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I am submitting herewith a thesis written by Ola Abdelmoniem Mostafa entitled " Lipodysregulation and Type 2 diabetes in TALLYHO/Jng Mice." I have examined the final electronic copy of this thesis for form and content and recommended that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Nutrition.

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Lipodysregulation and Type 2 diabetes in TALLYHO/Jng Mice

A Thesis
Presented for the
Master of Science Degree
The University of Tennessee, Knoxville

Ola Abdel Moniem Mostafa
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DEDICATION

This thesis is dedicated to my mother, Afaf Eldeeb, who inspired me all the time, and to my late father, Dr. Abdelmoniem Mostafa, who devoted his life to his children, and always encouraged them to achieve the best education. It is also dedicated to my husband Dr. Essam Laag for helping and supporting me throughout my studies, and to my wonderful children Mostafa, Abdelrahman& Salma.

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I have been truly blessed by having you all in my life.

Abstract

The prevalence of reported diabetes was 2.9 times higher in overweight than in non-overweight persons as proved by the National Health and Nutrition Examination Survey (NHANES III) data. The majority of individuals with type 2 diabetes develop insulin resistance associated with overall obesity. Several lines of evidence indicate that many of the metabolic derangements associated with obesity and type 2 diabetes originate from dysregulation of lipid metabolism.

TALLYHO/Jng (TH) mice are a newly established inbred polygenic model for obesity and type 2 diabetes. These mice are characterized by insulin resistance, hyperinsulinemia, diabetes (males) obesity, and dyslipidemia including hypertriglyceridemia (HTG). TH male mice rapidly develop HTG at a very young age before the onset of overt diabetes, and this HTG is accompanied by hyperinsulinemia, and preceded glucose intolerance.

The goal of this study is to characterize the lipodysregulation in TH mouse. In order to achieve this goal, we measured tissue triglyceride content in non-adipose tissue, and hepatic fatty acid β-oxidation rates in diabetic TH mice. TH male mice exhibit significantly elevated TG content and lipid accumulation in skeletal muscle, liver, and kidney. Attenuated hepatic fatty acid oxidation was also featured in male diabetic TH mice although it was not statistically significant. It is likely speculated that impaired fatty acid oxidation in liver may be in part responsible for the HTG, elevating TG content in peripheral tissue including the skeletal muscle, which may be attributable to the impaired insulin sensitivity and diabetes in male TH mice. Elucidating the mechanism of lipodysregulation in diabetic model of TH mice will provide insights into the causes and mechanisms underlying the metabolic complications of insulin resistance and diabetes and ultimately improved therapeutic strategies for prevention and treatment of type 2 diabetes.

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Nomenclature

Abbreviations

BMI	Body mass index [weight (kg)/hight $(m)^2$]
B6	C57BL/6J
CAD	Coronary artery disease
CPT1	Carnitine palmitoyl transferase 1
DAG	Diacyl glycerol
FFA	Free Fatty acid
Glut 4	Glucose transporter 4
HK	Hexokinase
HDL	High density lipoprotein
HTG	Hypertriglyceridemia
IMCL	Intramyocellular lipid
IRS	Insulin receptor substrate.
LDL	Low density lipoprotein
MRS	Magnetic resonance spectroscopy
NEFFN	Non estrified fatty acids
PDH	Pyruvate dehydrogenase
PKC	Protein kinase C
PPAR	Peroxisome proliferator activated receptor.
sdLDL	Small dense low density lipoproteins
SNS	Sympathetic nervous system
TG	Triglyceride
TH	TALLYHO/Jng
T2DM	Type 2 diabetes mellitus
TZDs	Thiazolidinediones
VLDL	Very low density lipoproteins

Chapter I: Introduction

The World Health Organization referred to the increased prevalence of obesity and diabetes as the 21st century epidemic (1). Obesity is the most common metabolic disease worldwide (2). More over, its incidence and prevalence are rising rapidly (3). The global incidence of type 2 diabetes mellitus (T2DM) is projected to be nearly 300 million people by the year 2025, and many of those affected will be young adults. Successful strategies to halt this epidemic will require a better mechanistic understanding of how the disease arises (4). Nearly all individuals with T2DM are insulin resistant, and the majority of them are obese (5). An increase in overall fatness, specifically of visceral as well as ectopic fat depot, is associated with insulin resistance (6). Over the last decade, major advances have been made to understand the pathophsiology and molecular biology of T2DM (7,8). T2DM is a bipolar disease where both insulin secretion and insulin action are defective. This complex interaction leads to a progressive increase of plasma glucose levels (9). It is also well established that the development of T2DM results from an interaction between subjects' genetic makeup and their environment, and that with the increasing prevalence of obesity, the prevalence T2DM is reaching epidemic proportions (10). Various organs play a crucial role in the pathophysiology of T2DM. Disruption of the cross talks between endocrine pancreas, liver, skeletal muscle, adipose tissue, and presumably gut and central nervous system may lead to impairment of homeostasis and T2DM (11,12).

Although most patients with T2DM are overweight or obese (10), the role of fat was initially neglected in the pathophysiology of the disease (11). The role of fat,

especially the interaction of non-esterified fatty acids with glucose metabolism started to be highlighted almost a decade ago (12). The crucial impact of fat distribution, especially the negative influence of intra-abdominal or visceral fat depot is now better recognized (13). The concept of lipotoxicity has developed recently, where the deleterious role of ectopic triglyceride storage in the development of defective insulin action and insulin secretion has been emphasized (14). In addition, adipose tissue can secrete adipokines that may affect glucose metabolism and insulin sensitivity, such as leptin, tumor necrosis factor (TNF)-α, resistin, and adiponectin (15).

The ectopic fat storage hypothesis states that if fat mass can not expand through proliferation and differentiation of adipocytes in times of positive energy balance, then adipocyte hypertrophy results, and the excess dietary fat will be shunted to the liver, skeletal muscles, and pancreatic β-cells. If fat oxidation capacity cannot be increased to compensate for the increased influx of lipids within these tissues, then intracellular accumulation of lipids occurs (16).

In obesity and T2DM, the lipid content within and around muscle fibers is increased. Changes in muscle in fuel partitioning of lipid between oxidation and storage of fat calories, contributes to the accumulation of intramyocellulr triglyceride (IMTG) and to the pathogenesis of both obesity and T2DM (17).

The prevalence of hepatosteatosis (increased lipid accumulation in the liver) is increased in T2DM. A recent clinical investigation on patients with T2DM treated with insulin, indicated that hepatic triglyceride content is a strong determinant of hepatic insulin resistance (5).

Various lipid disorders appear to be related to both diabetes mellitus and to insulin resistance. A high triglyceride and a low high-density lipoprotein (HDL) cholesterol level appear usually to coincide in individuals with diabetes and insulin resistance. These disorders are at least twice as prevalent as among other individuals (18).

The medical and socioeconomic burden of T2DM is caused by the associated complications (19,20), which impose enormous strains on health-care systems (Figure1). The incremental costs of patients with T2DM arise at least 8 years earlier before the diagnosis of the disease is established (21). The devastating complications of diabetes mellitus are mostly macrovascular and microvascular diseases as a consequence of accelerated atherogenesis. Cardiovascular morbidity in patients with T2DM is two to four times greater than that of non-diabetic population (22).

Animal models have been used as an adjunct to human studies to minimize difficulties encountered while studying obesity and T2DM in humans, largely by the capability of genetic and environmental controls (23-27). In addition, rodents and humans share biological and physiological characteristics, so it is possible to apply information discovered in rodents to humans (28,29).

TALLYHO/Jng (TH) mice are a newly established inbred polygenic model for obesity and type 2 diabetes. These mice are characterized by insulin resistance, hyperinsulinemia, diabetes (males) obesity, and dyslipidemia including hypertriglyceridemia (HTG). TH male mice rapidly develop HTG at a very young age before the onset of overt diabetes, and this HTG is accompanied by hyperinsulinemia,

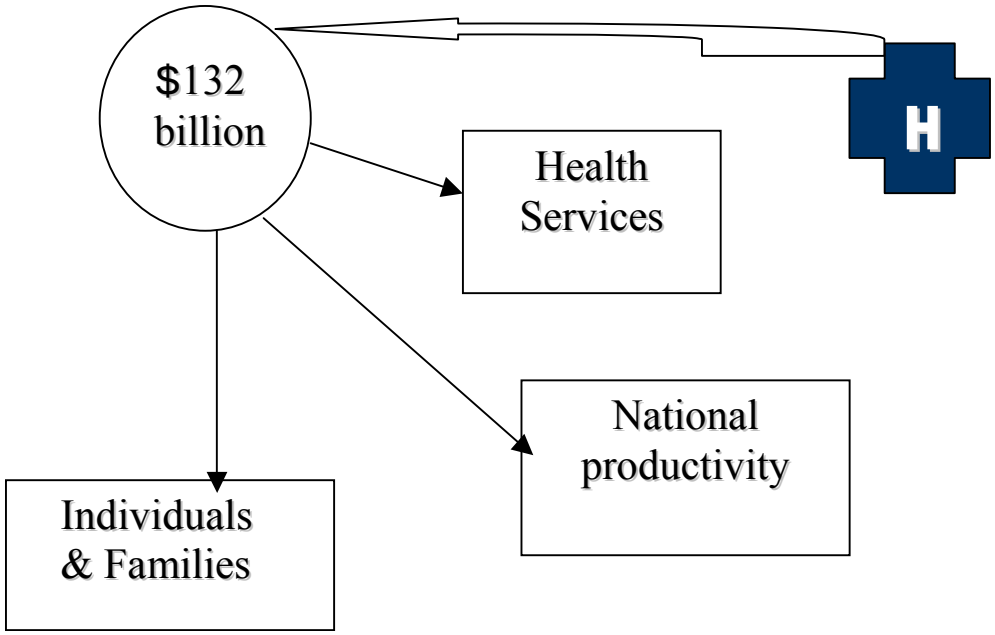

Figure 1: Economic impact of diabetes:
The cost of diabetes affects health services, national productivity as well as individuals and families. Hospital in- patient costs for treatment of complications are the largest single contributor to direct health care costs.

and preceded glucose intolerance.

The goal of this study is to characterize the lipodysregulation in TH mouse. Studying the pathophysiological mechanisms of lipodysregulation in TH mice will lead to better understanding of the disease process and will provide a framework that explains the links between lipid and glucose metabolism. It may as well lead to the development of new pharmacological strategies to treat T2DM

Chapter II: Literature Review

1) Obesity and type 2 diabetes

The prevalence of obesity [defined as a body mass index (BMI) above 30] is increasing rapidly in many countries, almost reaching epidemic proportions in some areas (30). The high prevalence of obesity has led to an increase in the co-morbid conditions of obesity especially type 2 diabetes (also known as non insulin dependent diabetes mellitus), hypertension, cardiovascular disease, and certain cancers (31,32). Type 2 diabetes is the most common metabolic disease in the world. In the United States, it is the leading cause of blindness, end-stage renal disease, and limb amputation, with associated health care costs estimated to exceed $130 billion per year (33).

Type 2 diabetes is a heterogeneous syndrome characterized by abnormalities of carbohydrate and fat metabolism. The causes of type 2 diabetes are multifactorial and include both genetic and environmental elements that affect beta cell function and various tissue (muscle, liver, adipose tissue, and pancreas) insulin sensitivity (34).

Positive energy balance in our obesigenic environment (where there is increased availability and over-consumption of foods high in energy, and a concomitant decline in activity dependent energy expenditure) leads to excess lipid storage in liver (35) and skeletal muscles (36,37) followed by insulin resistance, glucose intolerance and diabetes. When energy intake is greater than energy expenditure, excess dietary fat will lead to adipocyte hypertrophy, and fat storage in ectopic sites such as liver, skeletal muscle, and the pancreatic beta cells. Ectopic lipid decreases insulin action leading to hyperinsulinemia and predisposing to type 2 diabetes (38). It has been observed by using

magnetic imaging techniques such as computed tomography and magnetic resonance imaging that visceral fat accumulation is specifically associated with metabolic alteration of obesity, both in men and women (39-42). It was also noticed that obese women with greater proportion of upper body fat are more likely to have dyslipidemia, hyperinsulinemia, and glucose intolerance than obese women with a greater proportion of lower body fat (43).

2) Regulation of blood glucose level

According to the American Diabetes Association (175), criteria for the diagnosis of diabetes are shown in Table 1.

Table 1: Criteria for the diagnosis of diabetes.

Normoglycemia	**IFG or IGT**	**Diabetes**
FPG <100 mg/dl	FPG ≥ 100 and < 126 mg/dl (IFG)	FPG ≥126 mg/dl
2-h PG <140 mg/dl	2-h PG ≥140 and < 200 mg/dl (IGT)	2-h PG ≥ 200 mg/dl Symptoms of diabetes and causal PG ≥ 200 mg/dl

FPG; Fasting Plasma glucose, IFG; Impaired fasting glucose, IGT; Impaired glucose tolerance; 2-h PG two hours postload glucose: This test requires use of a glucose load containing the equivalent of 75 g anhydrous glucose dissolved in water.

Maintenance of normal blood glucose levels depends on a complex interaction between the insulin responsiveness of skeletal muscle and liver and glucose stimulated insulin secretion by pancreatic β-cells. Defects in the former are responsible for insulin resistance, and defects in the latter are responsible for progression to hyperglycemia (44).

Insulin signaling:

Insulin is the most potent anabolic hormone known. It promotes the synthesis of carbohydrates, fats, and lipids, and inhibits their degradation and release back into the Circulation (45). The insulin receptor is a tyrosine kinase that undergoes autophosphorylation upon insulin binding, and catalyzes the phosphorylation of several intracellular substrates including the insulin receptor substrate (IRS1 - IRS4) proteins, GAB-1, Sch (46), APS, P60 DOK, SIRPS, and c-Cb1 (47-52). Upon tyrosine phosphorylation, each of these subunits interacts with a series of signaling proteins containing Src-homology 2 (SH2) domains leading to initiation of different signaling pathways. Each of these pathways plays a separate role in the different cellular effects of insulin (Figure2). Initiation of these signaling pathways stimulates the trafficking of Glut 4 vesicles, their docking, and their fusion with the plasma membrane, and enhance glucose uptake by the cells (53).

3) Insulin resistance

Insulin resistance is a state of reduced responsiveness to normal circulating levels of insulin (54). It is an early feature of type 2 diabetes as indicated by the following: First, virtually all patients with type 2 diabetes are insulin resistant, and prospective studies have shown that this insulin resistant state develops 1-2 decades before the onset

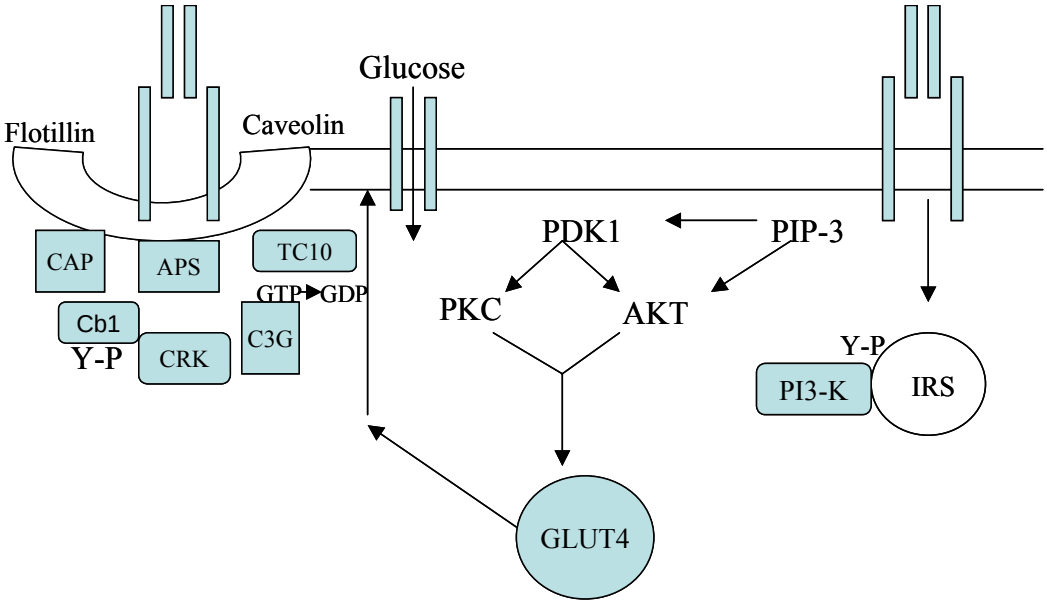

Figure 2: Insulin signaling pathway: A model for diverse signaling pathways in insulin action. Two signaling pathways are required for the translocation of the glucose transporter Glut 4 by insulin in fat and muscle cells. Tyrosine phosphorylation (Y-P) of the insulin receptor substrate (IRS) proteins after insulin stimulation leads to an interaction with and subsequent activation of the Src-homology 2 (SH2)-domain-containing protein phosphatidylinositol 3-kinase (PI3-K), producing the polyphosphoinositide phosphatidylinositol (3,4,5)-trisphosphate (PIP3), which in turn interacts with and localizes protein kinases such as phosphoinositide-dependent kinase 1 (PDK1). These kinases then initiate a cascade of phosphorylation events, resulting in the activation of Akt and/or atypical protein kinase C (PKC). A separate pool of the insulin receptor can also phosphorylate the substrates Cbl and APS. Cbl interacts with Cbl-associated protein (CAP), which can bind to the lipid raft protein flotillin. This interaction recruits phosphorylated Cbl into the lipid raft, resulting in the recruitment of the SH2/SH3 adaptor protein CrkII through an interaction of the SH2 domain of CrkII with phosphorylated Cbl. CrkII binds constitutively to the GDP–GTP factor C3G, which can catalyze the exchange of GTP for GDP on the lipid-raft-associated protein TC10. Upon activation, TC10 interacts with one or more effectors to initiate a separate signaling pathway that, along with the PIP3-dependent protein kinases, can stimulate the trafficking of Glut4 vesicles, their docking and their fusion with the plasma membrane (53).

of the disease (55-57). Second, insulin resistance in the offspring of parents with type 2 diabetes is the best predictor for later development of the disease (58). Lastly, perturbations that reduce insulin resistance prevent the development of diabetes (59).

Insulin resistance is said to be present when the biological effects of insulin are less than expected for both glucose disposal in skeletal muscle and suppression of endogenous glucose production primarily in the liver (60). In the fasting state, however, muscle accounts for only a small proportion of glucose disposal (less than 20%) whereas endogenous glucose production is responsible for all the glucose entering the plasma. Endogenous glucose production is increased in patients with type 2 diabetes or impaired fasting glucose (61,62). Because this increase occurs in the presence of hyperinsulinemia, at least in the early and intermediate disease stages, hepatic insulin resistance is the driving force of hyperglycaemia of type 2 diabetes (Figure 3).

Skeletal muscle and liver are the two key insulin-responsive organs responsible for maintaining normal glucose homeostasis, and their transition to an insulin resistant state accounts for most of the alterations in glucose metabolism seen in patients with type 2 diabetes (44).

Pathophysiology of hyperglycemia

In order to understand the cellular and molecular mechanisms responsible for development of type 2 diabetes, it is necessary to determine how glycemia is controlled. Insulin is the key hormone for regulation of blood glucose and, generally, normoglycemia is maintained by the balanced interplay between insulin action and insulin secretion.

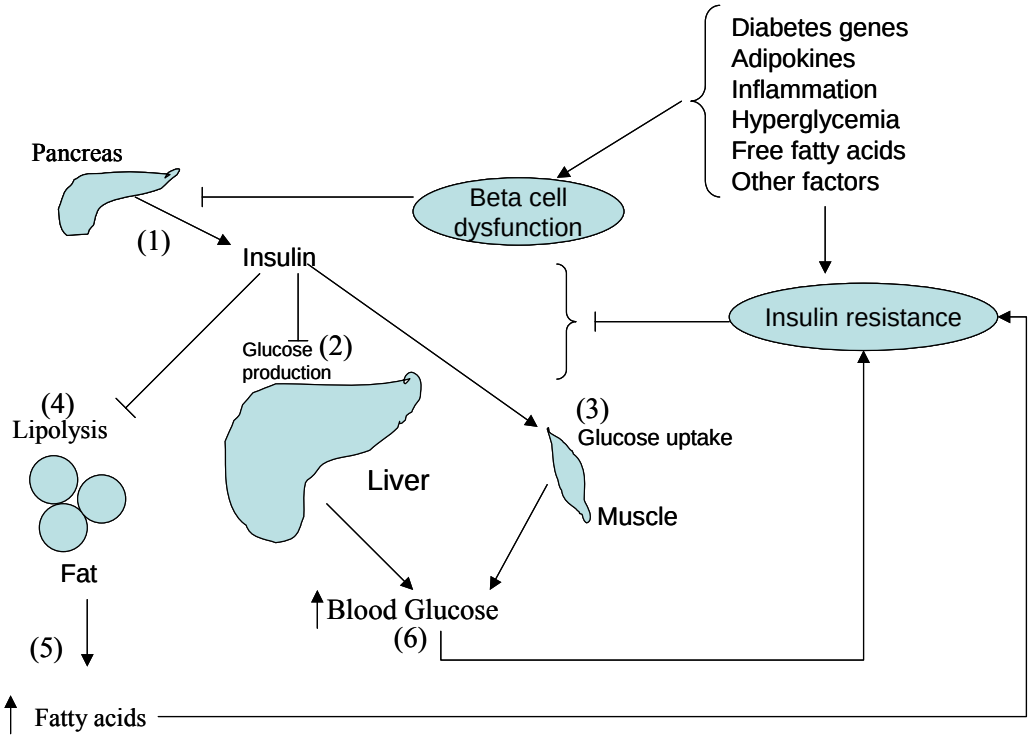

Figure 3: Pathophysiology of hyperglycemia and increased fatty acids in type 2 diabetes: Insulin secretion from the pancreas (1) normally reduces glucose output by the liver (2), enhances glucose uptake by skeletal muscle (3), and suppresses fatty acid release from fat tissue (4). The various factors shown that contribute to the pathogenesis of type 2 diabetes affect both insulin secretion and insulin action. Decreased insulin secretion will reduce insulin signaling in its target tissues. Insulin resistance pathways affect the action of insulin in each of the major target tissues, leading to increased circulating fatty acids and the hyperglycaemia of diabetes. In turn, the raised concentrations of glucose and fatty acids in the bloodstream will feed back to worsen both insulin secretion and insulin resistance. (Adapted from (22)).

Importantly, the normal pancreatic β-cell can adapt to changes in insulin action i.e., a decrease in insulin action is accompanied by up-regulation of insulin secretion (and vice versa) (63).

Deviation from this balanced mechanism, such as in the patients with impaired glucose tolerance and type 2 diabetes, occurs when β-cell function is inadequately low for a specific degree of insulin sensitivity. Thus, β-cell dysfunction is a critical component in the pathogenesis of type 2 diabetes. This concept has been verified in cross- sectional as well as longitudinal studies in Pima Indians progressing from normal to impaired glucose tolerance to type 2 diabetes (64), when insulin action decreases (as with increasing obesity) the system usually compensates by increasing β-cell function. However, at the Same time, concentrations of blood glucose at fasting and 2 hours after glucose load will increase mildly (64). This increase may first be small, but over time becomes damaging because of glucose toxicity, and may itself cause β-cell dysfunction. Thus, even with (theoretically) unlimited β-cell reserve, insulin resistance paves the way for hyperglycemia and type 2 diabetes (22)**.**

4) Fatty acid metabolism

When fuel molecules are available in amounts greater than energy needs, fatty acids are synthesized, esterified to glycerol and stored as triglycerides (TG) in adipose tissue. On the other hand, when there is an energy need, stored fatty acids must be released from triglycerol and transported to the mitochondria of peripheral tissues, where they are degraded. Catabolism of fatty acids is brought about by β-oxidation, a four-step process yielding acetyl CoA, which feeds into aerobic metabolism at the citric acid cycle (Figure 4).

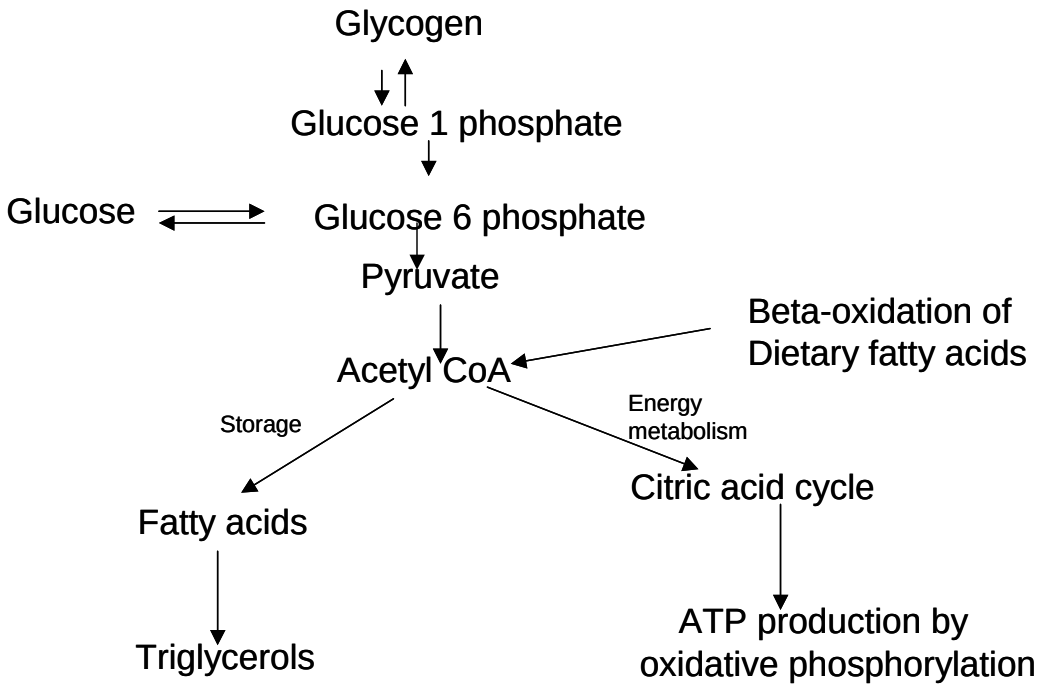

Figure 4: The linkage between carbohydrate and fatty acid metabolism. Actyl CoA is produced from β-oxidation of dietary fatty acids and from glycolysis plus pyruvate oxidation. Excess acetyl CoA may be used to make Fatty acids stored in triglycerols.

β-oxidation begins in the mitochondrial matrix after the entry of fatty acids. Carnitine acyl transferase II catalyses the final steps in the transport by linking the fatty acids to mitochondrial CoASH. β-oxidation of fatty acyl CoA takes place by recurring series of four steps:

1)Oxidation of the carbon-carbon single bond by FAD to form carbon-carbon double bond.

2) Addition of H_2O to the double bond, with the formation of a hydroxyl group on one carbon.

3) Oxidation of the hydroxyl group by NAD+ to produce a ketogroup

4) Carbon-carbon bond cleavage, releasing acetyl CoA.

Significance of β-oxidation

β-oxidation products are of a great metabolic importance. The net reaction for beta-oxidation of palmitic acid is:

Palmitic acid + ATP + 7 FAD + 7 NAD + 8 CoASH + 8 H_2O→ 8 acetyl SCoA + AMP+ 2 Pi + 7 $FADH_2$ + 7 NADH +7 H^+

The acetyl CoA is ready for further oxidation by the citric acid cycle. The enzymes needed for citric acid cycle are present in the mitochondrial matrix, the same location as the enzymes for β-oxidation.

The complete degradation of palmitoyl CoA requires seven turns of β-oxidation, and each round requires the entry of FAD, NAD, H_2O, and CoASH. The complete oxidation of palmitate to CO_2 and H_2O yields 129 ATP

5) Dyslipidemia and insulin resistance

Research has established healthy ranges for lipoprotein profiles. According to American Heart Association (65), table (2) illustrates the initial classification for the lipid profile.

Patients with DM, particularly those with type 2 diabetes, have characteristic abnormalities of plasma lipids and lipoprotein concentrations that almost certainly play a significant role in the increased risk for coronary artery disease (CAD). This dyslipidemia is characterized by higher plasma (TG) levels, both in fasting and the postprandial state, reduced levels of high density lipoprotein (HDL) cholesterol, and abnormal low density lipoprotein (LDL) particles. Plasma free fatty acid concentrations are increased in many insulin resistant states, including obesity and type 2 diabetes mellitus (66).

More recently, the concept of lipotoxicity involving the β cell has been put forward. Generally, in both non-diabetic and diabetic obese patients, non estrified fatty acids (NEFA) concentrations are raised as a result of enhanced adipocyte lipolysis. Fatty acids lead to enhanced insulin secretion in acute studies, but after 24 hours they actually inhibit insulin secretion (22).

Free fatty acids are insulinotropic (67), and this action is an important mechanism to protect against hypoinsulinemia during fasting, when glucose; the primary stimulant for pancreatic insulin secretion is not available. The availability of FFA, derived from

Table 2: Blood total cholesterol, HDL cholesterol, LDL cholesterol, and TG level classification (from American Heart Association) (65).

Total cholesterol	**Category**
< 200 mg/dl	Desirable level
200 to 239 mg/dl	Borderline high
240 mg/dl and above	High. More than twice the risk of Coronary heart disease
HDL cholesterol	
< 40mg/dl (for men) < 50 mg/dl (for women)	Low HDL. A major risk for heart disease.
60 mg/dl and above	High HDL. Considered protective against heart disease
LDL cholesterol	
< 100 mg/dl	Optimal
100-129 mg/dl	Near or above optimal
130-159 mg/dl	Borderline high
160-189 mg/dl	High
190 mg/dl and above	Very high
Triglyceride	
<150 mg/dl	Normal
150-199 mg/dl	Borderline high
200-499 mg/dl	High
500 mg/dl and above	Very high

HDL: High density lipoprotein, LDL: Low density lipoprotein

adipose tissue, ensures the secretion of a minimal level of insulin that is essential for prevention of unrestrained free fatty acid output from adipose tissue (68-70). Excess inflow of FFA and accumulation of TG in β-cells have a negative effect on β-cell function. The increased secretion pressure results in beta cell deterioration up to apoptosis (71). Understanding the pathophysiology underlying this dyslipidemia is crucial to optimizing therapy in patients with diabetes (72).

Mechanism of fatty acid induced insulin resistance

In 1963, the Randle hypothesis stated that fatty acids caused insulin resistance by a substrate competition mechanism (73,74). According to this hypothesis, increased fatty acid oxidation in muscles will lead to high production of intracellular acetyl CoA and citrate, which in turn inhibit 2 enzymes involved in glucose utilization (44). These enzymes are pyruvate dehydrogenase and phosphofructokinase (Figure 5). This in turn leads to increased intracellular glucose and glucose 6 phosphate concentration, which inhibits hexokinase and results in reduced insulin stimulated glucose uptake and oxidation (44).

Recent studies indicated that this mechanism for fatty acid induced insulin resistance does not apply in human skeletal muscles (75). These studies proposed that fatty acids cause insulin resistance by inhibiting insulin stimulated glucose transport activity through accumulation of intracellular fatty acyl CoA, and diacyl glycerol, and possibly ceramides. This accumulation leads to activation of critical signal transduction

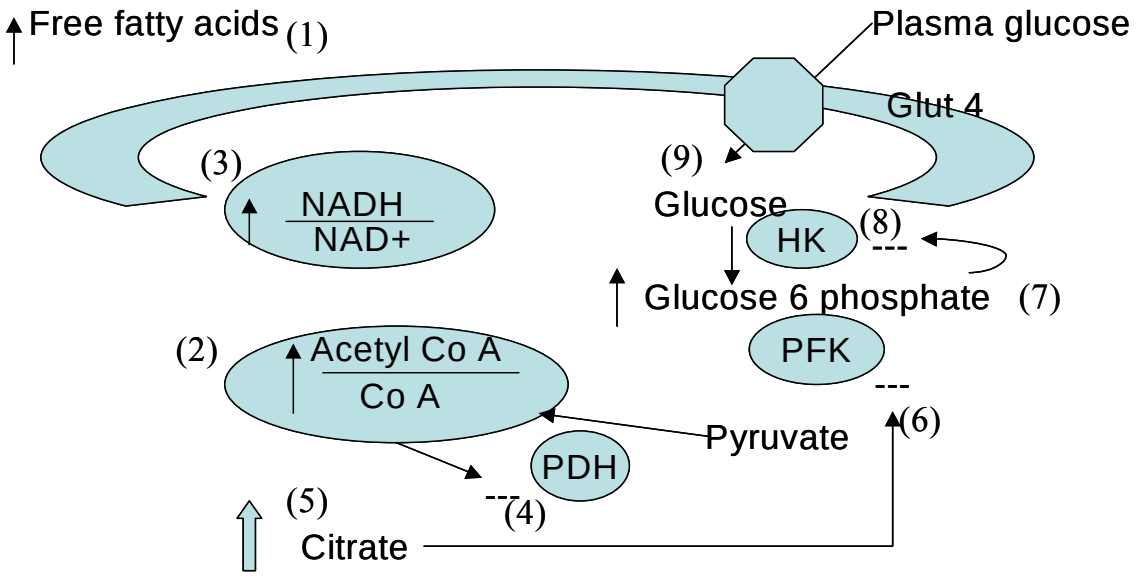

Figure 5: **Mechanism of free fatty acid induced insulin resistance in skeletal muscle. The Randle cycle**. The increased FFA concentration (1) results in an elevation of the intra-mitochondrial Acetyl CoA/Co A (2) and NADH/NAD+ (3) ratio, with subsequent inactivation of PDH (4).This in turn causes citrate concentration to increase (5), leading to inhibition of PFK (6). Subsequent increase in intracellular G6P concentration (7) will inhibit HK activity (8), which results in an increase in intracellular glucose concentratio(9), and decrease in muscle glucose uptake.Glut 4, glucose transporter 4; HK, hexokinase; G6p, glucose 6 phosphate; PFK, phosphofructokinase; PDH, pyruvate dehydrogenase;

pathways that ultimately suppress insulin signaling (76) (Figure 6). The proposed mechanism is as follows:

Fatty acid metabolites (long chain acyl CoA and diacyl glycerol) accumulate inside the cells because of increased fatty acid delivery or decreased mitochondrial β- oxidation, trigger serine/threonine kinase cascade (possibly involving new protein kinase C). This eventually induces serine/threonine phosphorylation of critical IRS-1 sites, therefore inhibiting IRS-1 binding and activation of PI3-kinase, resulting in reduced insulin stimulated glucose transport (77).

In the liver, the associated activation of pyruvate carboxylase by the high availability of acetyl CoA derived from fatty acid oxidation results in stimulation of gluconeogenesis (78).

6) Lipid lowering medications

Although behavioral interventions such as diet exercise, and smoking cessation can help to improve dyslipidemia, most patients need pharmacological therapy to reach treatment goals(79). There are several classes of medications used in the management of dyslipidemia associated with insulin resistance and type 2 diabetes including statins, fibrates, niacin, and thiazolidinediones (80).

Statins (HMG-CoA reductase inhibitors)

Statins lower LDL levels and can also to varying degrees lower plasma TG levels and raise HDL cholesterol. Statin mediates its action on plasma lipoproteins through increasing LDL receptor activity and reducing hepatic lipoprotein secretion (81).

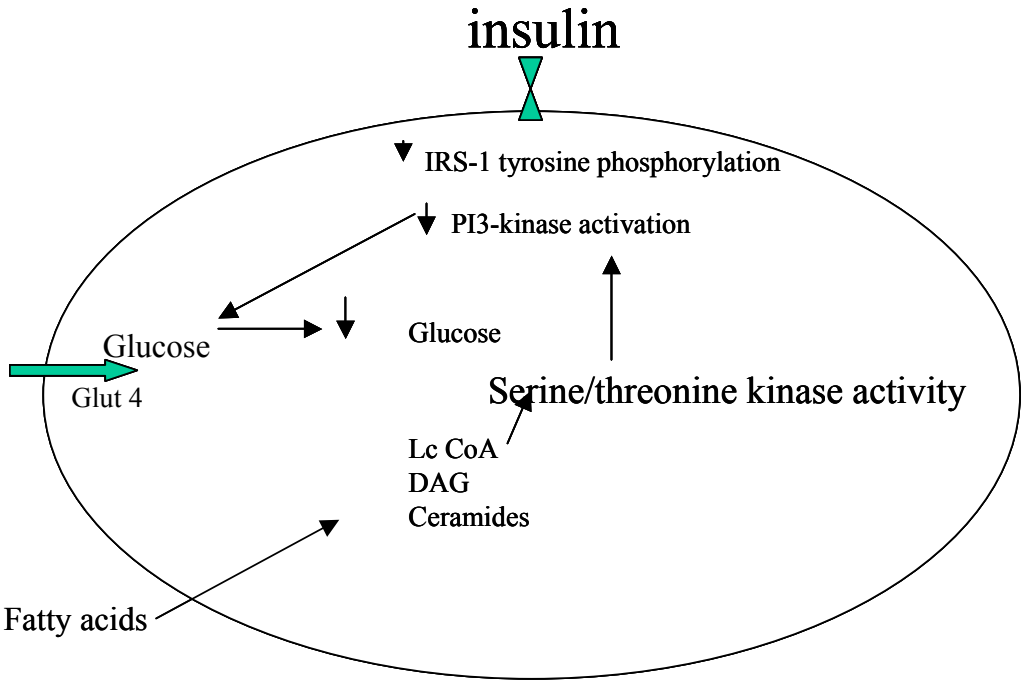

Figure 6: Proposed mechanism of fatty acid induced insulin resistance. Free fatty acid metabolites such as DAG, fatty acyl CoA, and ceramides activate a serine/threonine kinase cascade leading to phosphorylation of serine threonine sites on IRS-1 which in turn reduces the ability of IRS-1 to activate PI3-K with consequent decrease in glucose transport activity. LcCoA: long chain acyl CoA, DAG: Diacyl glycerol, S: Insulin receptor subunit, PI3- kinase: phosphatidyl inositol 3 kinase.

Fibrates (Peroxisome proliferator-activated receptor-α agonists)

Fibrates reduce plasma levels of TG rich lipoproteins. This effect is mediated by transcriptional regulation of genes that promote clearance of TG rich lipoproteins i.e.: It increases lipoprotein lipase and its activator apolipoprotein (apoCII), and inhibits (apoCIII), a protein that reduces lipolysis of TG rich lipoproteins and clearance of their remenants (82). Fibrates also raise HDL apparently through increasing production of HDL apoproteins and reducing transfer of cholesterol ester from HDL to VLDL (83,84). The effects of fibrates on LDL cholesterol are variable. A number of studies have shown that fibrates can reduce the levels of small dense LDL and reverse the small LDL phenotype (85-87). This effect might be due to the beneficial effects on TG rich lipoprotein metabolism as well as the reduced activity of cholesterol ester transfer Fibrate treatment can be effective in normalizing the atherogenic dyslipidemic picture in patients with type 2 diabetes (88,89).

Niacin (nicotinic acid)

Niacin significantly reduces TG levels, increases HDL levels, and increased LDL particle size, and therefore improves the atherogenic lipoprotein profile. Niacin suppresses the hepatic production of VLDL, and reduces fatty acid release from adipose tissue. It has been found that the HDL rising effect of niacin is potentiated by an increase in the effective half-life of HDL due to reduced uptake by the receptors responsible for intrahepatic degradation of HDL (90).

TZDs (peroxisome proliferator-activated receptor-γ agonists)

TZDS have an insulin-sensitizing effect that lowers glucose levels in patients with type 2 diabetes. It was observed that TZDs exert beneficial effect on lipoproteins and may improve some aspects of dyslipidemia associated with type 2 diabetes (91). Total cholesterol and LDL levels tend to increase with TZDs therapy, and there is a consistent increase in HDL cholesterol. TG levels decrease with poiglitazone therapy and also are reduced with rosiglitazone in patients with elevated baseline TG levels (92-94).

7) Ectopic fat and insulin resistance

Although standard definitions of insulin resistance still define it in terms of the effects of insulin on glucose metabolism, the last decade has seen a shift from the traditional "glucocentric" view of diabetes to an increasingly acknowledged "lipocentric"view point (77). This hypothesis holds that abnormalities in fatty acid metabolism may result in inappropriate accumulation of lipids in muscle, liver, and β-cells (68). It is further proposed that ectopic fat accumulation is involved in the development of insulin resistance in muscle and liver as well as impairing β- cell function (so called lipotoxicity) (95). Lipid accumulation within myocytes and hepatocytes is strongly associated with insulin resistance in diabetics (66), non diabetic relatives of patients with type 2 diabetes (a cohort at high risk of developing diabetes)(96), individuals with impaired glucose tolerance, and obese subjects (97).

In obesity, fat cells, which are already overloaded with TG, fail in their normal role of protecting other tissues from dietary fatty acid influx. The increased flux of FFA and TG in the circulation has an adverse effect on insulin sensitivity, as well as a longer term effect; that is accumulation of TG in glucose-metabolizing tissues such as skeletal

muscle, liver, and pancreatic β-cells. This accumulation of TG in β-cells leads to impairment of insulin secretion in response to glucose. This view has been consistent in a large number of both human and animal studies. Thus, the adipose tissue is now recognized as being a highly active, closely regulated metabolic tissue that performs the vital role of buffering FFAs flux into the circulation against the variable input of dietary fat from hour to hour. When this buffering capacity is overwhelmed by consistent intake of fat above the rate of oxidation, the dietary fat must go elsewhere, and its deposition in non-adipose tissue leads eventually to insulin resistance and beta-cell dysfunction (78). Reduction of ectopic fat lipid accumulation in the liver, muscle, and beta cells may prove to be an additional goal for future pharmacological interventions (98).

Muscle triglyceride and insulin resistance

TG content in skeletal muscle is increased in cases of obesity and type 2 diabetes, and is considered to be a strong predictor of insulin resistance (99,100). In a cross sectional study of healthy, young, lean offspring of type 2diabetic patients, there was an inverse relationship between intramyocellular lipid (IMCL) content as measured by H MRS and insulin sensitivity, consistent with the hypothesis that altered fatty acid metabolism contributes to insulin resistance in patients with type 2 diabetes (101). Increased IMCL is postulated to cause defects in insulin signaling in muscles and liver, reduce insulin stimulated muscle glucose transport activity and glycogen synthesis in muscles, and impair insulin suppression of hepatic glucose production (37).

Magnetic resonance spectroscopy has recently been developed as a noninvasive imaging method for assessing muscle lipid content and appears to be able to distinguish between intramyocyte and extramyocyte lipid (17). IM TG is increased in obesity and is

correlated with the severity of insulin resistance (102). A number of studies assessing muscle attenuation characteristics on computed tomography imaging have also indicated increased lipid content in association with obesity and type 2 DM and as a determinant of insulin resistance (99,103-105).

Increases in IMTG have also been found in non-obese, first-degree relatives of individuals with T2DM and was found to relate to insulin resistance in this population (106). These data suggest that the regional deposition of fat within skeletal muscle may be an early body composition abnormality in relation to insulin resistance, obesity, and type 2 diabetes rather than arising only as a late complication of excess adiposity. The fact that lipid accumulation can be seen relatively early in the development of insulin resistance adds to the concept that perturbed lipid metabolism by skeletal muscle may have a pivotal role in the development of obesity and type 2 DM (17).

Although the association between increased muscle TG and insulin resistance is compelling, the mechanism is still not yet clear. It has been shown that endurance athletes have high intramyocellular TG content despite the fact of being highly insulin sensitive (107). It has also been known that short-term exercise training in humans improves muscle insulin sensitivity without an accompanying measurable changes in muscle TG content (108). In order to account for this apparent discrepancy, it has been proposed that TG accumulation within the insulin resistant muscles is merely a marker for some other harmful fatty acid metabolites, particularly diacyl glycerol (109), ceramide (110), and long chain acyl CoA (111) are elevated in some insulin resistant muscle models. The proposed mechanism of action for each of these metabolites is

mediated through their negative effects on insulin signaling via activation of isoforms of protein kinase C (109-114), and inhibition of activation of protein kinase B (115-117).

Liver triglceride and insulin resistance

Ectopic fat storage in the liver is also of pathphysiological importance. Studies have shown that subjects with type 2 diabetes have significantly higher hepatic fat content as compared to fat, sex and age matched non-diabetic subjects. Hepatic fat content measured by nuclear magnetic resonance was also associated with insulin resistance (35). Fat accumulation in the liver was reported to be also associated with impaired insulin suppression of hepatic glucose production (118).

Examples of ectopic fat storage and insulin resistance

A. Lipodystrophy: This is a syndrome that is characterized by diabetes mellitus and decrease in adipose tissue mass. Due to this insufficiency of adipose tissue mass, the excess energy is stored as TG in the liver and skeletal muscle, followed by insulin resistance and diabetes (119,120). Transgenic animals models that lack adipose tissue development showed ectopic fat infiltration in the liver and skeletal muscles followed by insulin resistance and diabetes (121-123). Interestingly, adipose tissue transplantation back to these lipodystrophic animals reversed their hyperglycemia (124). These experiments indicate that inadequate adipose tissue mass is as deleterious as excess fat, and predisposes to insulin resistance and type 2 diabetes.

B. Obesity: Positive energy balance leads to metabolic disturbance that is similar to lipodystrophy in humans. This effect is excess lipid storage in the liver, and skeletal muscle (35-37), followed by glucose intolerance, insulin resistance and diabetes. The difference here is that adipose tissue stores are adequate or even large in these obese

patients, but is inadequate to sequester dietary lipid away from the liver, skeletal muscle, and pancreas. This explanation is supported by studies using thiazolidinedione which activates PPAR-γ, induced the differentiation of new fat cells in the subcutaneous adipose tissue, and decreased lipid infiltration in the liver and skeletal muscles. These observations suggest that pharmacological therapies designed to target ectopic fat storage may be used as a strategy to improve insulin resistance, and prevention and control of diabetes (125,126).

8) The link between fatty acid oxidation and ectopic fat storage

An alternative explanation of the excess fat storage is that the whole-body fat oxidation is impaired in obese subjects (16), leading to ectopic fat accumulation and eventually insulin resistance. So it is hypothesized that the machinery for fatty acid oxidation is insufficient to match the dietary fat load, or not properly activated in a timely fashion by signals to oxidize fats. Another possibility is that the mitochondrial fatty acid transporter availability is decreased. McGarry and coworkers studies on rodents indicated that inhibition of fatty acid oxidation increased intracellular lipids and decreased insulin action in vivo, and there was a strong correlation between them (127). Research on humans indicated that increased respiratory quotient (which is an indicative of decreased post absorptive fat oxidation) predicts weight gain (128-130) and is associated with deterioration of insulin sensitivity.

The control of fatty acid oxidation is the key for energy balance that regulates other metabolic processes, such as lipolysis and lipogenesis (131). When the capacity to oxidize fat is not properly activated by signals necessary to oxidize fat, or insufficient to match the dietary fat intake, the excess fat accumulates in the ectopic fat storage. Muscle

biopsies from patients with type 2 diabetes showed impaired mitochondrial capacity for fatty acid oxidation (16,132). The defect in mitochondrial oxidative capacity was found to be inherited since the lean offspring of type 2 diabetes patients proved to have the same defect (133).

Fatty acid oxidation may be also regulated by neural-endocrine mechanism. The use of synthetic beta-adrenergic agonists increased β-oxidation and improved insulin sensitivity in healthy young men (134,135). It is possible that the sympathetic nervous system (SNS) regulates fat oxidation, body weight, and insulin sensitivity. This was based upon the finding that low SNS activity has been linked to wait gain and decreased fat oxidation (136).

9) Pathogenesis of diabetic dyslipidemia

A significant component of the risk associated with type 2 diabetes is attributed to the characteristic lipid triad profile of raised small dense low density lipoproteins (sdLDL) levels, lowered high density lipoprotein (HDL), and elevated triglycerides (TG). There is now an increased evidence of the major importance of diabetic dyslipidemia as a cause of CVD rather than hyperglycemia (140).

Insulin resistance leads to impairment of the normal insulin mediated suppression of FFA release from visceral adipose tissue. This in turn leads to increased flow of FFA to the liver, which results in overproduction of very low-density lipoproteins (VLDL), and increased plasma TGs. This is further exacerbated by the reductions in lipoprotein lipase (LPL) activity and decreases in TG metabolism that are associated with insulin resistant state. In turn the hypertriglyceridemia causes lowered HDL-C levels and increased sdLDL (141).

The major disposal route for hepatic fatty acids and TG are the export of hepatic VLDL and the oxidation of fatty acids in the mitochondria, peroxisomes, and microsomes (142,143). Genetic and environmental factors are important factors. Impaired fatty acid β-oxidation capacity and defective VLDL transport system that impair lipid exporting capacity result in accumulation of hepatic triglyceride, and fatty liver (144,145).

Chapter|||: Experimental Section

1) Abstract

The TALLYHO/Jng (TH) mice are a newly established inbred polygenic model of type 2 diabetes and characterized by insulin resistance, hyperinsulinemia, hyperglycemia (males), obesity, and dyslipidemia. Our previous study demonstrated that hypertriglyceridemia is profoundly associated with insulin resistance and hyperglycemia in male TH mice. Therefore, we hypothesize that abnormalities in lipid metabolism may underlie the development of diabetes in male TH mice. In present study, we have determined triglyceride levels and accumulation in non-adipose tissue including liver, skeletal muscle, kidney, and pancreas of diabetic TH mice to assess the role of ectopic fat in diabetes of TH model. Also, we have evaluated fatty acid oxidation in liver and adipose tissue of TH mice in an attempt to understand the mechanism of lipodysregulation in these mice. Compared to C57BL/6J (B6) as control, diabetic TH mice exhibited significantly elevated triglyceride content in skeletal muscle and kidneys that was effectively reduced by bezafibrate, an agonist of peroxisome proliferators-activated receptor-α, treatment. Hepatic ^{3}H-palmitate oxidation rates were reduced in diabetic TH mice compared to B6 controls, although this difference did not approach statistical significance. Further, a marked accumulation of lipid droplets in liver was exhibited in TH mice. In summary, the development of insulin resistance and diabetes in TH mice may be associated with increased fat content of non-adipose tissue in these mice. The elevation of tissue lipid possibly resulted from the hypertriglyceridemia that may be in part caused by reduced hepatic β-oxidation in TH mice.

2) Introduction

Type 2 Diabetes is one of the fastest growing public health problems in the increasingly obese western society (33). By the year 2020, more than 250 million people will be affected worldwide, resulting in a substantial financial burden, with more than $100 billion spent annually in the United States alone (55,56,146). The relationship between obesity and diabetes is of a such great interdependence that the term diabesity has been applied (147). Ford et al., stated that for every kilogram weight gain, the risk of diabetes increases between 4.5-9% (147).

Cross sectional studies have demonstrated the presence of insulin resistance in virtually all patients with type 2 diabetes, and prospective studies have demonstrated the presence of insulin resistance one to two decades prior to the onset of the disease (55-57). Insulin resistance in the offspring of parents with type 2 diabetes has been shown to be the best predictor for the later development of the disease (58), and perturbations that reduce insulin resistance may prevent the development of diabetes (59). Therefore it is important to understand the pathogenic mechanisms of insulin resistance in order to identify novel targets for primary and secondary prevention (44). Alteration in lipid metabolism and in free fatty acid supply as well as lipid induced attenuation of insulin signaling received much less attention as a cause of diabetes (78). Indeed, both rodents and human studies observed a correlation between the degree of insulin resistance in vivo and the triacylglyceride (TG) content of non-adipose tissue including the skeletal muscle, the primary target for insulin stimulated glucose disposal.

Further, biochemical analysis of human muscle biopsies (148,149) and in vivo studies indicated that the obese, insulin resistance phenotype is associated with decreased

fatty acid oxidation capacity in the mitochondria characterized by decreased number of mitochondria, unusual mitochondrial morphology, decreased levels of mitochondrial enzymes (including succinate dehydrogenase (107,150), citrate synthase and carnitine palmitoyl transferase (132,151-153). It is therefore proposed that decreased fatty acid oxidation capacity may exacerbate lipid accumulation particularly in the presence of elevated plasma free fatty acid levels (154).

TALLYHO/Jng (TH) mice are a model for obesity and type 2 diabetes that are characterized by insulin resistance, hyperinsulinemia, hyperglycemia (males), obesity, and dyslipidemia associated with increased TG, free fatty acids (FFA) and cholesterol levels. TH females are normally not diabetic, and this gender dimorphism for diabetes seems to be commonly observed in mice (155-158).

In our previous study, it was observed that the plasma TG levels in TH male mice were significantly associated with insulin resistance and diabetes and preceded the development of diabetes in male TH mice. Therefore we hypothesized that lipodysregulation possibly caused by insulin resistance is responsible for the hypertriglyceridemia (HTG) that might lead to ectopic fat accumulation and the development of overt diabetes in TH male mice. To test this hypothesis in the present study we have evaluated β-oxidation and ectopic fat accumulation in TH mice.

3) Materials and methods

Animals

All animals were allowed free access to food and water in a temperature and humidity controlled room with 12:12- hours light-dark cycle. Mice were weaned onto standard rodent chow [4% fat, Harlan Teklad Rodent diet (w) 8604, Harlan Teklad;

Madison, WI]. All animal studies were carried out with the approval of the University of Tennessee Animal Care and Use Committee. Mice were euthanized by CO_2 asphyxiation.

Tissue Triglyceride

Age and sex matched groups of TH and C57BL/6J (B6) mice were euthanized with CO_2. Liver, kidney, muscles (soleus and gastrocnemius) and pancreas were dissected and stored in – 80°C. Lipid extracts were prepared from the tissues using the method of Hong Lan et al (159) and TG content was measured with an assay based on the detection of glycerol (160). Briefly, tissues (10-13 mg) were minced in chloroform and methanol (2:1 vol: vol) and incubated at –20° C overnight to release lipid before centrifugation at 1,200 rpm for 10 minutes at 4° C. The supernatants (the organic phase) were then collected and washed once with H_2O. Lipids in the organic phase were transferred into a new tube, dried, and re-dissolved in 200µl thesit solution (88317, Sigma). TG content was measured using colorimetric kit (Sigma, TR0100).

Bezafibrate Effect on Tissue Triglyceride

At 6 week of age, mice were fed customized bezafibrate (1.5 gm/kg of diet, Sigma) containing or regular chow diet for 17 weeks. At the end of the study mice were killed and tissue TG content was measured as described above.

Histological examination

Age and sex matched TH and B6 mice were euthanized with CO_2. Tissue samples from the liver and kidney were dissected, fixed in alcoholic formalin, cut into 2mm thick slices, washed well, and postfixed in 1% osmium tetoxide (OsO_4). Paraffin

sections, 4 microns thick, counter stained with nuclear fast red were examined under a light microscope.

^{3}H-Palmitate oxidation in hepatocytes

A. Hepatocyte Isolation

Hepatocytes were isolated following the method of A.W. Harman et al (161) in which the mice were anesthetized using tribromoethanol (Avertin 1.25%) (0.02 ml/gm body weight is the does for B6 mice and 0.03 ml/gm-body weight for TH mice). A longitudinal incision was made into the abdomen and chest cavity. Briefly, the liver was perfused in a retrograde fashion with a modified Hank's buffer (116 Mm NaCl, 5.4 Mm KCL, 0.8 mM $MgSO_4$, 3 mM Na2HPO_3, 26mM $NaHCO_3$, 20 mM Hepes), pH 7.3, and 37°C, containing 0.1 mM EGTA for 3 minutes. Perfusion was then continued for a further 8 minutes with modified Hanks buffer containing 1 mM $CaCl_2$, and collagenase (0.4 mg /ml). Flow rates were controlled by a perfusion pump (Variable flow mini pump, 13-876-1 Fischer) at 4ml/minute. After perfusion, the liver was excised and gently teased apart using a blunt spatula. The resulting cell suspension was filtered through two layers of nylon mesh (250 and 100 μm) to remove undigested material. The cell suspension was then centrifuged at 53 *g* for 2 minutes, and washed in the Hanks buffer containing 1 mM $CaCl_2$ for three times. The cells were then suspended into RPMI 1640 culture medium: HYQ RPMI-1640 medium, Cell culture reagents with 25mM HEPES with L- glutamate (SH 30255.01, Hyclone) containing 100U/ml penicillin, and 0.1 mg/ml streptomycin (15070-063, GIBCO). Cell viability was tested using Trypan blue exclusion test using 0.4% trypan blue. Cells were counted using a hemocytometer.

B. β- Oxidation of the liver

Freshly isolated hepatocytes were plated in a density of $(10)^5$ cells/well in 24 well culture plates. Plates were incubated 20 hours at 37°C in 5% CO_2. Palmitate oxidation was assayed using [9,10 ^{3}H] palmitate substrate (54 Ci/mmol) by the method of Moon and Rhead (162)& Dolittle (163). Cell monolayers were rinsed twice with phosphate buffer saline and incubated for 2 hours in 0.2 ml of substrate mixture containing 22 μm unlabelled palmitate, 5 μci (^{3}H) palmitate in Hanks buffer solution containing 0.5 mg/ml BSA. The reaction medium was collected, and treated with 0.2 ml 10% trichloroacetic acid. The cell monlayer was washed with additional 0.1 ml PBS and the wash was pooled with the initial reaction medium, and centrifuged at 13.000 Rpm. Supernatants were treated with 70 μl of 6 N NaOH, and applied to a 1 ml Dowex column. The ^3H_2O, which is an end product of oxidation, passes through the column. This sample was collected, together with 1 ml water wash to be directly quantitated as a measure of β-oxidation, using a β- counter.

[^{3}H] Palmitate oxidation in adipose tissue

Fresh fat pads were collected, cut into small pieces, and digested in KREBS buffer containing 1% collagenase (collagenase type 1, 1700-017, GIBCO). The digest was then filtered through gauze (600 μm) to remove debris. The adipocyte layer was collected, washed, and diluted to a total volume of 2 ml in RMPI for 2 (1ml) duplicate samples. Two hundred μl duplicate samples were collected in eppendorf tubes and stored at –80°C DNA measurement. Duplicate samples of 1ml volume were incubated with 1 μci [9, 10-(N)-^{3}H palmitate (P-1077, SIGMA) for one hour at 37°C in 5% CO_2 incubator.

After the addition of 1 ml trichloracetic acid, samples were extracted with 4 ml chloroform and applied to 1 ml dowex column over a layer of glass wool in a Pasteur pipette. Aliquots of the aqueous phase were collected in scintillation vials. Ten ml of scintillation cocktail were added, and samples were counted using a Beta counter (164).

Protein assay for hepatocytes

Twenty four well Plates were frozen to –80°C and thawed to 37° C. Protein content was determined by the modified Micro-Lowery method using a commercial kit (TP0300, SIGMA) with bovine serum albumin as a standard (690-A, Sigma).

DNA assay

DNA content in adipocytes was measured using CyQUAVT cell proliferation assay kit (C7026, Molecular Probes) was used. Cpm count was divided by DNA content.

Statistics

Statistical analysis for all the assays was conducted by ANOVA using Stat view (Abacus Concepts; Berkeley, CA). All data are presented as means ± SE.

4) Results

Tissue triglycerides

TH male mice exhibited significantly higher tissue TG levels in the kidney, muscle, and soleus muscle compared to age- and sex-matched B6 mice. No statistically significant differences were observed in liver and pancreas although increased trends were seen in TH mice. In females, TH exhibited a significantly increased TG content in pancreas compared to B6 females (all mice were 21 weeks old) (Figure 7).

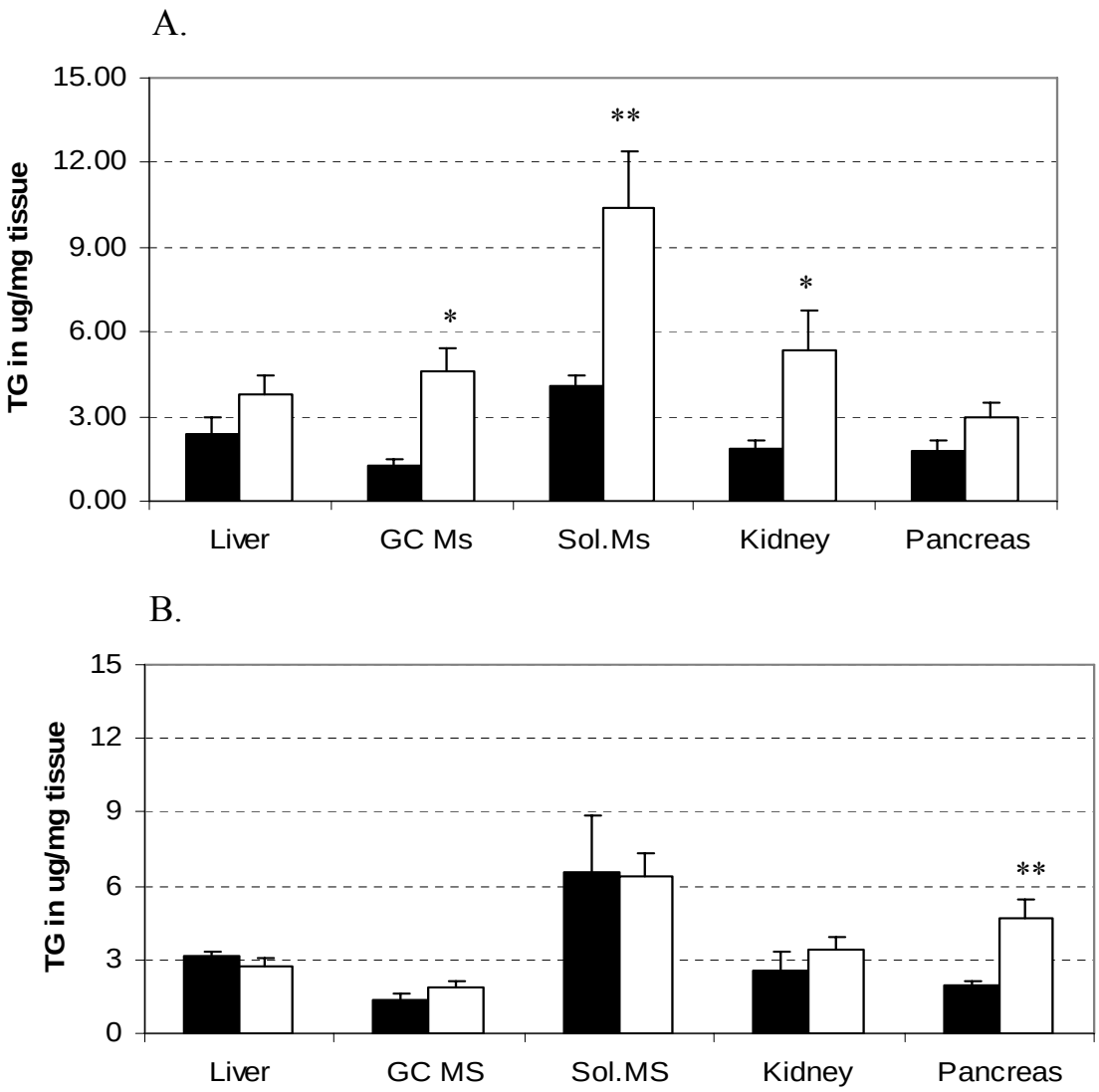

Figure 7: Tissue triglyceride content in 21 weeks old TALLYHO/Jng and C57BL/6J male (A), and female mice (B). A statistically significant high triglyceride levels were detected in the gastrocnemius (GC), soleus muscle (Sol.Ms), and kidney of TH males (n = 5) compared to B6 males (n = 7) and in the pancreas of TH females (n = 7) compared to B6 females (n = 6). * $p = < 0.05$ ** and $p = < 0.01$ vs. B6 in each tissue. B6 in each tissue. Black and white bars represent B6 and TH respectively.

Bezafibrate effect on tissue TG level

Bezafibrate treatments resulted in a significant reduction in tissue TG content in the liver, muscle and soleus muscle in TH male mice to a level that is close to that in B6 mice. There was no significant bezafibrate effect on TG content in the kidney, likely due to the large variation.

The effect of the bezafibrate containing diet on tissue TG levels in B6 mice in TH females was not consistent, possibly because the basal tissue TG levels in these groups were not elevated (Figure 8).

Histological examination

Microscopic examination revealed lipid accumulation throughout the hepatic paryncheme of male TH mice as indicated by OsO_4 staining, but in B6 mice, lipid accumulation was restricted to stellate cells (Ito) cells (Figure 9). In the kidney, proximal cortical tubular epithelial cells in B6 mice contained small apical cytoplasmic lipid droplets whereas larger accumulations were found in some renal epithelial cells of TH mice (Figure 9).

β- Oxidation of ^{3}H palmitate in hepatocytes

Although there was a trend that β-oxidation of TH male hepatocytes was lower than that of age- and sex- matched B6 mice, the difference was not statistically significant. Female TH mice also had a trend of lower β-oxidation, but the difference was not statistically significant (Figure 10 A).

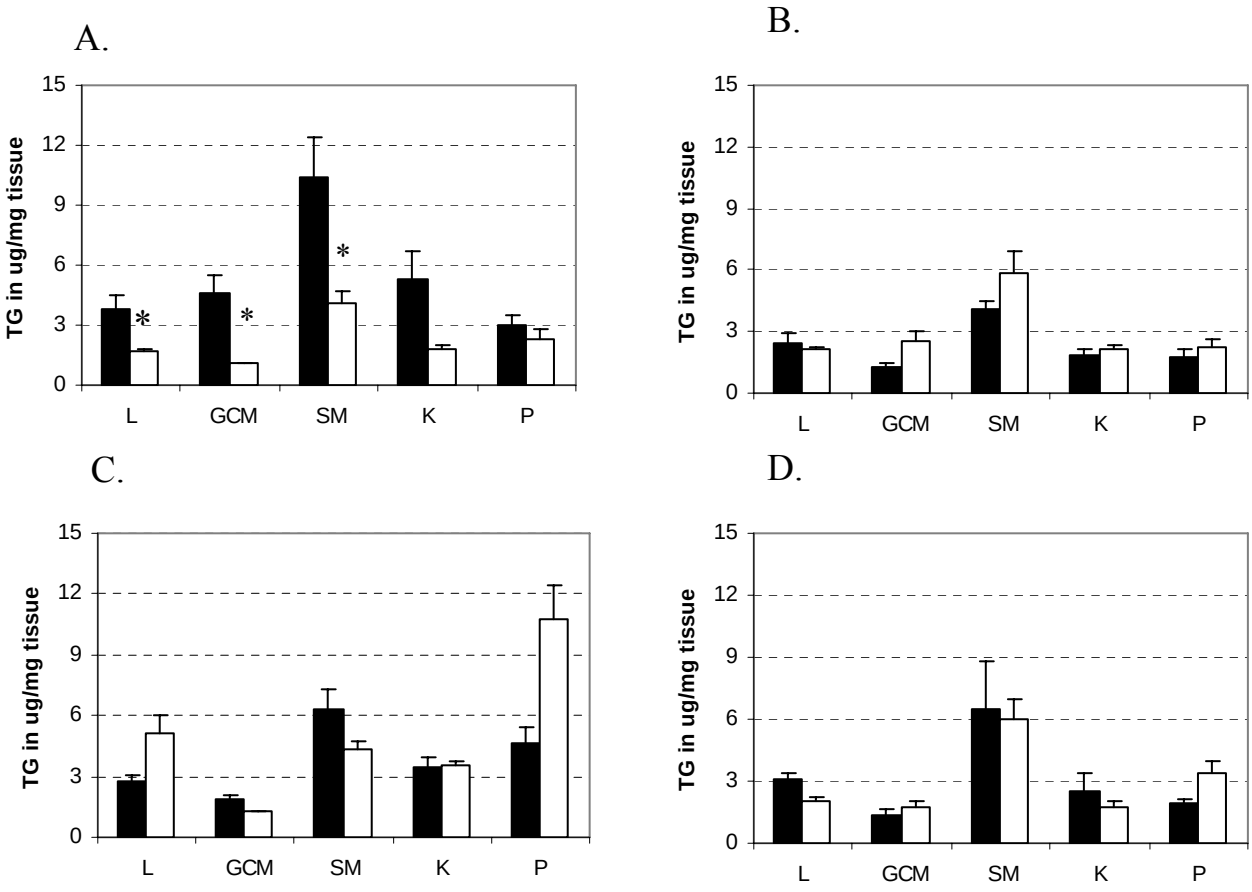

Figure 8: Bezafibrate effect on tissue triglyceride content in 21 weeks old TH and B6 mice. Sex and age matched groups of mice were fed regular chow diet or customized bezafibrate containing diet for 17 weeks. (A) TH males (n = 5 mice in each tissue), (B): B6 males (n =7, regular chow; n = 3, bezafibrate diet), (C) TH females (n = 7, regular chow; n = 4 mice, bezafibrate diet), and (D) B6 females (n = 7 mice, regular chow; n = 6 mice, bezafibrate diet). Black and white bars represent mice on regular chow diet and bezafibrate containing diet, respectively.
* $p < 0.05$ vs. chow diet in each tissue

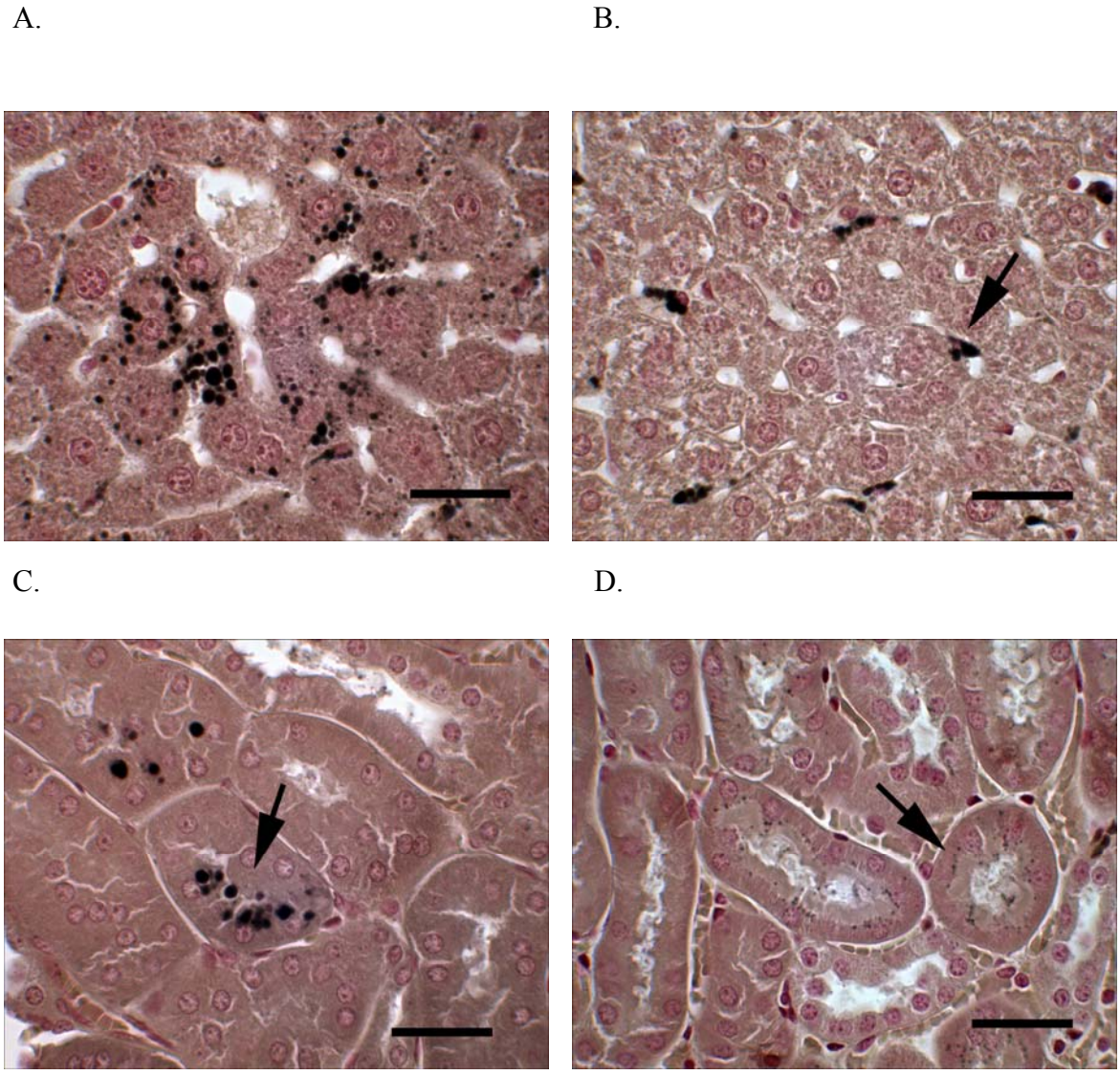

Figure 9: Osmium tetroxide staining of the liver and kidneys to detect lipid in 21 weeks old TH and B6 mice. In the liver, lipid was common in hepatocytes of TH mice (A) but was restricted to hepatic stellate (Ito) cells (arrow) in B6 mice (B). In the kidney, small lipid droplets were present in renal proximal convoluted tubules of B6 mice (arrow, D) and larger deposits were scattered in tubular epithelial cells of TH mice (Arrow, C). The scale bar indicates 50 μm.

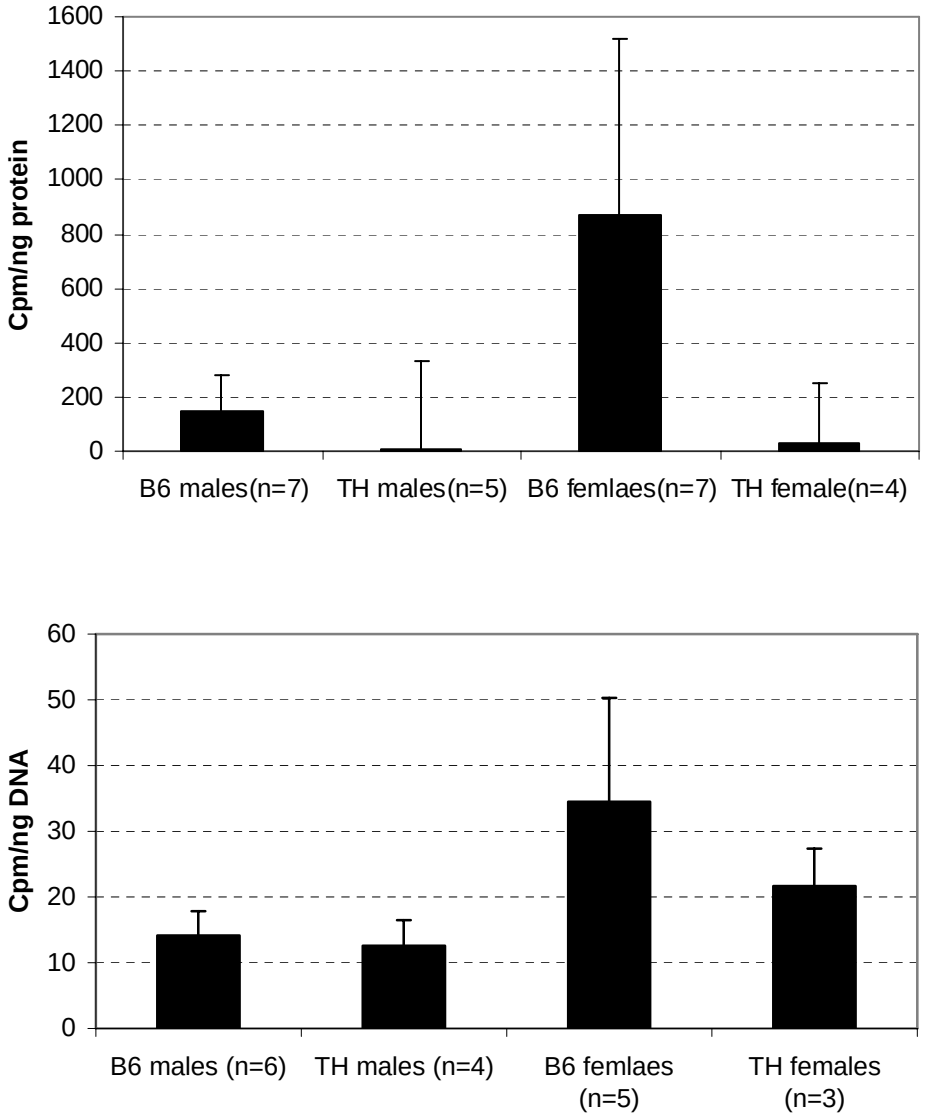

Figure 10: β-oxidation of ^{3}H palmitate in hepatocytes (A) and adipocytes (B) in 13 weeks old TH and B6 mice. Freshly isolated hepatocytes and adiopocytes were incubated with ^{3}H palmitate. The release of $^{3}H_2O$ was quantitated as a measure for β-oxidation.

β-oxidation of ^{3}H palmitate in adipocytes

Although there was a slight trend that β-oxidation rates in TH male mice were lower than age and sex matched B6 mice, the difference was not statistically significant (Figure 10 B).

5) Discussion

In the present study, we have demonstrated that diabetic TH male mice exhibit significantly elevated TG content and lipid accumulation in non-adipose tissue including skeletal muscle, liver, and kidney. Further, bezafibrate, known as a plasma lipid lowering agent, effectively reduces the tissue TG content in these mice. Attenuated hepatic fatty acid oxidation was also featured in male TH mice although it was not statistically significant.

Several human studies and animal studies using transgenic animal models with alteration in the muscle TG content have demonstrated a positive correlation of intramyocellular lipids (IMCL)-triglyceride content with insulin resistance. Conversely, studies on animal models with diminished IMLC showed improved insulin sensitivity (165,166).

Also, in human studies, intravenous lipid infusion increased IMCLs rapidly and high fat feeding for 3 days increased IMCL content modestly (167), and weight loss decreased IMLCs with concomitant improvement of insulin sensitivity (168). It has been hypothesized that IMCL is caused by decreased capacity of fatty acid β-oxidation in muscles (169) and the excess lipid stored is not mobilized, and may affect insulin sensitivity by the production of lipid peroxidation products such as 4-HNE and /or malondialdehyde (170).

Shulman et al., have also demonstrated that high fat feeding caused hepatic fat accumulation that is strongly associated with hepatic insulin resistance in the absence of peripheral fat accumulation or peripheral insulin resistance in male Spargue-Dawley rats. They speculated that fat induced hepatic insulin resistance might be due to activation of PKC∈and/or JNK1, which might lead to impairment of IRS-1and IRS-2 tyrosine phosphorylation. This block in insulin signaling limits the ability of insulin to activate glycogen synthase. They also found that fat accumulation increased the contribution of gluconeogenesis to total endogenous glucose production. Treatment of the high fat diet fed rat with low dose 2,3 dinitrophenol to increase energy expenditure resulted in prevention of hepatic fat accumulation and activation of PKC∈ and JNK 1. This in turn preserved the insulin signaling and prevented the development of hepatic insulin resistance(171). Therefore, it is speculated that the hypertriglyceridemia developed at young age may then lead to accumulation of intramyocellular and hepatic lipids which then contribute to the development of overt diabetes in male TH mice. Evaluation of altered lipid accumulation in these cells of male TH mice at pre-diabetic stage remains to be determined.

The pharmacological treatment with bezafibrate aimed at lowering plasma triglyceride showed also improvement in tissue TG accumulation in male TH mice. Whether this diminished tissue TG content may ameliorate insulin resistance and diabetes in TH male mice, however, remains to be evaluated.

Acute increase in free fatty acids has a stimulatory effect on pancreatic β-cells insulin secretion. In contrast, chronic FFAs elevation results in reduced glucose

stimulated insulin secretion. Prolonged in vitro exposure of β-cells to FFAs, TGs, or glucose leads to stimulation of lipogenesis and /of increased fatty acid estrification and intracellular fat deposition, which is associated with β-cell dysfunstion in animal studies.

A study on Zucker diabetic fatty rats indicated that abnormalities in insulin secretion in these rats are attributed to a 50-fold increase in islet TG content compared to normal control group. Furthermore, incubation of the islets in triglitazone, which simultaneously reduced islet TG content, resulted in improvement in insulin secretion. Another possible mechanism for TG induced β-cell lipotoxicity is the increased formation of nitric oxide which induced β-cell apoptosis(172-174).

In our study, pancreatic TG content was found to be elevated in TH mice compared to age and sex matched B6 mice, which might be a contributing factor in the development of insulin resistance and type 2 diabetes in these mice. Although TH females are not normally hyperglycemic (176) they had a significantly higher pancreatic TG content compared to age matched B6 females that still needs to be explained. Protection from diabetes in females has known to be attributed to low hepatic estrogen sulfotransferase activity in female mice (177). Sex dimorphism for diabetes appears to be commonly observed in mice (178-180).

Plasma levels of TG are largely controlled by TG synthesis and secretion from the liver in a cooperative manner with lipolytic activity in adipose tissue; for example, promotion of TG hydrolysis in adipose tissue by lowering insulin (*i.e.* through fasting) or by insulin resistance (to lowering lipolysis) increases plasma FFA that are then taken up by the liver and either re-esterifed to TG and secreted as TG-rich lipoprotein, very low density lipoproteins (VLDL), or oxidized (181). It is, therefore, plausible to speculate

that chronic inhibition of fatty acid oxidation in the liver might cause an increase in re-esterification of fatty acid uptaken to TG as a default, which in turn increases TG secretion as VLDL form as well as tissue accumulation in male TH mice. Investigating whether impaired β-oxidation is also attributable to the excess lipid accumulation in the skeletal muscle of male TH mice will further elucidate the link between lipodysregulation and insulin resistance in TH model and remains to be conducted. Interestingly, stimulation of β-oxidation by over-expression of CPT 1 significantly improved insulin response in L6 myocytes (154).

In summary, an excess lipid accumulation in non-adipose tissue becomes apparent in hypertriglyceridemic and diabetic TH male mice. It is likely that impaired fatty acid oxidation in liver may be in part responsible for the hypertriglyceridemia, leading to TG accumulation in peripheral tissue including the skeletal muscle in TH mice. This increased non-adipose triglyceride levels may be attributable to the impaired insulin sensitivity and diabetes in these mice.

Conclusions and Future Directions:

Our studies demonstrated a significant association between lipodysregulation and type 2 diabetes in TH mice. Further experimentation on TH mice tissue TG content at an earlier stage (younger age) before the onset of diabetes is required. Examining the effect of bezafibrate on β-oxidation, and whether it improves or prevents insulin resistance and hyperglycemia in TH mice, will be also valuable.

One notable finding from the present study is a drastic elevation of TG content in soleus muscle in TH male mice. The role of intramyocellular lipids content in mediating insulin resistance has been well demonstrated in humans and animal models. Therefore, our long-term goals include studying intramyocellular lipid deposits and the related insulin sensitivity and signaling in TH mice. This study will also be extended to investigation of mechanisms including fatty acid uptake and lipid oxidation in skeletal muscle.

Despite the enormous research work done to explore the pathogenesis of diabetes, the question of what causes type 2 diabetes might be one of the most frequently asked and least satisfactorily answered in the history of diabetes research. Elucidating the mechanism of lipodysregulation in diabetic model of TH mice will provide insights into the causes and mechanisms underlying the metabolic complications of insulin resistance and diabetes. This will ultimately lead to improved therapeutic strategies for prevention and treatment of type 2 diabetes.

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Appendix

Table 1 A: Body mass index (BMI)

< 18.5	Underweight
18.5 – 24.9	Normal weight
25 – 29.9	Overweight (pre-obesity)
30-34.9	Grade 1 obesity
35– 39.9	Grade 2 obesity
> 40	Grade 3 obesity (Morbid Obesity)

Body mass index is calculated by dividing the weight in kilograms (kg) by the height in square meters $(m)^2$.

Tissue triglyceride measurement

1) Mice are euthanized with CO_2.

2) Liver, kidney, muscles (soleus and gastrocnemius) and pancreas are dissected and stored in – 80°C.

3) Lipid extracts were prepared from the tissues using the method of Hong Lan et al (159):

- Tissues (10-13 mg) are minced in 3 ml chloroform and methanol (2:1 vol: vol). Use falcon blue cap tubes (Falcon, 352097).
- Incubate at –20° C overnight to release lipids.
- Centrifuge at 1,200 rpm for 10 minutes at 4° C.
- Collect the supernatants (the organic phase) and wash once with H_2O.
- Centrifuge at 1,200 rpm for 10 minutes at 4° C.

- Transfer Lipids in the organic phase into a new Falcon tube.
- Leave the tubes to dry under the hood. It takes up to 5 days to have the tubes completely dried.
- Re-dissolve the lipids in 200µl thesit solution (88317, Sigma).
- Measure TG content using colorimetric kit (Sigma, TR0100).

Osmium Tetroxide procedure for fat staining

Fixative:

Alcoholic formalin.

Reagents:

Osmium tetroxide (4% aqueous solution, 20816-12-0, Electron Microscopy sciences). Prepare 1% solution by dissolving osmium in 0.1 M Cacodylate Buffer. Store in a dark container (or wrap with foil) in the refrigerator. The vapor is harmful, so prepare and use the reagent under a fume hood.

Periodic acid solution 0.05% (S186-3202).

Procedure:

1) Trim buffered formalin-fixed tissue to 2mm thick and wash in running tap water for at least 30 minutes. It is important to cut the tissue thin as osmium has a very low penetrating power.

2) Rinse the tissue well in distilled water.

3) Place the tissue in 5 ml of osmium tetroxide 1% solution. Cap the container and leave for 1-2 hours. Periodically agitate the solution containing the tissue.

4) Rinse tissue in two changes of distilled water for 15 minutes each.

5) Differentiate by placing tissue in 10 ml of 0.5% periodic acid solution for 30 minutes. Agitate the solution periodically. The background tissue will clear and leave the fat stained black.

6) Wash the tissue in tap water for 30 minutes.

7) Place the tissue in a processing cassette (Omnisette tissue cassettes, Histoprep, Fischer scientific) with the darker stained side facing down.

8) Cover the cassette completely with 70% alcohol, and send to the histopatholgy lab for preparing paraffin sections and slides.

Hepatocyte isolation

Solutions:

1) Modified Hank's buffer containing EGTA:

116mM NaCl.

5.4 mM KCl

0.8mM MgSo4 .7H2O

0.4 mM KH2PO4

0.3 mMNa2HPO3

26 mM NaHCO3

20 mM Hepes,

0.1mM EGTA. Bring to PH 7.3, 37° C.

2) Modified Hanks buffer containing 1 mM $CaCl_2$ and collagenase 0.2 mg/ml (with no EGTA)

3) Modified Hanks buffer containing 1 mM $CaCl_2$ without collagenase for wash.

Procedure for hepatocyte isolation:

1) Oxygenate all the solutions and bring the temperature to 37°C using a water bath.

2) Anaesthetize the mouse with intraperitoneal injection of Avertin (1.25%). The dose is 0.02/mg for B6 mice, and 0.03 /mg for TH mice.

3) After the mouse is anaesthesized, lay it down on the back on a cork dissecting board (NC 9063641, Fischer scientific). Fix the limbs with 4 pins.

4) Make a longitudinal incision in the abdomen and chest cavity.

Occlude the vena cava with an artery clamp (John Hopkins Bulldog clam, 1½ inches straight, 34-2920, biomedical research instruments) in a position cranial to the hepatic vein.

5) Insert an IV catheter (EXE safe left catheter, 24G X 3/4″, 14-841-21) into the abdominal vena cava, caudal to the hepatic vein.

6) The portal vein is then severed using a superfine sissors (11-1020, 31/4 straight, needle points, 0.2mm X 0.2 mm, Biomedical research Instruments). The liver is perfused in a retrograde fashion with a modified Hank's buffer containing EGTA , for 3 minutes . Perfusion is then continued for a further 8 minutes with modified Hanks buffer containing 1 mM $CaCl_2$ and collagenase 0.2 mg/ml. Flow rates have to be adjusted at 4ml/minute. Flow is controlled by a perfusion pump (Variable flow mini pump, 13-876-1 Fischer).

7) After perfusion, the liver is excised and gently teased apart using a blunt spatula.

Filter the resulting cell suspension through two layers of Nylon mesh (250 and 100 um) to remove undigested materials.

8) Centrifuge the cell suspension at 53 g for 2 minutes.

9) Take the cell pellet, and re-suspend it in Hank's buffer (containing 1 mM $CaCl_2$).

10) Repeat the centrifuge and washing for 3 times.

11) The cells are suspended into RPMI 1640 culture medium (HYQ RPMI-1640 medium. Cell culture reagents with 25 mM HEPES with L- glutamate, SH 30255.01) containing100U/ml penicillin, 1 mg/ml streptomycin.

12) Cell viability testing: Use Trypan Blue exclusion test.

13) Cell counting: Use a hemocytometer.

Reference: Isolation of hepatocytes from postnatal mice, Andrew W. Harman. 1986.

Assessment of Cell viability with Trypan Blue

Trypan blue is enables easy identification of dead cells. Dead cells take up the dye and appear blue. In contrast, living cells repel the dye and appear refractile and colorless.

Procedure:

1) Prepare the hemocytometer for use by cleaning all surfaces and cover slip. Completely dry using non-linting tissue (or air dry). Center the cover slip on the hemocytometer.

2) Transfer 50 µl of Trypan blue (0.4% solution in phosphate, 1691049) into a clean eppendof tube.

3) Add 50 µl of the cell suspension in the tube containing the stain.

4) Mix the solution thoroughly, but gently. Cut the tips of the pipette to avoid cell damage if narrow tips are used.

5) Allow the mixture to set for 2-3 minutes after mixing. Do not let the cells sit in the dye for more than 5 minutes because both the living and dead cells will take up the dye after 5 minutes.

6) Pipette 9 µl of the Trypan blue cell suspension mixture into one of the two containing chambers. Fill the chamber slowly and steadily. Avoid injecting bubbles into the chamber.

7) If > 60% of cells are viable, count the cells using a hemocytometer.

Procedure for cell counting:

1) Pipette 9 µl of the cell suspension in one of the two counting chambers as above.

Allow the suspension to settle for 10 seconds.

2) Count the cells in each of the four 1mm3 corner squares of the hemocytometer. Do count the cells touching the top or left borders. Do not count the cells touching the bottom or right borders.

Determine the cell count:

1) Calculate the total cells counted in the four corner squares.

2) If the total cell count is less than 100, or if more than 10% of cells counted appear to be clustered, carefully remix the original cell suspension and repeat the counting.

3) If the total cell count is greater than 400, dilute the suspension, and repeat the counting.

4) Calculate the cell count using the equation: cell/ ml =(n) x104

n = the average cell count per square of the four corner squares counted

5) Determine the total number of cells in the total suspension volume:

Determine the total volume of the cell suspension.

Multiply the volume of the cell suspension by the “cells/ml” value calculated above.

β- oxidation of the liver:

Freshly isolated hepatocytes were plated in a density of $(10)^5$ cells/well in 24 well culture plates (multiwell, 24 wells, 353047, Falcon). Plates were incubated 20 hours at 37 °C in 5% CO_2. Palmitate oxidation was assayed using [9,10 ^{3}H] palmitate substrate (54 Ci/mmol) by the method of Moon and Rhead. The release of ^{3}H2O was quantitated as a measure of the rate of β-oxidation.

1) Cell monolayers are washed twice PBS.

2) Bring to a final concentration of 22 μM ^{3}H palmitate and BSA (0.5mg/ml)(Bovine serum albumin, A8022-50G) with Hanks buffered saline solution (HBSS X1, 14175-095, GIBCO, Grand Island,N.Y.).

Reaction medium:

For each sample, triplicates of both sample and negative controls are needed. So each sample will require 6 wells. Total reaction medium is 200 μl . (For 6 wells 200x6) + additional 200ul.ie 1400 ul

Take 2 ml Hanks& add 1mg BSA (0.5 mg BSA/1ml Hanks).

Take 1400 ul and add 14 μl of unlabelled palmitate.

Add the radiolabel led palmitate (5.9 μl /well)i.e. 40.6 μl for 7 wells.

1) Apply 200 μl of this mixture to wells containing monolayer.

2) Cells are incubated in the above reaction mixture for 2 hours at 37°C in humidified 5% CO_2 in 95% air.

3) The reaction mixture is removed and added to an eppendorf tube containing 200 μl of 10% wt/volume trichloroacetic acid.

4) Each well is rinsed with 100 μl PBS which is added to the tubes.

5) After 2 minutes at room temperature, the reaction mixture is centrifuged at 13.000 RPM in a Beckman centrifuge tube for 5 minutes.

6) Remove the supernatants immediately, and then add 6 N of NaOH (70 µl).

7) Apply to a 1 ml Dowex-1 column (Dowex 1x2 Cl- form strongly basic, 44290, Sigma Aldrich) in a Pasteur pipette lined with a bottom layer of glass wool (P13-019-2).

8) Columns are rinsed with 1 ml of distilled water.

9) The eluate is collected in a scintillation vial containing 10 ml of scintiverse. The samples are counted using a Beckman β-counter.

10) Add 0.5 ml H_2O to each well and keep in -80°C. In order to determine protein content, freeze and thaw (-80°C and 37°C three times)before measuring protein content.

11) The protein content is determined using total protein kit, Micro Lowry, Peterson's modification, Tp0300, Sigma Aldrich, St. Louis, MO). Readings are measured fluorometrically (Spectrophotometer 336009-86, Spectrosonic GENESYS).

<u>β- oxidation in adipose tissue:</u>

1) Reconstitute KREBS buffer. Prepare 1% BSA solution by dissolving 1 gm of BSA in 100 ml of KREBS buffer.
2) Prepare 1% collagenase (collagenase type 1, 1700-017, GIBCO) solution (depending on the number of samples ex, for each sample you will need 4 ml of 1% collagenase. So take 4 ml of solution in step 1 in which you dissolve 40 mg collagenase type 1).
3) Dissect the mouse and get the fat pads. Mince into little pieces and add to the 50 ml orange cap tube containing 4 ml of 1% collagenase.

4) Incubate in 37°C water bath for 40-60 minutes.

5) The digest was then filtered through gauze (600 μm) to remove debris. Stand for 10 minutes so that the fat cell layer floats.

6) Aspirate the lower phase using a glass Pasteur pippete, leaving the fat cell layer in the tube.

7) Add 10 ml of 1% BSA KREBS buffer without collagenase and swirl gently.

8) Stand for 10 minutes and aspirate the lower phase as in step 6. Repeat the wash twice.

9) Add 2 ml of KREBS buffer without collagenase of BSA. Swirl gently.

   Take 200 Two hundred μl duplicate samples were collected in eppendorf tubes and stored at –80°C DNA measurement.ul in eppendorf tube and keep in – 80°C for DNA assay.

10) Aspirate the lower phase leaving the adipose tissue layer in the tube.

11) Add 2 ml RMPI containing 10% FBS and divide into 2 tubes to perform a duplicate sample experiment. Duplicate samples of 1ml volume are incubated with 1 μci [9,10-(N)-^{3}H palmitate (P-1077, SIGMA) for one hour at 37°C in 5% CO_2 incubator. Leave the caps loose during incubation.

12) Add I ml trichloacetic acid to each tube.

13) Add 4 ml chloroform to each tube.

14) Take the upper phase (aquous phase).

15) Apply to 1 ml dowex column over a layer of glass wool in a Pasteur pipette.

16) Aliquots of the aqueous phase were collected in scintillation vials. Ten ml of scintillation cocktail were added, and samples were counted using a Beta counter (164).

17) DNA assay is then performed . Cpm is divided by DNA content for each sample.

Vita

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