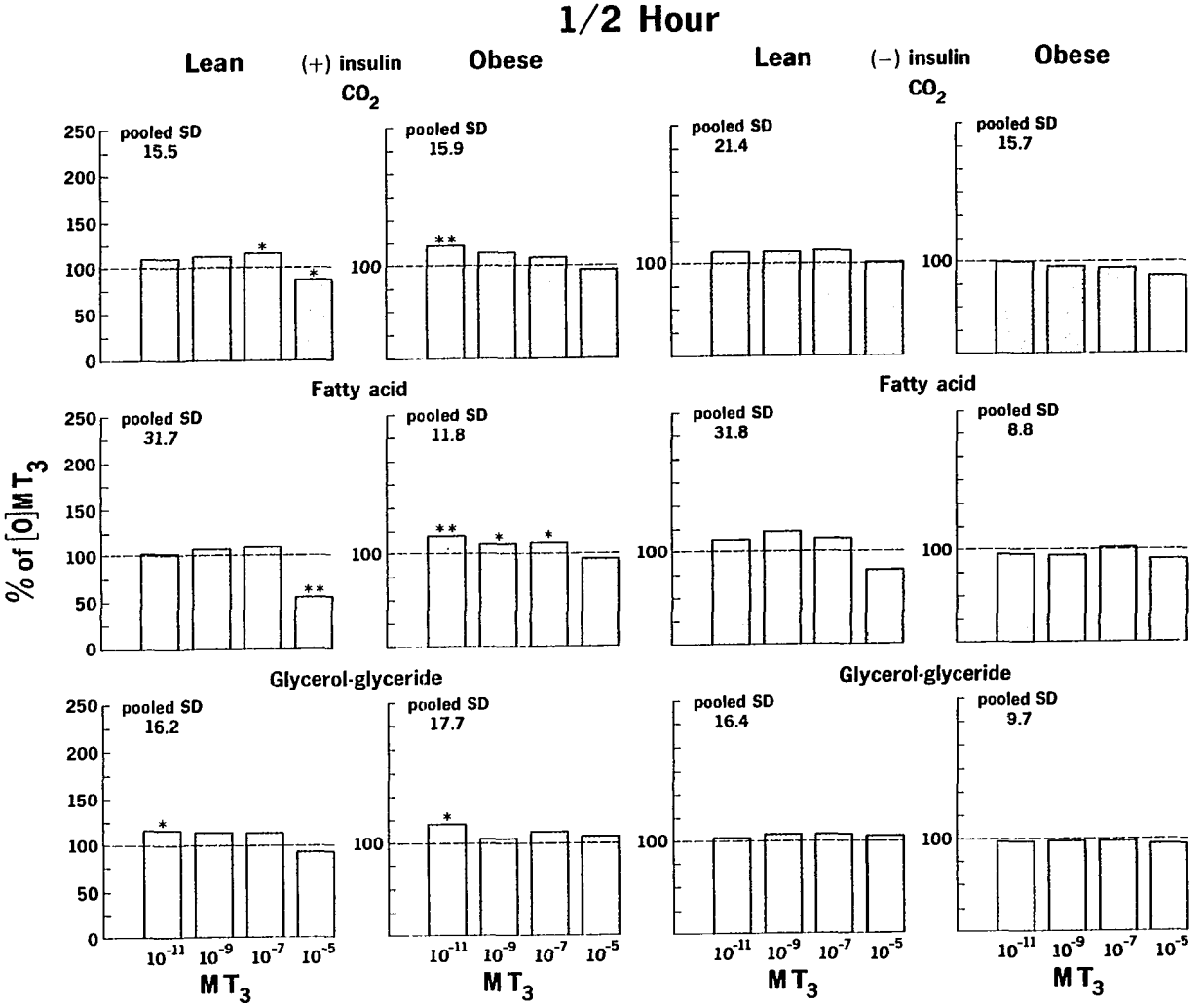

0.5 mM Glucose
(+) Insulin
(-) Insulin
1.0 mM Glucose
(+) Insulin
(-) Insulin
5.0 mM Glucose
(+) Insulin
(-) Insulin
Lean
Obese
CO_2
Fatty acids
Glycerol-glyceride
Nanomoles glucose converted / 10^6 cells / hr
0
10^{-11}
10^{-9}
10^{-7}
10^{-5}
MT3

adipocyte preparations. To best demonstrate the effect of T_3 on glucose utilization and to eliminate the daily variability between adipocyte preparations, the results were expressed as a percent change from the zero T_3 levels.

The effect of a 30 minute pre-treatment with T_3 on glucose utilization in isolated adipocytes is shown in Figure 2. Insulin was present in these experiments at 1000 μU/ml. There was a significant increase in fatty acid synthesis at the 10^{-11}, 10^{-9} and 10^{-7} M T_3 concentrations in the obese rats. When compared to basal, the 10^{-5} M T_3 concentration tended to decrease glucose utilization in adipocytes from lean and obese rats, except in the obese glycerol-glyceride production. This effect was much greater in the lean (22%) than in the obese (2%). When insulin was not present in the media, the effect of T_3 on glucose utilization was blunted (Fig. 2). The glucose utilization response of the obese adipocytes was very flat when insulin was absent from the media.

The next step in this study was to determine what effects a longer exposure time of adipocytes to T_3 would have on glucose metabolism. Isolated adipocyte glucose metabolism was measured from the same batch of cells after 1, 2 and 3 hours and no differences were found (data not shown). These experiments were carried out on both insulin stimulated and non-stimulated cells. Therefore, the metabolism of the cells was unaltered for at least 3 hours after the cells were isolated. Treatment of adipocytes with the physiological inactive isomer, D-T_3 resulted in

Figure 2. Effect of triiodothyronine on glucose utilization by adipocytes from lean and obese rats. Values are expressed as a percent of the [0] M T_3 values. Each value is the mean of 6 observations. Adipocytes were pre-incubated for 30 minutes with or without T_3 at 37 °C before the addition of 5 mM glucose and U-[^{14}C] glucose, which initiated the 1 hour incubation. Insulin, when present during the 1 hour incubation, was at 1000 μU/ml. The nmoles glucose converted to product/10^6 cells/hr for the 100% values of the various groups are: lean (+)insulin- 83.7 CO_2, 49.2 FA, 212.9 GG; obese (+)insulin- 470.4 CO_2, 654.1 FA, 269.9 GG; lean (-)insulin- 18.4 CO_2, 8.0 FA, 92.1 GG; obese (-)insulin- 208.8 CO_2, 280.3 FA, 241.8 GG. Incubations and extractions of metabolic products were performed as described in "Materials and Methods". Values were statistically significant from [0] M T_3: * $P<.05$, ** $P<.01$.

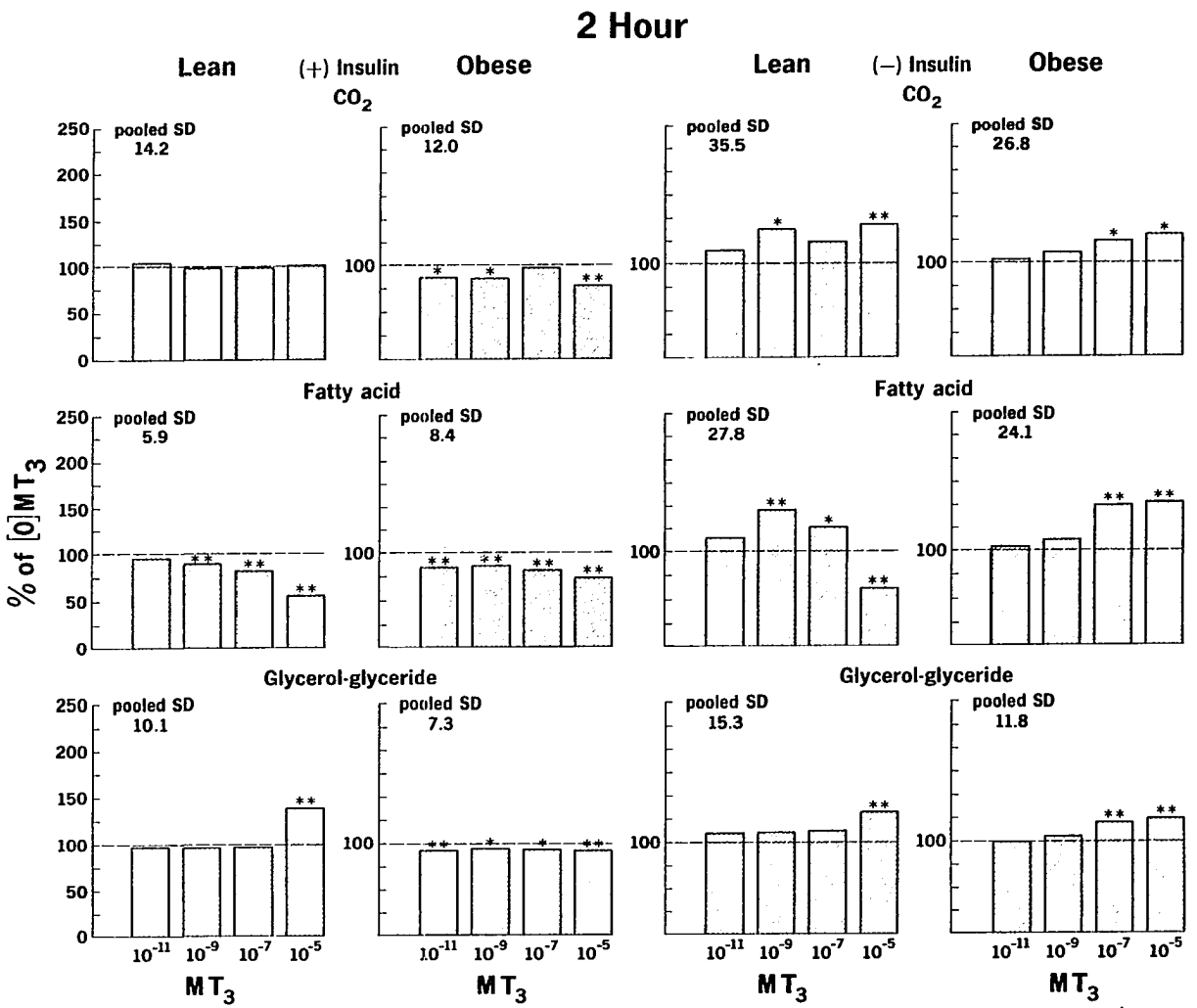

1/2 Hour
Lean
(+) insulin
Obese
Lean
(−) insulin
Obese
CO_2
Fatty acid
Glycerol-glyceride
% of [0]MT_3
pooled SD
15.5
15.9
21.4
15.7
31.7
11.8
31.8
8.8
16.2
17.7
16.4
9.7
250
200
150
100
50
0
10^{-11}
10^{-9}
10^{-7}
10^{-5}
MT_3

no change in glucose utilization (data not shown). This is in agreement with earlier studies performed in this laboratory (51).

Isolated adipocytes were pre-incubated for 2 hours with T_3 and with or without insulin. The results of the adipocyte incubations in the presence of insulin are shown in the two left panels of Figure 3. Fatty acid formation significantly decreased ($p<.01$) with increasing T_3 concentrations. Glycerol-glyceride formation was significantly increased at 10^{-5} M T_3. Obese adipocytes showed a significant decrease in glucose metabolism at nearly all levels of T_3.

Very different results were obtained from fat cells subjected to varying levels of T_3 for 2 hours in the absence of insulin (the two right panels of Fig. 3). CO_2 and glycerol-glyceride formation tended to increase with increasing T_3 concentrations in both the lean and obese. Fatty acid synthesis in the lean adipocytes increased at the 10^{-9} and 10^{-7} M T_3. Fatty acid synthesis in the obese adipocytes increased with increasing concentrations of T_3. A significant decrease from basal glucose utilization was observed in fatty acid synthesis in the lean adipocytes exposed to 10^{-5} M T_3. With insulin present, physiological levels of T_3 resulted in decreased fatty acid synthesis in both lean and obese adipocytes. These results indicate that treatment of adipocytes with T_3 alters glucose utilization. These effects are pathway specific and variable between the genetically obese and lean rat. In addition, insulin appears to modulate the effect of T_3.

Figure 3. Effect of triiodothyronine on glucose utilization by adipocytes from lean and obese rats. Values are expressed as a percent of the [0] M T_3 values. Each value is the mean of 6 observations. Adipocytes were pre-incubated for 2 hours with or without T_3 at 37 °C before the addition of 5 mM glucose and U-[^{14}C] glucose, which initiated the 1 hour incubation. Insulin, when present during the 1 hour incubation, was at 1000 μU/ml. The nmoles glucose converted to product/10^6 cells/hr for the 100% values of the various groups are: lean (+)insulin- 58.6 CO_2, 35.3 FA, 199.5 GG; obese (+)insulin- 682.7 CO_2, 921.8 FA, 541.0 GG; lean (-)insulin- 24.4 CO_2, 7.2 FA, 124.6 GG; obese (-)insulin- 188.4 CO_2, 208.2 FA, 247.9 GG. Incubations and extractions of metabolic products were performed as described in "Materials and Methods". Values were statistically significant from [0] M T_3: * $P<.05$, ** $P<.01$.


2 Hour
Lean
(+) Insulin
Obese
Lean
(−) Insulin
Obese
CO_2
CO_2
pooled SD 14.2
pooled SD 12.0
pooled SD 35.5
pooled SD 26.8
Fatty acid
Fatty acid
pooled SD 5.9
pooled SD 8.4
pooled SD 27.8
pooled SD 24.1
Glycerol-glyceride
Glycerol-glyceride
pooled SD 10.1
pooled SD 7.3
pooled SD 15.3
pooled SD 11.8
% of [0] MT_3
250
200
150
100
50
0
10^{-11} 10^{-9} 10^{-7} 10^{-5}
MT_3

To measure the effect of T_3 on the first step in glucose metabolism, glucose transport, 2-deoxyglucose uptake was measured. Insulin significantly ($p<.0001$) stimulated 2-deoxyglucose transport in adipocytes isolated from lean rats. The adipocytes were pretreated for 1/2 hour with 0 or 10^{-9} M T_3 or 0 or 10^{-5} M T_3 (Fig. 4). Insulin had no effect on 2-deoxyglucose uptake by adipocytes from obese rats.

The 1/2 hour pretreatment of lean adipocytes with 10^{-5} M T_3 consistently decreased 2-deoxyglucose transport, however at no point was it statistically significant (Fig. 4). The same treatment of obese adipocytes produced a statistically significant ($p<.05$) decrease in 2-deoxyglucose uptake at 10^{-5} M T_3 (Fig. 4). Pretreatment of both lean and obese adipocytes with 10^{-9} M T_3 had no effect on 2-deoxyglucose transport (Fig. 4).

Lean adipocytes pretreated for 2 hours with 0, 10^{-9} or 10^{-5} M T_3 had nearly identical 2-deoxyglucose transport rates (Fig. 5). In contrast to the 1/2 hour results, the 2 hour pretreated lean adipocytes were insensitive to insulin stimulation. The obese adipocytes that were pretreated with T_3 in the absence of insulin had a statistically significant ($p<.01$) increase in 2-deoxyglucose transport compared to the insulin stimulated cells. This is a good indication that down regulation of the insulin receptor occurs in the obese adipocytes subjected to insulin at 1000 μU/ml for 2 hours.

Figure 4. The effect of triiodothyronine and insulin on 2-deoxyglucose uptake by adipocytes from lean and obese rats. ●, triiodothyronine present; o, triiodothyronine absent; ——, insulin present; ---, insulin absent. Values are expressed as the nanomoles 2-DG uptake/10^6 cells/time. Each value is the mean of 8 observations for the [T3]=10^{-9} data and 5 observations for the [T3]=10^{-5} data. Adipocytes were pre-incubated for 30 minutes with or without T_3 at 37 °C before measuring 2-DG uptake. Insulin, when present, was at 1000 μU/ml. See "Materials and Methods" for details of the protocol. Values were statistically significant from [0] M T_3: * $P<.05$.

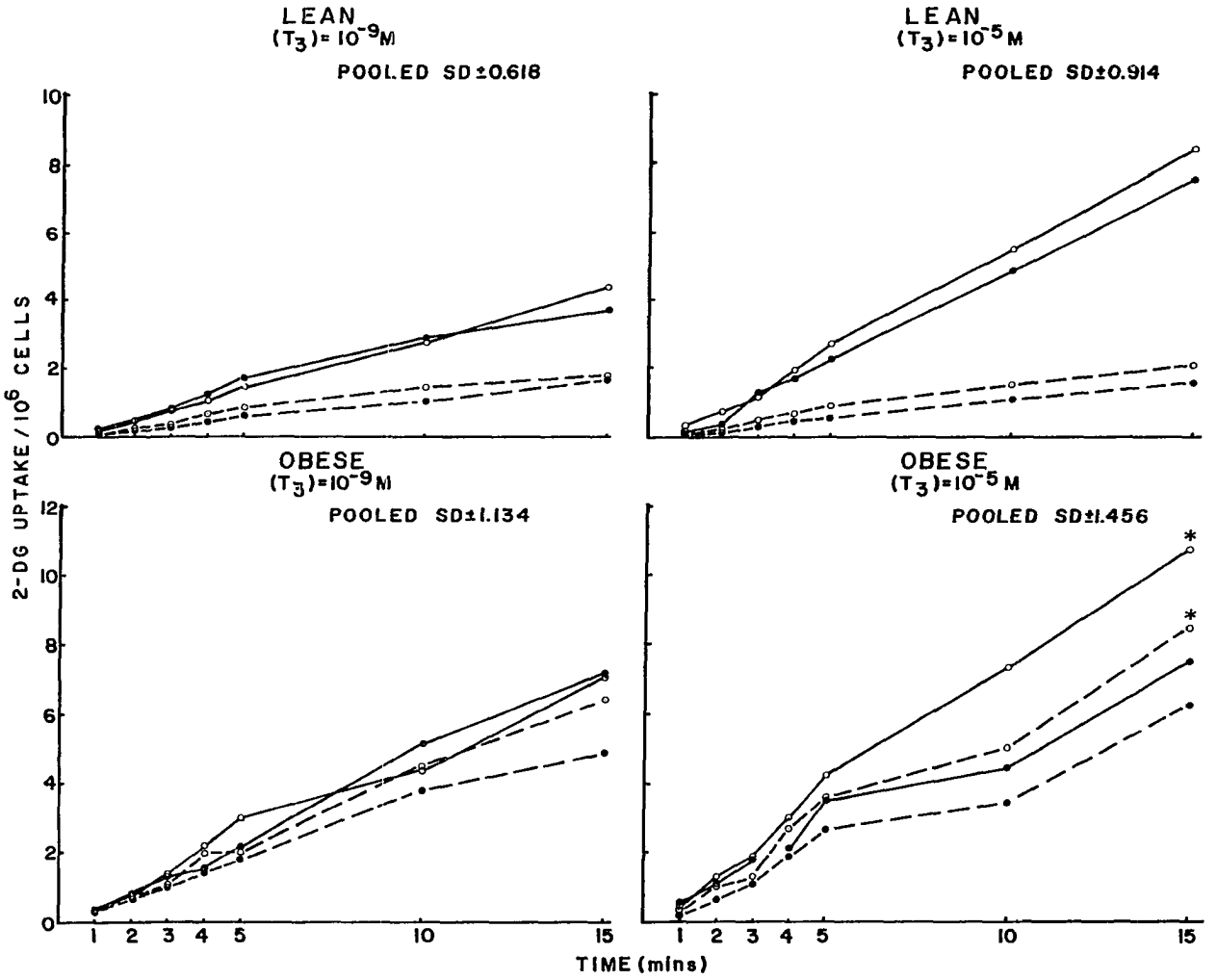
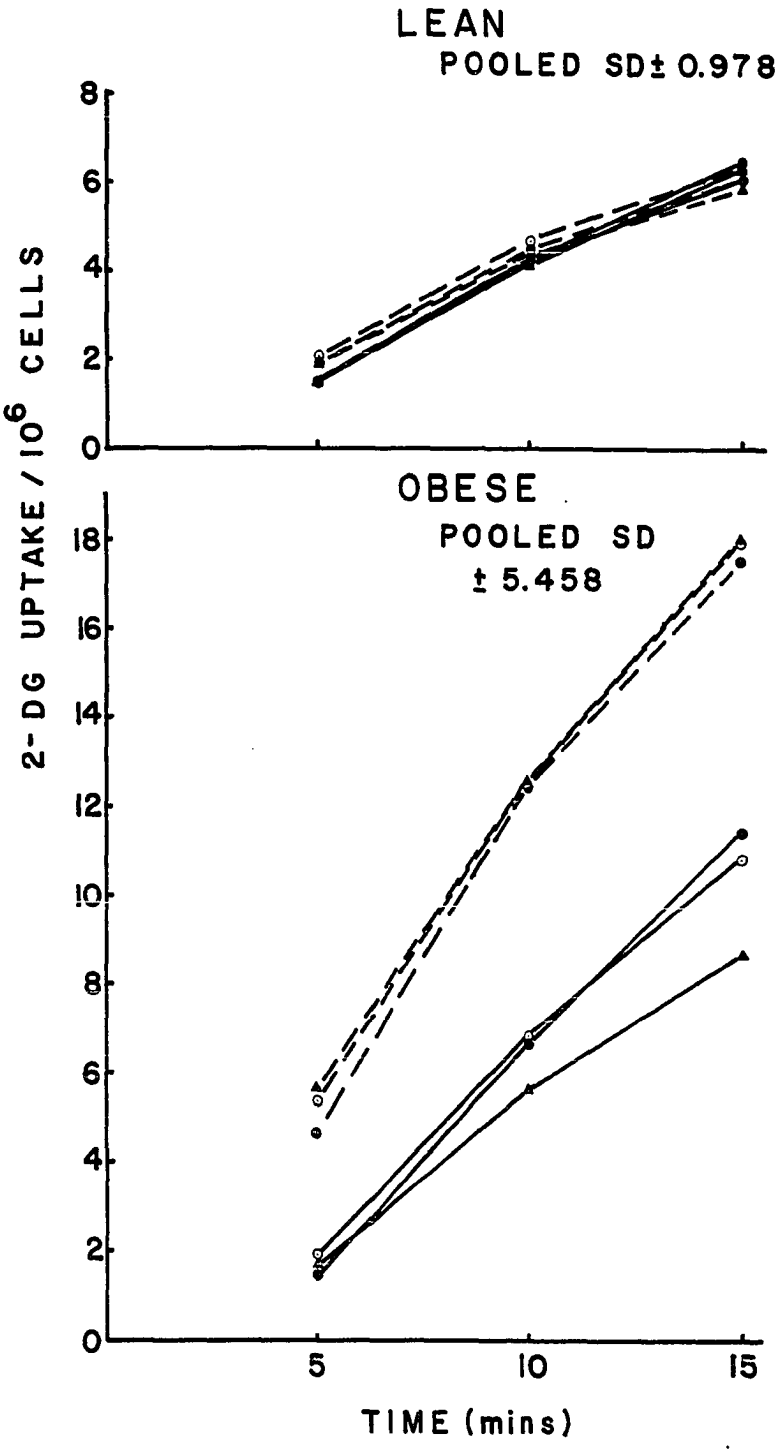

LEAN
(T3)=10-9M
POOLED SD±0.618
LEAN
(T3)=10-5M
POOLED SD±0.914
OBESE
(T3)=10-9M
POOLED SD±1.134
OBESE
(T3)=10-5M
POOLED SD±1.456
2-DG UPTAKE / 10^6 CELLS
TIME (mins)
0
2
4
6
8
10
12
1
2
3
4
5
10
15
*
*

Figure 5. The effect of triiodothyronine and insulin on 2-deoxyglucose uptake by adipocytes from lean and obese rats. ▲ 10^{-5} M T_3; ● 10^{-9} M T_3; o 0 M T_3; —— insulin present; --- insulin absent. Values are expressed as the nanomoles 2-DG uptake/10^6 cells/time. Each value is the mean of 6 observations. Adipocytes were pre-incubated for 2 hours with or without T_3 at 37 °C before measuring 2-DG uptake. Insulin, when present, was at 1000 μU/ml. See "Materials and Methods" for details of the protocol.


LEAN
POOLED SD± 0.978
OBESE
POOLED SD
± 5.458
2-DG UPTAKE / 10^6 CELLS
0
2
4
6
8
10
12
14
16
18
5
10
15
TIME (mins)

DISCUSSION

The primary mode of action of thyroid hormones is postulated by many investigators to take hours or days. This is because the action of thyroid hormones is believed to be largely mediated by binding to nuclear receptors (46,56,65), with a subsequent increase in mRNA and protein synthesis. Recent reports support the physiological relevance of the direct action of thyroid hormones on extranuclear events. Sterling et al. (63,64) reported the direct stimulation of mitochondrial oxidative phosphorylation after intravenous injection of T_3 at physiological doses. Plasma membrane T_3 receptor sites are reported for thymocytes (60), hepatocytes (50), fibroblasts (23,42) and adipocytes (48). The plasma membrane T_3 binding sites in fat cells are identified as high affinity, low capacity (1,48), which indicates that a strong barrier to the entry of T_3 into adipocytes exists. This may help explain why pharmacological concentrations are usually required to obtain any acute physiological effect of thyroid hormone administration (10,11,51).

In this study, the insulin and glucose concentration of the media had profound effects on glucose utilization. Treatment of lean adipocytes with increasing concentrations of glucose (0.5, 1.0, 5.0 mM) resulted in increasing glucose utilization (Fig. 1). The adipocytes isolated from obese rats responded in the same way as the lean adipocytes, although the magnitude of change was not as great. This is because the basal levels of glucose utilization were much higher in the

obese. Therefore, glucose metabolism may occur near the maximum rate. Other investigators (14,43) present evidence for a maximum capacity of adipocytes for glucose metabolism, especially for de novo fatty acid synthesis. Insulin increased glucose utilization in both the lean and obese adipocytes (Fig. 1). These observations are not novel as rat epididymal adipocytes are reported to have maximal insulin stimulation within the physiological concentration range of the hormone (13,28,44). The magnitude of insulin stimulation was much greater in the lean than the obese. This again may be due to the elevated basal glucose utilization found in the obese which may be nearly maximally stimulated. Increased fat cell size is accompanied by the progressive development of a marked resistance to insulin (14,43) which may also explain the decreased magnitude of stimulation in obese cells. The large adipocytes of obese rats appear to be locked in an insulin-stimulated-like state which may be a consequence of the hyperinsulinemia associated with obesity (31). The concomitant insulin insensitivity and alteration of glucose transport and metabolism may reflect the consequence of normal growth processes that ultimately prevent the cell from expanding its triglyceride stores beyond some maximum volume (33). It is interesting to note the amount of stimulation found in the different pathways and the difference in the magnitude of change between the lean and obese (Fig. 1). CO_2 and fatty acid production were approximately 6 fold and 12 fold greater in the obese than the leans, respectively. The amount of glycerol-glyceride produced was approximately the same in the lean

and obese. The production of CO_2 and fatty acids was more sensitive to increases in media glucose concentration than was glycerol-glyceride.

The results described in this paper indicate that T_3 has an acute effect on glucose metabolism in adipocytes, and insulin can modulate this effect. Alternatively, T_3 may act to modulate insulin action. A 1/2 hour pre-incubation of cells with T_3 followed by a 1 hour incubation with radiolabeled glucose and T_3 results in significant changes in glucose utilization (Fig. 2). When T_3 was present at approximately 10,000X the physiological concentration, 10^{-5} M, glucose utilization was decreased by an average of 22% in adipocytes derived from lean rats. The same trend was seen in the obese adipocytes, however, the magnitude of change was not as great. This is indicative of a possible T_3 resistance by obese adipocytes. The effect of this pharmacological dose of T_3 was more pronounced in CO_2 and fatty acid formation, indicating that the T_3 effects are pathway specific.

When the adipocytes were incubated with T_3 in the absence of insulin, there was no statistically significant effect of T_3. This implies that T_3 requires the presence of insulin to have an effect. These results are supported by Gharbi-Chihi et al. (24) and Anselmet et al. (1) who found enhanced de novo fatty acid synthesis in ob 17 cells with chronic exposure to physiological levels of T_3. These investigators found that insulin and T_3 had a synergistic effect on fatty acid synthesis.

The adult obese Zucker rat exhibits hyperinsulinemia (6) and hypothyroidism (20,8). Many studies have been performed to investigate the relationship between this altered state of insulin and thyroid hormone metabolism. Most (15,57,61), but not all, reports (67) indicate that adipocytes obtained from hyperthyroid animals are incapable of responding to insulin either by increasing glucose uptake or the rate of lipid formation from glucose. Mariash and Oppenheimer (39) suggested that the insulin stimulated intracellular metabolism of glucose could produce a factor which interacts with the nuclear T_3 signal. Arner et al. (2) observed a large increase in insulin binding in isolated adipocytes from hypothyroid patients and a marked decrease in insulin binding in adipocytes from hyperthyroid patients. Experimentally induced hypothyroid animals become hyperinsulinemic, and hyperthyroid animals have a lower circulating insulin level (38). These results, in addition to ours, further support the significance of insulin-T_3 interaction on glucose metabolism.

To investigate the effects of a longer exposure time of adipocytes to T_3, glucose metabolism was measured after 2 hours (Fig. 3). Preliminary studies showed that glucose metabolism was unaltered for at least 3 hours after isolation of the adipocytes. The pathway specificity of T_3 action is even more pronounced in the 2 hour studies. The lean CO_2 production was unaltered by T_3 treatment (Fig. 3). Fatty acid formation decreased with increasing T_3 concentrations and glycerol-glyceride formation was unaltered except at the 10^{-5} M T_3

concentrations, where significant ($P<.01$) increases occurred. This would suggest that lipogenesis is being inhibited at the 10^{-11}, 10^{-9} and 10^{-7} M T_3 concentration. The nearly 45% decrease in fatty acid synthesis concomitant with a significant increase in glycerol-glyceride production at 10^{-5} M T_3 would suggest that triglyceride turnover is accelerated in these adipocytes.

The obese rat adipocytes had reduced levels of glucose utilization following a 2 hour pretreatment with T_3 (Fig. 3). These results suggest that when cells from a hyperinsulinemic animal are subjected to high levels of insulin in vitro concomitant with T_3, the effect is an overall reduction in glucose metabolism. These results further support the hypothesis that the insulin status of the cells affects the action of thyroid hormone on glucose metabolism.

When insulin is not present in the lean adipocytes pretreated with T_3 for 2 hours, fatty acid synthesis was significantly increased at approximately physiological concentrations of T_3, 10^{-9} M T_3. However, at the 10^{-5} M T_3, fatty acid synthesis was depressed with an increase in glycerol-glyceride and CO_2 production. This would suggest that at this very high level of T_3, lipogenesis is decreased with an increase in triglyceride turnover in the adipocytes.

In contrast to when insulin was present, the obese adipocytes had increased levels of glucose utilization with increasing concentrations of T_3 when insulin was absent during the 2 hour preincubation (Fig. 5). This even more dramatically exemplifies the importance of the insulin status of the cells on the effect of T_3.

The only occasion when the 1/2 hour pretreatment with T_3 significantly affected 2-deoxyglucose transport was when it was present at 10^{-5} M levels in the obese adipocytes (Fig. 4). In this case, the T_3 significantly ($P<.05$) decreased glucose transport in both the insulin stimulated and nonstimulated cells. Glucose utilization was not affected by T_3 (Fig. 2) in these cells. Therefore, glucose transport does not appear to limit the rate of glucose metabolism; rather, T_3 affects a postreceptor event which alters the glucose utilization rate. The results of Schoenle et al. (57) show that a change in glucose transport is not necessarily followed by a change in glucose utilization. They found that basal glucose transport in hypophysectomized rat adipocytes proceeds at a very rapid rate, similar to that observed in normal cells under maximal stimulation by insulin. However, these cells do not incorporate more glucose than do normal fat cells.

Insulin stimulated 2-deoxyglucose transport was significantly ($P<.001$) increased in the lean adipocytes pretreated for 1/2 hour with T_3. Adipocytes from obese animals were insulin insensitive in the 1/2 hour transport experiments. This result is in agreement with other investigators (14,43,68) who have described insulin resistance in genetically obese rats.

Lean adipocytes were insulin insensitive to 2-deoxyglucose transport after a 2 hour preincubation (Fig. 5). Kono et al. (36) noted that extended mechanical treatment or hard centrifugation can cause an

"insulin-like" effect on glucose transport. This was found to be due to a shift in the distribution of glucose transporters from a nonfunctional site to a functional site in the plasma membrane. The extended period of time these cells were in a metabolic shaker may cause this "insulin-like" effect in these cells. The 2 hour pretreatment of obese adipocytes with T_3 did not affect 2-deoxyglucose transport (Fig. 5). The obese cells that were in contact with insulin during the 2 hour pretreatment had a significant decrease in glucose transport. This is an indication of down regulation of the insulin receptor. This down regulation may explain why glucose utilization was decreased in these cells in the presence of insulin (2 left panels of Fig. 3) and increased in the absence of insulin (2 right panels of Fig. 3).

From the results of this study, it would appear that T_3 has an acute and direct action on adipocyte glucose metabolism. The action of T_3 can vary depending upon the prior thyroid hormone and insulin status of the organism and the in vitro treatment of the adipocytes. Adipocytes from thyroidectomized rats lose their lipolytic capacity and these same cells have increased rates of lipogenesis (12,66). These results agree with the data presented here and suggest that thyroid hormones might modulate critical regulatory step(s) common to lipogenesis and lipolysis. The role of thyroid hormones may be to affect lipolysis and lipogensis depending upon energy needs of the organism. Adipose tissue is unique in its ability to store large amounts of energy, and thyroid hormone may act in conjunction with other hormones to maintain energy equilibrium.

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PAPER II. EFFECT OF TRIIODOTHYRONINE AND INSULIN ON ADIPOSE TISSUE EXPLANTS FROM LEAN AND OBESE ZUCKER RATS MAINTAINED IN CULTURE

ABSTRACT

The effect of insulin and triiodothyronine (T_3) on glucose utilization in adipose tissue explants from 6 week old lean and obese Zucker rats was investigated. The tissue explants were maintained in a chemically defined media for 18 hours. Isolated adipocytes from the adipose tissue explants were insensitive to the acute effects of insulin. Glucose utilization was stimulated in lean explants subjected to 200 μU/ml insulin in the explant media. Glucose utilization was not significantly stimulated at this level of insulin in the obese explants. This indicates that these cells are insulin resistant. Supplementation of T_3 to lean adipose tissue explants media increased lipogenesis at physiological concentrations. Lipogenesis was increased in the obese explants only when T_3 was present at pharmacological concentrations. The addition of T_3 concomitant with insulin to the explant media tended to decrease lipogenesis in both the lean and obese. This effect was more striking in the obese than in the lean. Glycerol-glyceride formation in these adipocytes was increased at pharmacological concentrations of T_3. When insulin was present and T_3 was supplemented at physiological through pharmacological concentrations, adipose tissue explants from hypothyroid (obese) rats had decreased lipogenesis and reesterification of triglycerides may be accelerated. Insulin was shown to modulate the effect of thyroid hormone on adipocyte glucose metabolism.

KEY WORDS

Adipose tissue, triiodothyronine, tissue explant, insulin, obesity, Zucker rat, glucose metabolism

INTRODUCTION

The action of thyroid hormones on various tissues has received extensive attention over the years. A more than casual relationship between abnormal circulating levels of thyroid hormone and a number of diseases has been established; including, diabetes (32) and obesity (6,12). Hypothyroidism is clearly associated with some forms of obesity in man and obese animal models. Some investigators report a direct correlation between the degree of obesity and decreased T_3 concentration in the serum (3,6,12). Hypothyroidism may precede the onset of hyperinsulinemia, hyperphagia and insulin resistance associated with most types of obesity (39). These results exemplify the importance of studying the role of thyroid hormones on metabolism of adipose tissue, one of the major target tissues in obesity.

The genetically obese Zucker rat is an excellent model for early onset hypercellular-hypertrophic human obesity. In comparison to the nonobese rat, the obese (fa/fa) rat exhibits hyperphagia (4), hyperinsulinemia (2), a lower maintenance energy requirement (23), defective thermic capacity to low environmental temperatures (19) and diet (40) and decreased serum levels of thyroid hormones (6,12).

Thyroid hormones are generally considered as gene activating hormones. A number of investigators (7,27,31) report the existence of nuclear receptor sites for T_3. Since thyroid hormones are known to increase total RNA synthesis and modify several specific mRNA's, the nuclear binding of T_3 is generally regarded as the major site of thyroid

hormone action (26). However, recent evidence suggests that the thyrometabolic state of a single cell is the result of the simultaneous action of T_3 at the nucleus, mitochondria and plasma membrane (15,22,24,28,33).

Results presented in the first paper suggest that T_3 has an effect on adipocyte glucose metabolism within 30 minutes. This effect is variable depending on the prior insulin and thyroid hormone status and the in vitro treatment of the cells. Some of the results obtained after a 2 hour treatment of the adipocytes with T_3 were inconsistent with the 1/2 hour results. These inconsistencies may reflect stimulation from the animal's environment or may reflect a long term action of T_3. The present investigation was conducted to elucidate the 18 hour effects of T_3 and possible interaction of T_3 and insulin on glucose metabolism in adipose tissue from lean and obese Zucker rats in an environment not influenced by the aberrations found in the obese rats.

There are limited methods to investigate the metabolic changes of adipose tissue exposed to variations in the biochemical environment for more than a few hours. The use of tissue explants offers one such possibility. Adipose tissue explants have been maintained in culture for 30 weeks with minor morphological changes from that of freshly excised tissue (34). The measurement of glucose transport and utilization in isolated cell suspensions has been criticized because the mechanical agitation and use of digestive enzymes in the fat cell isolation procedure are known to affect these processess (13,14,17,20).

In addition, isolated fat cells are viable only for several hours. Even though some metabolic alterations may occur with adipose tissue explants (1,8,21,35), this technique is a valuable and important research technique.

Tissue explants were maintained in culture in the absence of bovine serum albumin and serum. In this procedure, the culture medium is chemically defined.

The purpose of this study was to examine the 18 hour effects of triiodothyronine and insulin on adipose tissue. Tissue explants from lean and obese Zucker rats were used as the model system.

MATERIALS AND METHODS

Animals

Six week old male lean (?/+) and obese (fa/fa) Zucker rats were obtained from the breeding colony at Iowa State University. Animals were housed in wire cages and allowed free access to water and a commercial lab diet (Simonsen Mill Inc., Quimby, Iowa). The rats were subjected to a 12 hour light-dark cycle and maintained at 24 °C and 50% relative humidity.

Materials

Bovine serum albumin (fraction V, RIA grade), porcine insulin, and 3,3'-5-triiodo-L-thyronine (sodium salt) were obtained from Sigma Chemical Co., (St. Louis, MO). Collagenase (type IV) was obtained from Cooper Biochemical (Freehold, NJ). Osmium tetroxide (OsO_4) was obtained from Electron Microscopy Sciences (Fort Washington, PA). Nylon filtration screen was obtained from Tetko (Elmsford, NY). 2,4,6-trimethylpyridine was obtained from Aldrich (Milwaukee, WI). Omnifluor scintillant was obtained from New England Nuclear (Boston, MA). D-(U-^{14}C) glucose and the radioimmunoassay kits for insulin and triiodothyronine were obtained from Amersham (Arlington Heights, IL). Tissue culture medium 199 with Earle's salts was obtained from Gibco Laboratories (Grand Island, NY). Petroleum ether (bp 38 - 54.5 °C), scintiverse II scintillation solution and all other chemicals were obtained from Fisher Scientific Co. (Springfield, NJ).

Cell isolation protocol

Eight lean or obese Zucker rats were fasted 20 hours. The animals were decapitated and the abdominal area was surgically scrubbed with an iodine based surgical disinfectant and 70% ethanol. Within a few minutes the epididymal fat pads were aseptically removed and rinsed with 10 ml of medium 199 with Earle's salts. The fat pads were minced with sterile scissors until the tissue pieces were less than 5 mg (approximately 2 minutes). An approximately equal aliquot of the tissue pieces were placed into cotton plugged, sterile 25 ml polycarbonate flasks. Each flask contained 10 ml of medium 199 with Earle's salts and various levels of 3,3'-5-triiodothyronine (T_3) or insulin. All the tissue removal and isolation procedures were carried out within a laminar flow hood to assure sterile conditions. The flasks were incubated for 18 hours at 37 °C in a metabolic shaker and gassed with 95% O_2-5% CO_2.

At the end of the incubation period the mixture was filtered through a 250 μm nylon screen. The media were saved and frozen (-80 °C) in the insulin dose response study. The media were assayed to determine the amount of remaining insulin and T_3 using radioimmunoassay (RIA) procedures as described in the Amersham RIA kits. The adipose tissue pieces were placed into 50 ml polypropylene beakers and adipocytes were isolated by the collagenase digestion procedure of Rodbell (29). The collagenase digestion mixture contained 30 mg bovine serum albumin (BSA) and 1 mg collagenase/ml Krebs Ringer bicarbonate buffer (KRB), pH 7.4.

Two ml of the digestion mixture were used per gm of tissue. The beakers were placed in a gyrotory metabolic shaker at 37 °C and gassed for 1 hour with 95% CO_2-5% O_2. The digestion mixture was filtered through a 250 µm nylon screen with two KRB rinses of the screen. The crude cell suspension was centrifuged (50 X g for 1 minute), collected and washed twice by resuspension in KRB containing 3% BSA, pH 7.4. The number of isolated cells and cell viability were determined microscopically using a cell counting chamber. Exclusion of 0.6% trypan blue was used as an indication of cell viability. In all cases the cell viability was found to be greater than 93%. The volume of the cell suspension was adjusted so that it contained approximately 5 X 10^5 cells/ml. An aliquot of the cell suspension was fixed in 2,4,6-trimethylpyridine buffer containing 2% osmium tetroxide according to the method of Hirsch and Gallian (18). The fixed adipocytes were filtered consecutively through a 250 µm mesh and 25 µm mesh nylon screens. Adipocytes were then rinsed and taken up in a known volume of 0.9% NaCl-0.004% Triton X-100 for counting in a Coulter Counter Particle Size Analyzer.

Glucose utilization

Approximately 5 X 10^5 adipocytes were added to 25 ml polycarbonate flasks that contained various levels of T_3 and insulin. Measurement of glucose utilization was initiated by the addition of 5 mM D-glucose and 0.5 µCi/ml D-(U-^{14}C) glucose. The incubation flasks were capped with rubber stoppers containing suspended plastic wells with 3 cm^2 filter paper strips. After 60 minutes, 0.1 ml of 25% KOH was injected onto the

paper strips in each flask, followed immediately by the addition of 0.5 ml of 2N H_2SO_4 into the media to stop the reaction. The released CO_2 was trapped on the paper strips during an additional 1 hour incubation.

The filter paper strips were placed in scintillation vials and air dried for 2 hours. Ten ml of toluene based scintillation fluid (4 g Omnifluor, 240 ml methanol and 760 ml toluene/l) were added to the vials, and radioactivity was determined by liquid scintillation spectrometry. The radiolabeled lipids were extracted using the method of Dole and Meinertz (11) with the following modifications. Five ml of an extraction mixture (20% petroleum ether: 80% 2-propanol) were added to the contents of the flasks and shaken at 25 °C for 2 hours. The contents of the flasks were then quantitatively transferred to screwcapped glass tubes with two 5 ml rinses. Nine ml of petroleum ether and 9 ml of water were added and the tubes were mixed. After the phases separated, the upper organic phase was removed and placed in new tubes. Nine ml of water were added and the tubes were mixed and centrifuged at 500 X g at ambient temperature. The organic phase was transferred to new tubes and evaporated at 60 °C. The extracted lipids were saponified by the addition of 6 ml of ethanolic KOH (5 g KOH/100 ml of 95% ethanol) and heated at 80-85 °C for 45 minutes. Six ml of water were added and the non-saponified lipids were extracted three times with 5 ml petroleum ether. Two ml of 12.4 N HCl were added to the tubes. The fatty acids were quantitatively extracted 3 times with 5 ml petroleum ether and collected into scintillation vials and air dried.

Ten ml of scintillation fluid were added to the vials and radioactivity was determined as stated above. Radioactivity in the glycerol-glyceride moiety (the remaining aqueous fraction), was determined by placing a 2 ml sample of the aqueous phase into 15 ml of scintiverse II scintillation solution and measurement of the remaining volume of the aqueous phase.

Statistics

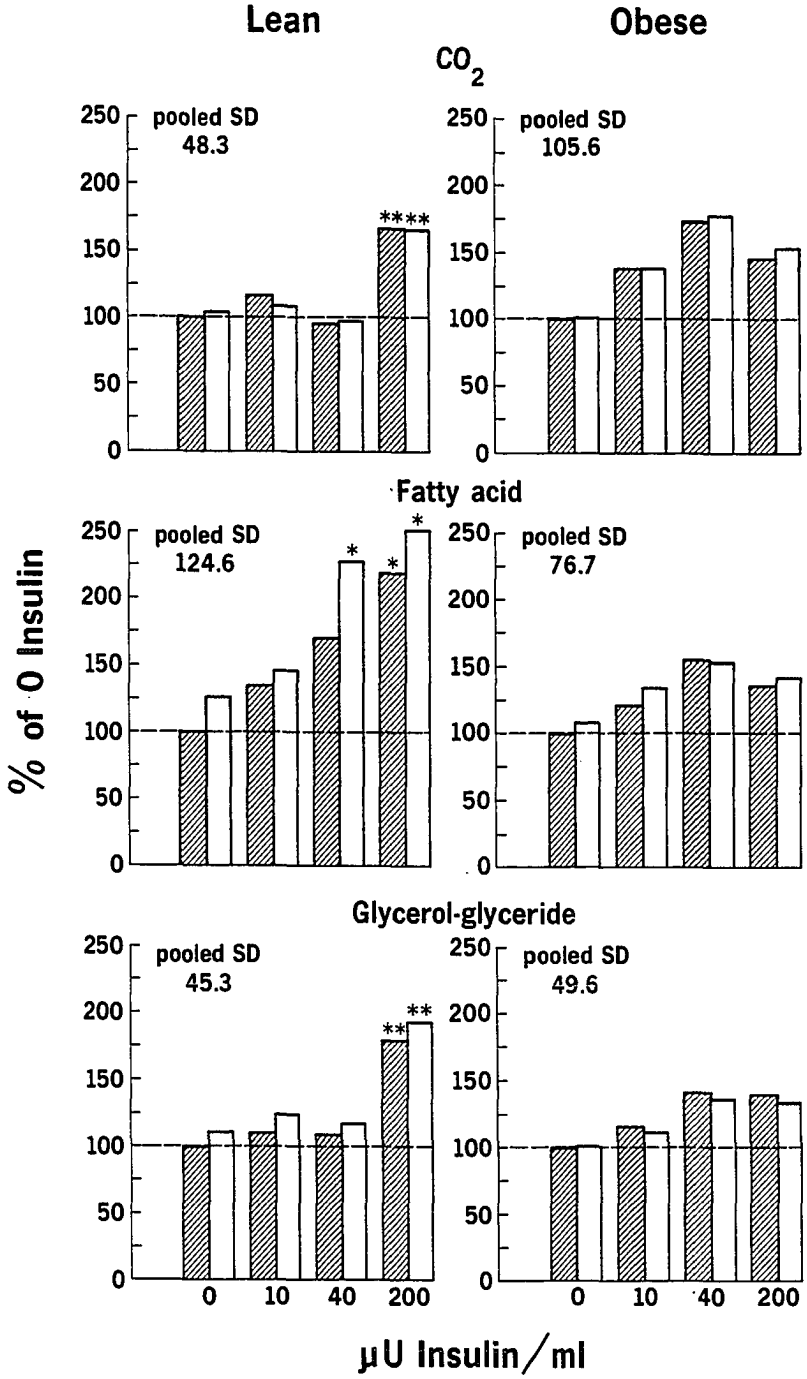
All statistical analyses were done using the Statistical Analysis System (SAS) at the Computer Center at Iowa State University. The main effects of the glucose utilization experiments including genotypes and hormone levels were analyzed by analysis of variance (ANOVA). As noted in the first paper, a large daily variability in the basal glucose utilization was evident. This was due to daily variability between adipocyte preparations. To best demonstrate the effect of T_3 on glucose utilization and to eliminate the daily variability between adipocyte preparations, the results were expressed as a percent change from zero T_3 levels within each preparation. The t test (36), using the pooled mean squared error term, was used to measure differences between the levels of T_3 used in the glucose utilization experiments. A probability of 0.05 or less was taken to be statistically significant.

RESULTS

Insulin dose response

The responses of isolated adipocytes from lean and obese adipose tissue explants to changes in media insulin concentration were similar (Fig. 1). Increasing the concentration of insulin in the media tended to increase the amount of glucose incorporated into CO_2, fatty acid and glycerol-glyceride. The addition of insulin during the 1 hour incubation did not significantly increase glucose utilization. Non-insulin stimulated glucose utilization was elevated to approximately the same levels as insulin stimulated glucose utilization. Insulin dose response studies were carried out on tissue explants in the presence of 10^{-9} M T_3 (data not shown). The results obtained were similar to those obtained in the absence of triiodothyronine (Fig. 1). The amount of T_3 remaining in the T_3 supplemented explant media after 18 hours was less than 10^{-11} M in both the lean and obese. The amount of insulin remaining after 18 hours in the insulin supplemented media was less than 10 μU/ml. This also was found when the media was supplemented with 200 μU/ml of insulin. We chose to supplement the media with 200 μU/ml of insulin in all subsequent explant studies. In the adipocytes from lean rats, this level of insulin caused a significant increase in glucose utilization compared to the absence of insulin in the incubation media (Fig. 1). The obese tissues did not show this dramatic increase in glucose utilization.

Figure 1. Effect of insulin on glucose utilization by adipocytes from lean and obese rats. Values are expressed as a percent of the 18 hour incubations without added insulin. Each value is the mean of 5 observations. Adipose tissue explants were incubated for 18 hours in media 199 with 0, 10, 40 or 200 μU insulin/ml at 37 °C. After this time, adipocytes were isolated and a 1 hour incubation with 5 mM glucose and U-[^{14}C] glucose was initiated. Insulin was either absent (dashed bars) or present (open bars) during the 1 hour incubation. Insulin, when present during the 1 hour incubation, was at 1000 μU/ml. The nmoles glucose converted to product/10^6 cells/hr for the 100% values of the various groups are: lean- 35.3 CO_2, 10.5 FA, 60.0 GG; obese- 191.9 CO_2, 258.9 FA, 181.8 GG. Incubations and extractions of metabolic products were performed as described in "Materials and Methods". Values were statistically significant from 0 added insulin: * $P<.05$, ** $P<.01$.


Lean
Obese
CO_2
pooled SD
48.3
pooled SD
105.6
Fatty acid
pooled SD
124.6
pooled SD
76.7
Glycerol-glyceride
pooled SD
45.3
pooled SD
49.6
% of 0 Insulin
250
200
150
100
50
0
****
*
**
0
10
40
200
μU Insulin/ml

18 hour effect of T_3

The effect of triiodothyronine on glucose utilization in adipose tissue explants is shown in Figure 2. These explants were maintained in the absence of insulin. After the individual adipocytes were liberated, they were subjected to the same levels of T_3, with or without insulin, for the 1 hour glucose utilization measurement. The acute presence or absence of insulin did not alter glucose utilization in the cells (Fig. 2). CO_2 production and fatty acid synthesis in lean adipocytes were significantly increased ($P<.05$) when the T_3 concentrations were 10^{-9} M and 10^{-7} M. Fatty acid production in the leans at 10^{-5} M T_3 was reduced to nearly the same level as when T_3 was absent from the media. Triiodothyronine treatment of adipocytes isolated from obese explants did not have a significant effect on fatty acid synthesis or CO_2 production. However, there was a trend towards increased fatty acid synthesis at the 10^{-5} M T_3 concentration. Glycerol-glyceride synthesis in the obese was significantly increased at the 10^{-5} M T_3 level.

18 hour effect of insulin and T_3

The effect of triiodothyronine on adipose tissue explants maintained in culture for 18 hours in the presence of insulin is shown in Figure 3. As shown previously (Figs. 1 and 2), insulin does not appear to have an acute effect on glucose utilization. CO_2 production and fatty acid synthesis from radiolabeled glucose tended to be lower at all levels of supplemented triiodothyronine when compared to non-T_3 supplemented cells. This is in opposition to the results seen when no

Figure 2. Effect of triiodothyronine on glucose utilization by adipocytes from the epididymal fat depots of lean and obese rats maintained for 18 hours without insulin. Values are expressed as a percent of the 18 hour incubations without added T_3. 100% represents [0] M T_3 level to which all other rates are relative. Each value is the mean of 8 observations. Adipose tissue explants were incubated for 18 hours in media 199 with or without T_3 in the absence of insulin at 37 °C. After this time, adipocytes were isolated and a 1 hour incubation with 5 mM glucose and U-[^{14}C] glucose was initiated. Insulin, when present during the 1 hour incubation, was at 1000 μU/ml. The nmoles glucose converted to product/10^6 cells/hr for the 100% values of the various groups are: lean (+)insulin- 46.6 CO_2, 24.5 FA, 93.2 GG; obese (+)insulin- 426.7 CO_2, 640.7 FA, 268.7 GG; lean (-)insulin- 39.3 CO_2, 17.2 FA, 79.3 GG; obese (-)insulin- 403.3 CO_2, 634.0 FA, 285.5 GG. Incubations and extractions of metabolic products were performed as described in "Materials and Methods". Values were statistically significant from [0] M T_3: * $P<.05$.

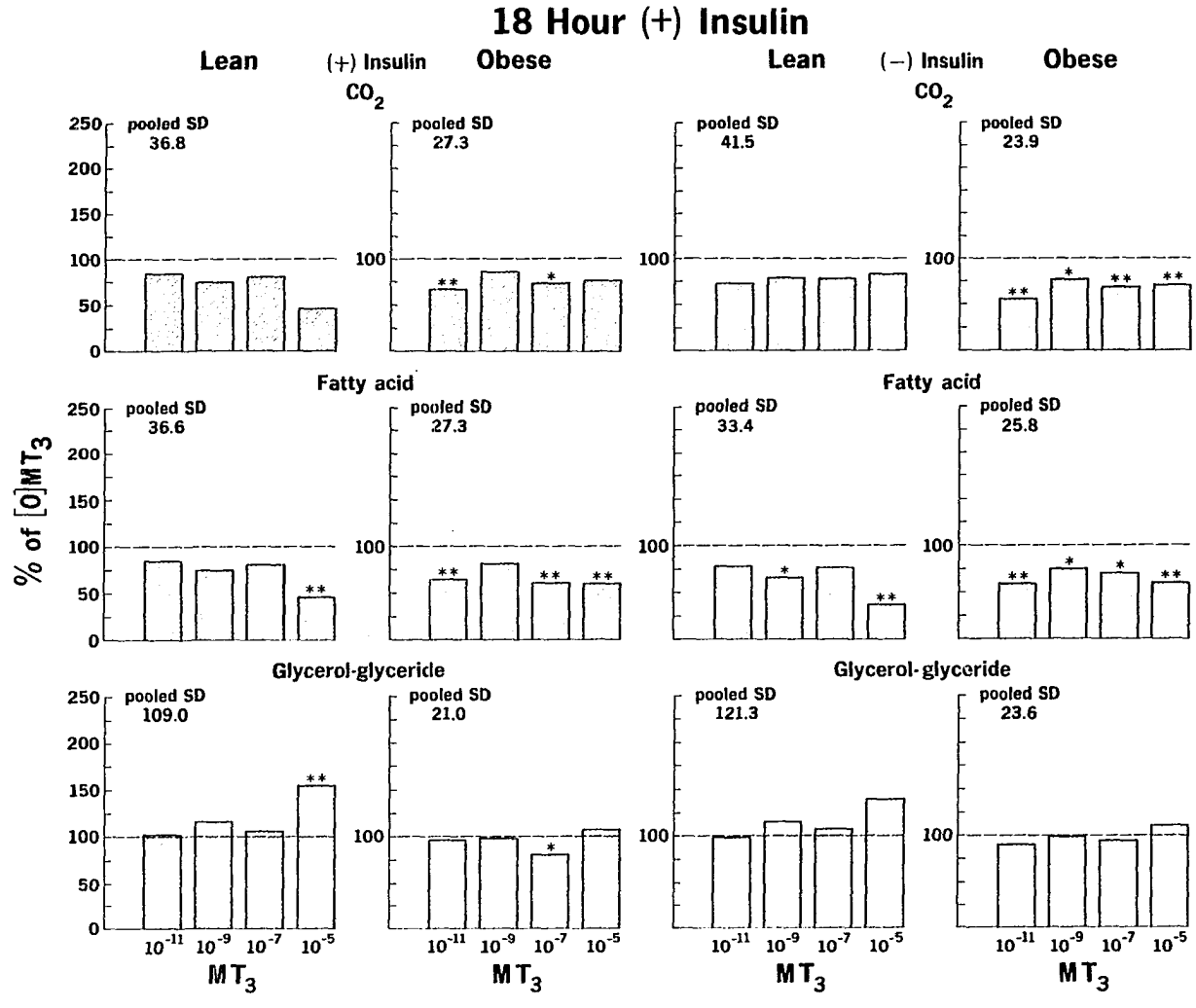
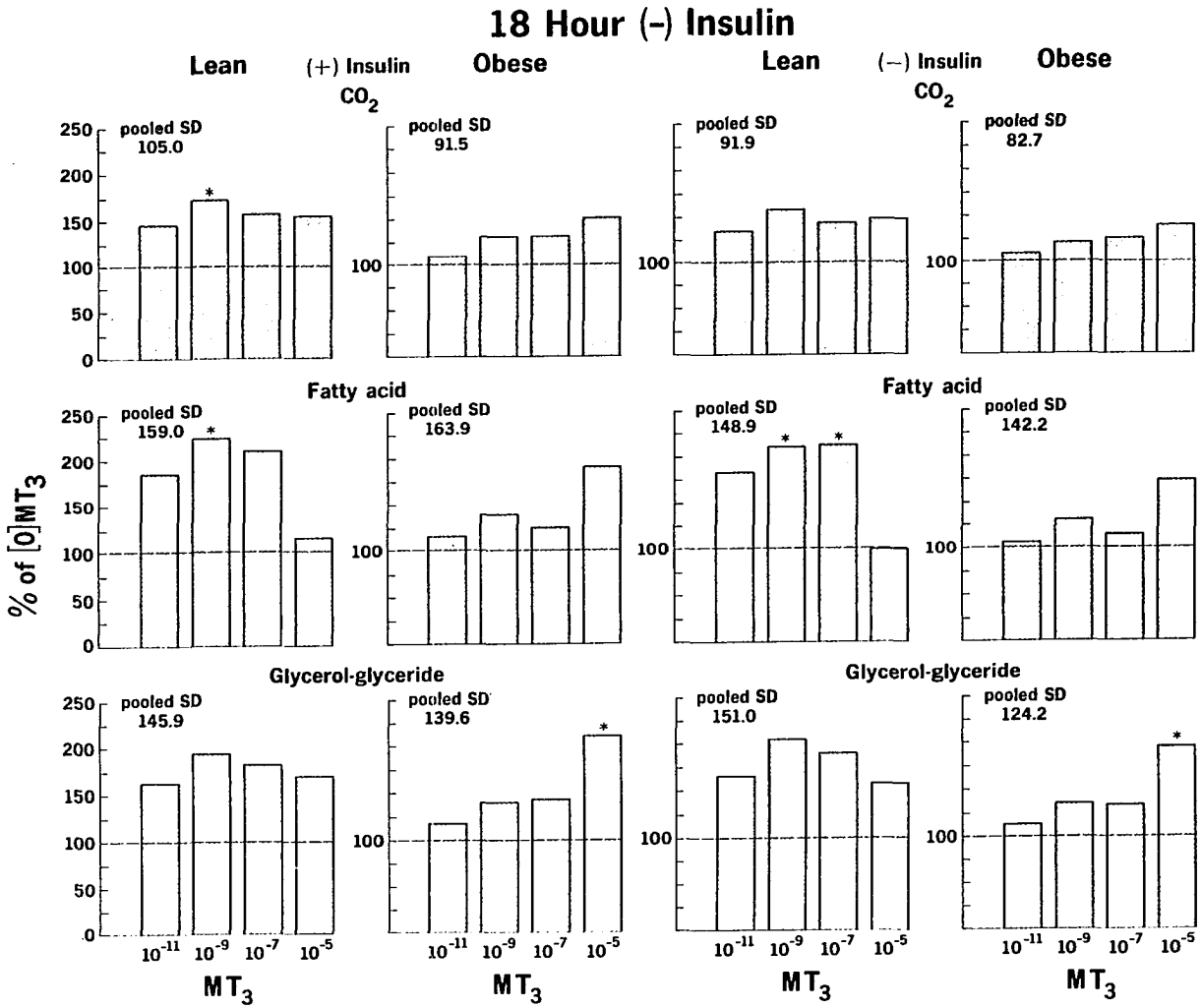

18 Hour (+) Insulin
Lean
(+) Insulin
Obese
Lean
(−) Insulin
Obese
CO2
CO2
pooled SD 36.8
pooled SD 27.3
pooled SD 41.5
pooled SD 23.9
Fatty acid
Fatty acid
pooled SD 36.6
pooled SD 27.3
pooled SD 33.4
pooled SD 25.8
Glycerol-glyceride
Glycerol-glyceride
pooled SD 109.0
pooled SD 21.0
pooled SD 121.3
pooled SD 23.6
% of [0]MT3
250
200
150
100
50
0
10-11 10-9 10-7 10-5
MT3

Figure 3. Effect of triiodothyronine on glucose utilization by adipocytes from the epididymal fat depots of lean and obese rats maintained for 18 hours with insulin. Values are expressed as a percent of the 18 hour incubations without added T_3. 100% represents [0] M T_3 level to which all other rates are relative. Each value is the mean of 6 observations. Adipose tissue explants were incubated for 18 hours in media 199 with or without T_3 in the presence of insulin (200 μU/ml) at 37 °C. After this time, adipocytes were isolated and a 1 hour incubation with 5 mM glucose and U-[^{14}C] glucose was initiated. Insulin, when present during the 1 hour incubation, was at 1000 μU/ml. The nmoles glucose converted to product/10^6 cells/hr for the 100% values of the various groups are: lean (+)insulin- 55.7 CO_2, 37.3 FA, 109.0 GG; obese (+)insulin- 343.1 CO_2, 434.2 FA, 307.4 GG; lean (-)insulin- 56.5 CO_2, 34.1 FA, 119.5 GG; obese (-)insulin- 359.6 CO_2, 399.9 FA, 312.8 GG. Incubations and extractions of metabolic products were performed as described in "Materials and Methods". Values were statistically significant from [0] M T_3: * $P<.05$, ** $P<.01$.


18 Hour (-) Insulin
Lean
(+) Insulin
Obese
Lean
(−) Insulin
Obese
CO_2
pooled SD 105.0
pooled SD 91.5
pooled SD 91.9
pooled SD 82.7
Fatty acid
pooled SD 159.0
pooled SD 163.9
pooled SD 148.9
pooled SD 142.2
Glycerol-glyceride
pooled SD 145.9
pooled SD 139.6
pooled SD 151.0
pooled SD 124.2
% of [0]MT_3
250
200
150
100
50
0
10^{-11} 10^{-9} 10^{-7} 10^{-5}
MT_3

insulin was present in the incubation media (Fig. 2). CO_2 production and fatty acid synthesis in the obese were significantly decreased at most of the levels of T_3 used. Fatty acid synthesis in the lean adipocytes was significantly ($P<.01$) reduced at 10^{-9} M and at the pharmacological level (10^{-5} M) of T_3. In contrast, glycerol-glyceride production was significantly ($P<.01$) increased at the 10^{-5} M level of T_3. Overall, the effect of triiodothyronine treatment on glucose utilization was more striking in the obese than in the lean.

DISCUSSION

Insulin stimulates lipogenesis in 6 week old lean and obese Zucker rats. Bray et al. (5) reported that insulin increased fatty acid production from radiolabeled glucose 6-fold in lean adipocytes and 2-fold in obese adipocytes. Isolated adipocytes from tissue explants maintained in culture for 18 hours are insensitive to the acute lipogenic effects of insulin (1,35). Results from this study agree with these findings. Glucose utilization in acutely isolated insulin-stimulated cells was not significantly different from that of insulin-stimulated and nonstimulated adipocytes from explants (data not shown). The non-insulin stimulated (basal) adipocytes from tissue explants had glucose utilization levels similar to those of insulin stimulated cells. One possible explanation for the "insulin-like" effect of explants maintained in culture may be the mechanical agitation of the gyrotory water bath. The "insulin-like" effect of vigorous mechanical treatment on adipocytes has been described by Vega and Kono (38). Alternatively, an unknown factor may be missing from the chemically defined media. The addition of insulin to the tissue explant maintained in culture media for 18 hours significantly increased glucose utilization (Fig. 1). The increased glucose utilization was only consistently significant at the 200 μU/ml level in the lean adipocytes. For this reason, we chose to supplement the culture media with 200 μU/ml of insulin in subsequent experiments. RIA determination of the concentration of insulin remaining in the explant media after 18 hours revealed that less than 10

μU/ml of insulin remained. Therefore, the metabolism of insulin occurs at a fairly rapid rate in adipose tissue explants.

Supplementation of the culture media with insulin did not significantly increase glucose utilization in obese adipocytes (Fig. 1). These cells appear to be insulin resistant. Results from the first paper show that acute insulin-stimulation of glucose utilization in obese adipocytes is similar to lean adipocytes. However, the magnitude of change was not as great in the obese because the basal levels of glucose utilization were elevated in the obese. Other investigators (10,25) present evidence for a maximum capacity of glucose metabolism in adipocytes. These investigators also found that increased fat cell size is accompanied by the progressive development of a marked resistance to insulin. The insulin resistance seen in the obese adipocytes (Fig. 1) indicates that the adipocyte metabolic profile is probably most influenced by the most recent hormonal environment from which the cells are isolated.

The addition of T_3 to lean adipose tissue explants maintained in media 199 increased CO_2 production and fatty acid synthesis when the T_3 concentrations were 10^{-9} and 10^{-7} M. Further increases in the T_3 concentration decreased fatty acid synthesis. The high level of media T_3 concentration (10^{-5} M) may increase the intracellular concentration of this hormone. This increased intracellular concentration of T_3 may act to slightly retard fatty acid synthesis in euthyroid adipocytes. Lean Zucker rats are euthyroid and obese Zucker rats are considered

hypothyroid (6,12). Glucose utilization in adipocytes isolated from obese rat explants was unaltered except at the 10^{-5} M T_3 concentration. Glycerol-glyceride production was significantly increased and fatty acid synthesis tended to increase. This is an indication that the obese adipose tissue may be T_3 resistant. The 10^{-5} M level of T_3 may increase intracellular levels of T_3 in the tissue of the hypothyroid obese rat. This may cause the intracellular concentration of thyroid hormone to approach normal with a concomitant rise in glucose utilization.

The addition of T_3 concomitant with insulin to the tissue explant media tended to decrease CO_2 and fatty acid formation from radiolabeled glucose (Fig. 3). This effect was more striking in the obese than in the lean adipocytes. This is exactly opposite to the findings obtained when insulin was absent from the incubation media (Fig. 2). Glycerol-glyceride formation was significantly ($P<.01$) increased at 10^{-5} M T_3 in adipocytes isolated from lean explants. In these same cells, fatty acid production was significantly ($P<.01$) decreased and CO_2 production was decreased approximately 50%. Therefore, it appears that when T_3 is present at pharmacological levels (10^{-5} M) in the presence of insulin, lipogenesis is retarded in lean adipocytes and reesterification of triglyceride turnover may be accelerated. When the T_3 concentration is 10^{-9} M in the absence of acute insulin, this same trend is present. The effect of thyroid hormone on epinephrine-stimulated lipolysis in adipose tissue is well documented (16,30), enhanced in hyperthyroidism and markedly reduced or abolished in hypothyroidism. Correze et al. (9) and

van Inwegen et al. (37) showed that fat cells from thyroidectomized rats lose their lipolytic capacity. These same cells had an increase in lipogenesis. Results from the experiments presented in this paper agree with those investigators in that T_3 appears to affect lipogenic and lipolytic pathways.

Adipose tissue explants provide a good alternative to cell culture and isolated cell suspensions to study the effects of changes in the biochemical environment on adipose tissue metabolism. Isolated cell suspensions are only viable for a few hours, and under these circumstances the environment that the cells were subjected to in the animal may profoundly influence the results. Experiments using cell culture are disadvantaged in that these studies only involve one cell type. Adipose tissue contains a variety of cell types, including endothelial cells, fibroblasts, mast cells and other connective tissue elements. The acute effects of insulin on glucose utilization are not seen in explants because the unstimulated or basal cells have glucose utilization rates similar to insulin stimulated rates. This is probably due to a change in the sugar transporters which causes an increase in sugar permeability. In this study, insulin appears to modulate the effect of thyroid hormone on glucose utilization. Thyroid hormone increases glucose utilization in adipose tissue explants maintained in culture for 18 hours. In terms of the low T_3 status of the obese Zucker rat, when insulin and thyroid hormone are both present at physiological through pharmacological levels in the explant media, fatty acid

synthesis is decreased and reesterification of triglycerides may be accelerated.

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GENERAL DISCUSSION AND CONCLUSIONS

Triiodothyronine (T_3) was shown to have an acute and chronic effect on glucose metabolism in adipocytes. Insulin modulated the action of T_3. T_3 does not have an effect on glucose metabolism when adipocytes are subjected to T_3 for 1/2 hour in the absence of insulin. In lean adipocytes treated with insulin, pharmacological levels of T_3 decreased lipogenesis and physiological levels increased lipogenesis in obese adipocytes. A longer pretreatment of adipocytes with T_3 further exemplifies the importance of the insulin status of the cells as well as the hormonal environment from which the cells are isolated on glucose metabolism. In the presence of insulin, T_3 decreased lipogenesis in both the lean and obese adipocytes. When insulin was absent during the 2 hour T_3 treatment, lipogenesis was increased in the lean adipocytes at physiological concentrations and increased at pharmacological concentrations in the obese. This indicates that the cells from the obese rats (hypothyroid) are thyroid hormone resistant. The effects of thyroid hormone on glucose metabolism appear to be postreceptor, as glucose transport was uneffected by T_3 treatment. Adipose tissue explants maintained in culture for 18 hours also showed the variability of the effect of T_3 on glucose metabolism depending upon the insulin status of the media and the environment from which the cells were obtained.

Hypothyroidism is associated with obesity and diabetes. The importance of this defect to the etiology of these disease states is as

yet unanswered. Adipose tissue is a major tissue involved in these disease states. This dissertation has furthered the understanding of the action of thyroid hormone and the interaction of thyroid hormone and insulin on adipose tissue metabolism. In addition, because of this work, future investigators may be able to elucidate the role of these hormones in obesity and other associated disease states.

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