GLYCOLYSIS AND GLYCERONEOGENESIS IN ADIPOCYTES: EFFECTS OF ROSIGLITAZONE, AN ANTI-DIABETIC DRUG

By

SOREIYU UMEZU

Bachelor of Science in Biochemistry and
Molecular Biology
Oklahoma State University
Stillwater, OK
2005

Submitted to the Faculty of the
Graduate College of the
Oklahoma State University
in partial fulfillment of
the requirements for
the Degree of
DOCTOR OF PHILOSOPHY
July, 2010

GLYCOLYSIS AND GLYCERONEOGENESIS IN ADIPOCYTES: EFFECTS OF ROSIGLITAZONE, AN ANTI-DIABETIC DRUG

Dissertation Approved:

Dr. Jose L Soulages

Dissertation Adviser

Dr. Chang-An Yu

Dr. Andrew Mort

Dr. Jack Dillwith

Dr. A. Gordon Emslie

Dean of the Graduate College

ACKNOWLEDGMENTS

First, I would like to immensely thank my advisor, Dr. Jose Soulages, for his support and guidance throughout my graduate studies. Without Dr. Soulages extensive expertise and knowledge along with his sense of humor I would not have achieved my goal of obtaining my Doctorate in Biochemistry. I would also like to thank Dr. Estela Arrese for her kind and generous advice. Both Dr. Soulages and Dr. Arrese have made a tremendous impact on me personally and professionally.

I would like to thank all the members of my committee: Dr. Chang-An Yu, Dr. Andrew Mort, and Dr. Jack Dillwith who have devoted their time reading my dissertation and their patience for my procrastination. Their support and guidance has been invaluable and extremely appreciative.

Most importantly, I want to acknowledge my parents: (Dr.) Mrs. Fumie Umezu and (Dr.) Mr. Yasuiki Umezu, for their unwavering support of me throughout my time in the U.S. and for suffering me while I'm doing whatever I want in my life.

Lastly, I want to thank to my good friends, Alisha Howard, Philip Verghese, Laticia Rivera, Noah Burns, and Masakazu Hamada. They have provided supports and smile to overcome obstacles in my graduate life.

TABLE OF CONTENTS

Chapter Page

LIST OF TABLES

Table Page

LIST OF FIGURES

Figure Page

CHAPTER I

INTRODUCTION

Diabetes Mellitus

Diabetes mellitus is the metabolic disorder affecting metabolism of carbohydrates, lipids, proteins in every type of cells in the body. This metabolic disorder is characterized by insensitivity to insulin, insufficient/absent insulin secretion, or both. Currently, around 23.6 million people (7.8% of the population) are affected by this metabolic syndrome in the United States [1]. Moreover, more than 246 millions are estimated to have diabetes worldwide, and this number is expected to increase in the next few decades [2-4]. In the U.S alone, the cost of diabetes, including medical care and social costs, reached around $174 billion in the year of 2007 [5]. This cost is also expected to increase drastically in next few years, therefore diabetes mellitus is now considered as epidemic.

There are several forms of diabetes. Among these cases of diabetes, type I and type II are the ones that affect most of the patients (95 to 99%). Type I diabetes (T1D) is caused by pancreas deficiency in insulin production [6, 7] whereas type II diabetes (T2D) is characterized by insulin resistance with deficiency in insulin secretion and hyperglycemia [8].

T1D is triggered by the autoimmune β-cells (pancreas) failure in insulin production. Because immune system tries to destroy insulin releasing factors in pancreas, 90% of the patients suffering from T1D display signs of antibodies against insulin and β-cells [7]. Autoimmune destruction is caused by either genetic or environmental factors, however, the exact mechanism is not well characterized [9]. Many cases of T1D are found among children although some adults also develop pancreas failure. Essentially, T1D patients require periodic insulin injection to maintain proper glucose concentration in plasma since they cannot produce the hormone by themselves.

.

T1D and T2D together comprise most of the population affected by diabetes. Only 5-10% of them have T1D. T2D is the most commonly diagnose case of diabetes which accounts for 90-95% of diabetic patients in the U.S [1]. T2D patients develop the conditions in which body/cells do not properly respond to existing insulin over period of time. T2D can be developed from multiple causes. These include high cholesterol, inactive lifestyle, high fat diet, and aging [3, 10]. However, to date the mechanisms by which people develop insulin resistance remain unclear.

Type II Diabetes Mellitus

Multiple factors seem to participate to the development of T2D although direct factors or the starting points of the T2D development are not characterized. The most prevalent condition induced by the development of insulin resistance is fasting hyperglycemia because of the lack of adequate response to insulin signaling in the cells to clear up glucose. Insulin resistance and hyperglycermia together define T2D. Individuals who have glucose concentration of more than 7.8mmol/L after 2 hours from food intake are considered to have developed insulin resistance and T2D. Insulin resistance in muscle and liver is most prevalent conditions account for glucose intolerance in T2D patients. Muscle in healthy individuals clears up the majority of glucose taken by peripheral tissues (muscle and peripheral glucose uptake) [11, 12]. However, this ability in T2D patients is severely impaired. In addition, hepatic glucose production (gluconeogenesis) in T2D-affected liver is also altered, and the organ overproduces glucose in the presence of insulin and hyperglycemia [12].

Excess glucose in plasma alone is known to cause several pathogenic effects. Hyperglycemia was first shown to damage β-cells in 1940 [13]. Other known pathological consequences associating to hyperglycemia include retinopathy, neuropathy, nephropathy, atherosclerosis [14-16], congenital birth defect [17], and fungal infection [18].

In addition to hyperglycermia and its pathogenic effects, multiple pathogenic consequences are related to T2D. These include higher blood fatty acid (FA) concentration, increased muscle triacylglycerol (TG, fat) content, and higher level of VLDL (very low density lipoprotein, known to increase cholesterol level in plasma) [19, 20]. When these conditions persist for longer periods of time, individuals develop overt

T2D with increased production on ketone body. Prolonged circulation of ketone body would eventually cause ketosis and acidosis that induce coma and death. Individual factors such as high TG concentration and low HDL increase risks of the development of cardiovascular diseases. Thus, T2D is a metabolic syndrome that can induce complex pathogenic effects.

Adipose Tissue and Adipocytes

Obesity is one of the major risk factors relating to the development of T2D [21]. In fact, more than 80% of the patients affected by type II diabetes are in the obese/overweight group [22]. In parallel to increased number of obese individuals, both adults and children, the number of individuals affected by T2D seems to keep increasing. However, it is important to note that the degree of insulin resistance is not much different among normal/lean weight T2D patients and obese/overweight patients [11].

Adipose tissue is the largest reservoir of energy as the form of fat in mammalian body. Since obesity is one of the major contributors of the development of T2D, adipose tissue must be the one of key organs participating in the development of T2D.

As individuals get obese, the adipose tissue often displays changes including sizes of adipocytes and altered metabolic activities, and the changes are particularly significant in the adipose tissue associated with internal organs. Obesity is also considered as an inflammatory condition of adipose tissue because of the increased entry and accumulation of macrophages in adipose tissue. These changes seem to deteriorate proper functions of the adipose tissue and increase the risks of cardiovascular disease and insulin resistance. On the other hand, fatless mice are also reported to be extremely susceptible to insulin resistance, hyperglycemia, fatty liver, and higher concentrations of TG and FA in circulation. Transplant and regeneration of adipose tissue into these fatless mice reversed those metabolic disorders, indicating an important metabolic role of adipose tissue [23, 24].

This tissue consists of several cell types including adipocytes (fat cells), macrophages, stromal cells, blood cells, and endothelial cells. There are two types of adipose tissue: brown and white adipose tissue. Brown adipose tissue is capable of thermogenesis, and adipocytes in brown adipose tissue contain a higher number of mitochondria. White

adipose tissue occupies the majority of whole adipose tissue mass in the body and play roles in whole body energy homeostasis by equilibrating lipid catabolism and lipid synthesis. Adipocytes in white adipose tissue can storing large amount of FAs as TG and can expand their sizes around 25 to 200 um in diameter depending on the volume of stored lipids (mainly TG) [25], and they serve as a major energy storage place in the body. Also, adipocytes can carry out “futile cycle”, in which they re-esterify (re-incorporate) FAs generated from lipolysis of stored TGs into newly synthesized TGs, depending on physiological needs.

In human adipocytes, at least 30% of FAs can be re-esterified into TG [26]. This ability indicates adipocytes’ dynamic regulation of lipolysis and FA re-esterfication to release adequate an amount of FA to plasma. Besides this lipid buffering function, adipocytes function as an endocrine organ and communicate with other organs by releasing peptide hormones such as resistin, leptin, and adiponectin in addition to adipokines including TNF-α and IL-6. These adipose tissue derived molecules are involved in several important physiological processes such as endocrine function (leptin, growth factors) [27], immune-inflammatory reactions (TNFs, IL-6) [28], metabolic processes (NEFA, adiponectin, resistin) [29-31], and cardiovascular functions (NEFA, PAI-1) [32, 33]. Therefore, nowadays adipose tissue has gained recognition as not only the energy storage site or connective tissue but also the metabolically active organ which plays important physiological functions.

Fatty Acid Toxicity

One of the adipose-related factors that appears to have important physiological roles in type II diabetes is plasma free FAs (non-esterified fatty acids, NEFAs), which can be generated either from food intake or lipolysis. Both normal/lean weight and obese T2D patients display prolonged elevation of NEFA level in plasma [19].

Normally NEFA level increases after meals and is either used as energy or esterified into TG in adipocytes depending on physiological requirements. NEFA is acutely used as an energy source and facilitates insulin release from pancreatic β-cells [34, 35]. However, if elevated levels of NEFA chronically persist, it impairs insulin release from β-cells thereby worsening insulin resistance. Elevated levels of NEFA also interfere with glucose utilization in muscle and liver besides the reduction in β-cell insulin release. Also the

condition appeared to be sufficient to cause insulin resistance and pro-inflammatory response in liver [36, 37]. Moreover, it impairs hepatic glucose production (gluconeogenesis) and muscle glucose uptake causing hyperglycemia. Lowering NEFA concentration in type II diabetes patients has been shown to improve insulin sensitivity on these affected tissues thereby lowering hyperglycemia [38-40]. Thus NEFA is a key contributor to insulin resistance and T2D. The capacity of adipocytes to store large amount of lipids and buffer FA flux, gives the adipose tissue a unique ability of protecting other organs and tissues from NEFA. This FA absorbing ability is often impaired in malfunctioning adipocytes, which display larger cell sizes compared to healthy ones. It has been also indicated that the number of adipocytes is not proportional to the degree of obesity but that the adipocyte size is probably the problem [3]. Therefore adipocytes' FA incorporating capacity is likely the one possible explanation why obesity is the risk factor of the development of T2D.

The Origin of NEFA: Synthesis and Hydrolysis of Triglycerides

The concentration of NEFA in blood is subjected to rapid variations associated to the ingestion of food and the nature of the food ingested. However, aside from these factors, the average NEFA concentration in plasma is mostly determined by the ratio between the adipose tissue output and uptake of NEFA. Thus, a proper balance between the rates of synthesis and degradation of TG in adipose tissue appears as major determinant of the probability to develop T2D.

The rates of TG synthesis and degradation are hormonally controlled. Among the hormones insulin, glucagon and nor-epinephrine play major roles [41, 42]. However, several other factors and hormones influence the rates of TG synthesis and hydrolysis. Among these are the concentration of glucose [43], tumor necrosis factor NF-kappa beta (NF-kβ) [44], adiponectin [45], HGH, and so on. The rate of TG hydrolysis is determined by the activity of two lipases, hormone sensitive lipase (HSL) and adipose TG lipase (ATGL) [46, 47]. These enzymes are directly (HSL) or indirectly (ATGL) regulated by reactions of phosphorylation/dephosphorylation catalyzed by kinases. A major kinase involved in HSL regulation is cAMP-acitivated protein kinase (PKA) [48], which is activated at low insulin levels and the presence of β-adreneric receptor agonist such as isoproterenol. ATGL is regulated by CGI-58 [49]. Apart from the regulation by kinases the expression levels of ATGL appear to play a major role in the rate of lipolysis.

The rate of synthesis of TG can be regulated at several enzymatic steps involved in the conversion of FA into TG. One of the main control points of the rate of TG synthesis could reside in the availability of G3P [50]. Because under most physiological conditions adipocytes do not express glycerol kinase, the capability of adipose tissue to phosphorylate glycerol and re-use it for the synthesis of TG is negligible [51]. For this reason, most of the glycerol produced during lipolysis is released from adipose tissue and re-utilized in the liver, which can phosphorylate and incorporate it into the glycolytic or gluconeogenic pathways.

It has been suggested that when insulin is not sufficient (fasting) or when the adipose tissue presents insulin resistance the availability of glucose is limited and the synthesis of G3P from glucose would not be sufficient to support the required synthesis of TG… It has been proposed that under these conditions glyceroneogenesis would supply the equivalents of G3P required. Investigation of the relative contributions of glycolysis and glyceroneogenesis to the synthesis of glyceride-glycerol (Glycerol constituting the backbone of TG) is a major goal of our work.

Metabolic Effects of Rosiglitazone, a PPAR-γ Activator, Commonly Used to Treat T2D: Role of Glyceroneogenesis and Glycerol Phosphorylation

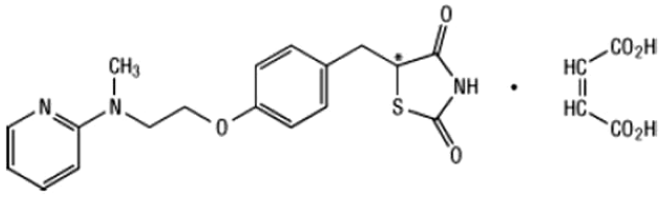
Since controlled diet and regular exercise are insufficient to improve insulin resistance and hyperglycemia [52], pharmacological strategies play important roles in the treatment of T2D. The anti-diabetic drugs released to the market in past few decades have one or more of following effects: (a) stimulate insulin release from pancreas [53, 54], (b) improve insulin sensitivity in peripheral tissues (liver, muscle, and adipose tissue) [55, 56], (c) prevent glucose uptake from intestine [57], and (d) inhibit hepatic glucose production [58]. Several combinations of these agents are used to maximize therapeutic effects to improve diabetic conditions.

Rosiglitazone (RGZ, Avandia) is a hypoglycemic drug used for the treatment of T2D and it is a member of the thiazolidinediones (TZDs) class of chemicals. RGZ improves insulin sensitivity of peripheral tissues to enhance glucose disposal. TZDs are agonists of peroxiosome proliferator-activated receptor-γ (PPAR-γ), a member of nuclear receptor transcription factors that is primary expressed in adipocytes [59]. Normally PPAR-γ

expression is induced during differentiation of pre-adipocytes into mature adipocytes (adipogenesis) [60], and individuals with the dominant-negative mutation in PPAR-γ expression appear to be severely insulin resistant and lipodystrophyic [61].

PPAR-γ activation by TZDs in mouse adipocytes has been reported to up-regulate expression of several adipose genes encoding proteins involved in energy metabolism, including proteins related to FA transport, oxidative phosphorylation, lipid catabolism and TCA cycle [62]. These observations imply that the primary target of TZDs is adipocytes/adipose tissue. However, insulin-sensitizing effects on other peripheral tissues such as liver and muscles are also observed. For example, muscle cells barely express PPAR-γ, but RGZ promotes a major improvement on insulin sensitivity and glucose disposal on skeletal muscle [63]. This action of the drug is thought to be mediated in part by the effect of RGZ on the concentration of circulating NEFA.

PPAR-γ works with several different transcriptional factors as a co-activator or co-repressor of transcriptions of various genes. RGZ has shown to have insulin-sensitizing and glucose lowering effects on patients with T2D.



Rosiglitazone

Glyceroneogenesis

Glyceroneogenesis is a truncated version of gluconeogenesis (Fig.1) and is a largely unexplored metabolic pathway [64]. In glyceroneogenesis, non-glucose precursors such as lactate, pyruvate, and alanine are used to produce glycerol 3-phosphate (G3P) [50]. It has been shown that in lipolytic conditions, such as fasting, pyruvate carobxylase (PC) and cytosolic phosphoenolpyruvate carboxykinase C (PEPCK-C) are up-regulated in adipose tissue. PEPCK-C converts oxaloacetate generated from pyruvate into phosphoenolpyruvate, and subsequent reactions produce G3P. The rate of synthesis of G3P in adipocytes is thought to be important in determining the capacity of adipose tissue

to esterify FA and, ultimately, the rate of NEFA clearance from plasma. Enhanced G3P production in adipocytes could facilitate the rate of FA esterification promoting a decrease of NEFA in plasma and, thus, improving insulin sensitivity of peripheral tissues.

PEPCK-C activity is observed in several different tissues including kidney, liver, and muscle, but the enzyme is barely active in adipocytes. Studies have shown that RGZ treatments up-regulate PEPCK-C mRNA expression and increase the enzymatic activity in mouse and human adipose tissues in fasting lipolytic condition [65]. The studies also reported that increased incorporation of radioactivity (cpm) of [$^{14}C_1$]-pyruvate into lipid upon activation of PEPCK-C activity in RGZ-treated adipocytes. Thus, some studies have proposed that RGZ improves the health of type II diabetic patients by promoting glyceroneogenesis in adipocytes [66-69].

However, transgenic mice overexpressing PEPCK-C in adipose tissue appeared to be more susceptible to diet induced insulin resistance and weight gain, and showed no changes in NEFA concentration in plasma [70]. This observation did not support the beneficial effect of glyceroneogenesis and/or a key role of PEPCK in controlling the rate of glyceroneogenesis.

A full understanding of the metabolic effects of RGZ in adipose tissue is lacking. Therefore one of the main goals of our studies is to gain insights into the metabolic consequences of RGZ treatment (Chapters III & IV).

Glycerol Kinase

An alternative mechanism to synthesize G3P is by direct phosphorylation of glycerol in a reaction involving glycerol kinase (GK). This enzyme is expressed in several tissues but it is specifically active in liver and absent in adipose tissue (Fig. 1). A relatively recent study reported that induction of GK expression in response to TZD treatment in human and mouse adipose tissue [71]. This is surprising since GK expression was thought to be absent in white adipose tissue. Moreover, the study reported that RGZ enhances GK activity and increases the incorporation of [^{14}C]-glycerol into lipids. Therefore, it was proposed that GK expression could be a key contributor to the anti-diabetic effects of TZD's. On the other hand, a follow up study failed to replicate the findings and could not demonstrate changes in GK expression upon RGZ treatment in mouse and human adipocytes [72]. Moreover, as in the case of glyceroneogenesis, the study supporting a

role of GK in TG synthesis did not provide quantitative estimates of the rates of incorporation of glycerol into lipids.

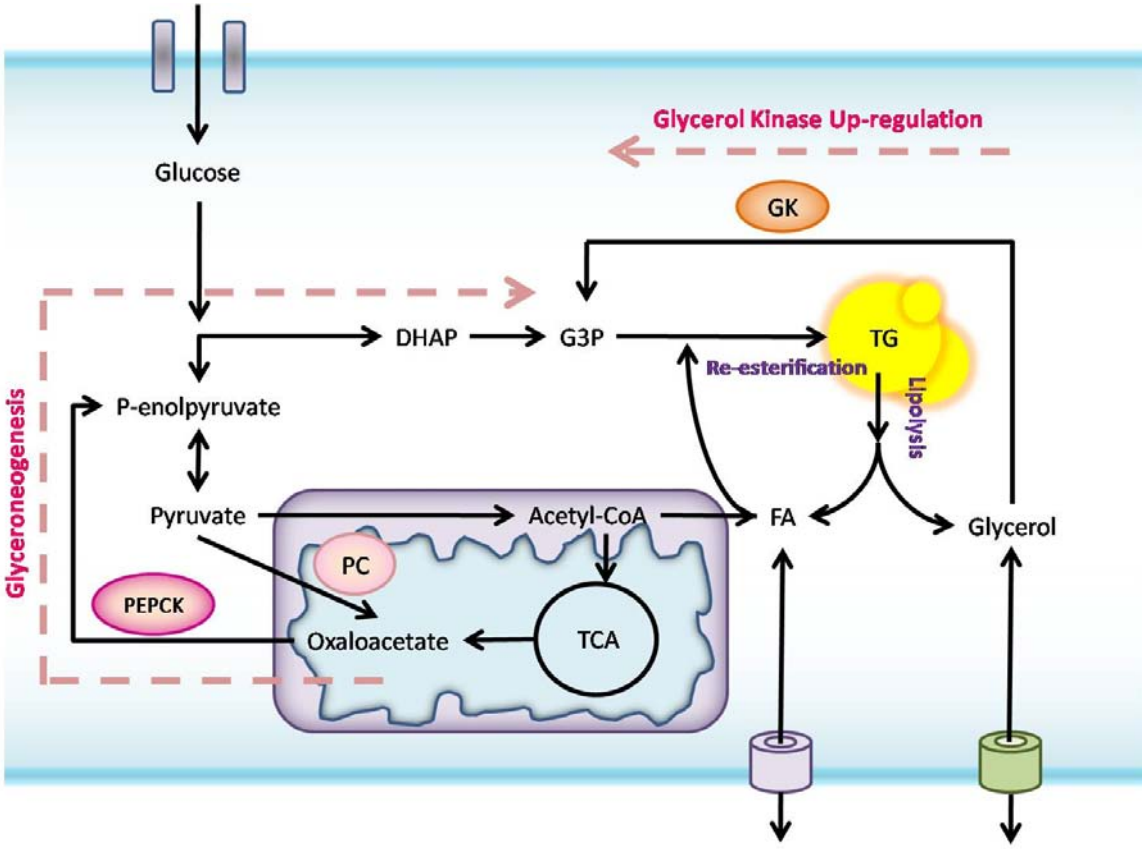


Figure 1: Metabolic routes involved in the synthesis of glyceride-glycerol in adipocytes. The figure illustrates an abbreviated version of the metabolic pathways involved in the synthesis of glyceride-glycerol (G-G) that could contribute FA lowering action of RGZ in adipocytes. Glycerol 3-phosphate (G3P, which eventually serves as G-G of TG) synthesis though glyceroneogenesis requires up-regulation of phosphoenolpyruvate carboxylase-C (PEPCK) and pyruvate carboxylase (PC). Glycerol kinase (GK) is required for the phosphorylation of glycerol which can be generated from lipolysis or taken up from plasma. Lipolysis and FA re-esterification are also emphasized.

Research Objectives and Significance

This project was based on the notion that previous studies have not addressed in a quantitative fashion the metabolism of glucose and pyruvate in adipocytes. We have hypothesized that it is possible to determine the contribution of glucose and pyruvate carbons to the synthesis of the glycerol backbone of TG. If this is possible, then, we could also gain a better understandings of the mechanisms by which TZDs, and RGZ in particular, have an anti-diabetic effect. In this context we have pursued the following specific aims:

- Study the metabolism of glucose in adipocytes and determine the rate of glyceride-glycerol (G-G) synthesis from glucose under different metabolic conditions (Chapter II).

- Investigate some of the possible causes of the metabolic changes induced by RGZ (Chapter III).

- Investigate the changes induced by RGZ on the metabolisms of glucose and triglycerides in adipocytes (Chapter IV).

- Estimate the contribution of pyruvate to the rate of synthesis of G-G in adipocytes under different physiological conditions (Chapter IV).

- Determine the overall contribution of glyceroneogenesis to the synthesis of glyceride-glycerol in adipocytes under different physiological conditions. Determine the effect of RGZ treatment on the rate of glyceroneogenesis and its overall contribution to the synthesis of glyceride-glycerol (Chapter IV).

Given the major role of adipose tissue in the development of diabetes type II, it is important to improve our understanding of the metabolism of glucose and triglycerides in adipocytes. Although many studies have previously addressed several aspects of the metabolism of TG and glucose, the present studies intend to fill some gaps that are considered of significance for the understanding of adipose tissue physiology and its role in the development of diabetes. Moreover, a full understanding of the metabolic effects of RGZ in adipose tissue is lacking. One of the main goals of our studies is to gain insights into the metabolic consequences of RGZ treatment (Chapters III & IV). By gaining knowledge on the effects of a drug that is beneficial to the treatment of diabetes, we may learn something significant for the understanding of the development and/or prevention of T2D.

CHAPTER II

ESTIMATION OF METABOLISM OF GLUCOSE AND TG IN ADIPOCYTES

Introduction

Adipose tissue is a very active organ in the regulation of the availability of substrates for energy production. TG is the major lipid component of adipocytes and the source of FA to other tissues when the supply of glucose is limited. Since an excess of FA has pathogenic effects on tissues and organs, the rate of FA output must be tightly regulated. Regulation of the synthesis of glyceride-glycerol (G-G) in adipocytes is essential to maintain proper NEFA concentration in plasma.

In this chapter we estimated the rates of utilization of glucose, and the fate of glucose carbons, in adipocytes in the presence and in the absence of insulin. We also describe a method that allows the estimation of the rate of incorporation of glucose carbons into the glycerol backbone of TG.

Experimental Procedures

Materials. 3T3L-1 mouse cell line was obtained from American Type Cell Culture (Manassas, VA). Fatty acid free bovine serum albumin (FA-free BSA), isobutyl methyl xanthine (IBMX), dexamethasone (DEX), trypsin, biotin, sodium pyruvate, insulin, streptomycin and penicillin were purchased from Sigma Chemicals Co. (St. Louis, MO). Fetal bovine serum (FBS) and calf bovine serum (CBS) were obtained from Hyclone (Logan, UT).

Dulbecco's modified Eagle's medium (DMEM) was purchased from Cellgro Mediatech, Inc (Herndon, VA). Rosiglitazone (RGZ) and [U-^{14}C]-glucose were purchased from American Radiolabeled Chemicals (St. Louis, MO).

Cell culture and differentiation. To obtain a homogeneously differentiated culture of adipocytes we used an experimental procedure as described by Rubin et al.[73] 3T3L-1 pre-adipocytes were maintained at 37°C in 8% CO_2 atmosphere in high glucose DMEM supplemented with 10% CBS and 0.01% antibiotics. Upon confluence, differentiation from preadipocytes into mature adipocytes was induced by addition of medium containing DMEM/10% FBS, IBMX (111 μg/ml), DEX (0.46 μg/ml), and insulin (1.5 μg/ml) supplemented with biotin (4 μg/ml) and sodium pyruvate (100 μg/ml). After 72 hours, the cells were incubated in DMEM/10% FBS containing insulin, biotin, and sodium pyruvate for another 72 hours. Once differentiation was completed, the cells were maintained in the DMEM/10% FBS medium. All metabolic studies were performed in fully differentiated cells. This is achieved between 9 and 15 days after initiation of the differentiation.

Metabolism of glucose and lipids in adipocytes. Mature adipocytes were incubated in the DMEM/10% FBS medium. Cells were briefly washed by incubating them in Krebs Ringer Bicarbonate (KRBH) medium containing 6mM glucose and 0.12% FA-free BSA. The wash medium was replaced with the KRBH experimental medium containing either insulin (1μg/ml), isoproterenol (1μg/ml), or DMSO (control) in addition to 6.0mM glucose and 0.12% albumin. Immediatly after replacing the wash medium with experimental medium, [U-^{14}C]-glucose (1.5 to 6μCi/well, depending on plate size) was added. The incubation in the presence of radiolabeled glucose proceeded for 1h. After this time, the medium was removed and placed on ice and the cells were homogenized in 150 mM Tris buffer containing 50 mM sodium chloride (pH 7.5).

Medium analysis. The levels of fatty acids, glycerol, glucose and lactate in the incubation medium were determined by spectrophotometric methods. NEFA and glycerol concentration were measured with the enzyme coupled colorimetric kits (NEFA: NEFA-C; Wako, Richmond, VA, glycerol: Infinity Triglyceride; Sigma, St.

Louise, MO). Lactate was quantified based on its oxidation by NAD^+ catalyzed by LDH [74].

Lipid analysis. Total lipids were extracted from cell homogenates using the Folch method [75]. Lipid classes, phospholipid (PL), diglyceride (DG), FA, and TG, were separated by thin layer chromatography (TLC) on silica gel 60 Å plate (Whatman, Maidstone, UK) using hexane: ethyl ether: formic acid (70:30:3 by vol) as developing solvent.[76] Lipid spots were visualized with iodine vapor, and the radioactivity associated to each spot was determined by scintillation counting.

TG hydrolysis was performed to determine percentages of radioactivity incorporated into the glyceride (G-G) backbone and fatty acid chains of TG. Triglyceride spots were scrapped from the TLC plates and the lipid extracted from the silica using Folch solvent. TG extracts were dried and hydrolyzed by incubation with 0.5 M potassium hydroxide in ethanol at 65 °C for 1 hour. The solution was acidified with 12 M hydrochloric acid, and glycerol and fatty acid were separated based on their solubility in water and Folch solvent (chloroform:methanol = 1:1 by vol). Radioactivity in glycerol and fatty acids was determined by scintillation counting. The percentages of radioactivity in total lipid extract, different lipid types, and glyceride-glycerol (G-G) backbone and FA chain of TG were used to determine rates of glucose conversion into lipids.

Glycogen content. Cellular glycogen content was assayed using the samples which were frozen immediately after homogenization. Briefly, cell homogenate was boiled and centrifuged to remove protein pellet. Supernatant was treated with amylo-glucosidase in 0.8M acetate buffer (pH. 4.5) to digest glycogen to glucose. Glucose content was measured using HK glucose kit (Sigma-Aldrich, St. Louise, MO) based on oxidation of NADH.

Intracellular specific activity of glucose. The specific activity of lactate (SA*lac*, cpm/nmol) derived from [U-^{14}C]-glucose was estimated to determine the intracellular specific activity of glucose (SA*glu*). The amount of lactate produced (1h) was calculated from the difference between initial and final concentrations of lactate in the cell culture medium. The radioactivity associated with lactate in the cell culture medium was determined after separation of the lactate by TLC. Briefly, 5 µl of medium

was loaded onto a silica gel 60 Å TLC plate (Whatman, Maidstone, England), and developed with a solvent mixture containing methanol: water: ammonium hydroxide (60:39:1=v:v:v). The spots from lactate and other radiolabeled compounds were visualized by autoradiography with Fuji RX film (Fuji Medical Systems, Stevenson Court, IL) after 12 hours to 1 week of exposure time. The spot corresponding to lactate was scraped, and radioactivity was counted using a scintillation counter.

Calculations. Rates of glyceride-glycerol (G-G) backbone synthesis (total TG synthesis = de novo TG synthesis + TG re-esterification) from glucose were calculated from the radioactivity incorporated into lipids and the specific activity of glucose. The rates of incorporation of glucose carbons into TG, G-G and fatty acids (FA-chains), were calculated from the distribution of radioactivity (percentages) among lipid species, and between the fatty acyl chains and G-G backbone of TG.

Other methods. The protein concentration in cell homogenates was determined spectrophotometrically in the presence of 10% SDS [77].

Results

Utilization of glucose carbons for lipid synthesis. To study the metabolism of glucose and lipid, adipocytes were incubated in KRBH medium (9.5 mM glucose/ 0.12 % BSA in KRBH) with [U-^{14}C]-glucose. Three different metabolic conditions were generated and analyzed by incubating the cells in KRBH medium containing: a) Insulin, to induce a low lipolytic state and promote lipogenesis; b) Isoproterenol, which stimulates lipolysis and; c) DMSO (control medium), which is the solvent used to dissolve isoproterenol. Exactly one hour after the addition of radioactive glucose, medium in each well was separated and cells were homogenized and frozen. The effect of the hormones on the metabolic state of adipocytes was confirmed by measuring the levels of certain medium metabolic parameters. As shown in Table I, insulin promotes a decrease in the release of glycerol to the medium, indicating a decrease in the rate of lipolysis (TG hydrolysis). As expected, isoproterenol significantly enhanced the lipolytic rate of adipocytes. These effects are

also reflected in the levels of NEFA. The increase in lactate levels observed with both insulin and isoproterenol indicated that these hormones enhance the rate of glycolysis.

Table I: Effects of insulin and isoproterenol on the metabolism of adipocytes.

nmol/mg.h	**Control**	**Insulin**	**Isoproterenol**
Glycerol	13.38 ± 1.9	7.23 ± 0.5	92.15 ± 1.8
Lactate	182.34 ± 19.1	258.04 ± 7.2	421.87 ± 26.7
NEFA	2.38 ± 0.5	0.13 ± 0.04	11.95 ± 1.16

Glycerol, lactate, and NEFA released into the experimental medium were spectrophotometrically measured after the 1 hour incubation with DMSO (control), insulin, and isoproterenol in KRBH medium. The initial components of the experimental medium besides the corresponding hormones included 9.5 mM glucose, 0.12 % FA-free BSA, and KRBH. The data shown represent the means (nmol/mg.h) ± SD (n=3).

The incorporation of glucose carbons into lipids and the distribution of radioactivity among cellular lipid classes were determined in the lipids extracted from cell homogenates. The amount of radioactivity incorporated in total lipids was highest in the cells incubated with insulin. In control and insulin containing media most of the radioactive glucose was incorporated into PL (~30 %) and TG (~60 %). However, about 80% of the [^{14}C]-glucose incorporated into lipids was found in TG when the cells were incubated in the medium containing isoproterenol. This result suggests a relationship between TG synthesis and hydrolysis and the rate of glucose metabolism in 3T3L-1 adipocytes (Fig. 2A and B).

To calculate the rate of glucose carbon utilization for the synthesis of glyceride-glycerol (glycerol backbone), TG isolated by TLC were subjected to alkaline hydrolysis to cleave FA chains from a glyceride backbone. Glycerol and FA were separated based on their solubility in organic and aqueous solvents. The fraction of [^{14}C]-glucose used for the synthesis of FA and glyceride-glycerol were calculated from the radioactivity associated with FA and glycerol. The Figure 2C shows that in the presence of insulin a large proportion of the glucose carbons incorporated into lipids was used for fatty acid synthesis (~ 65%), whereas ~35% is used for the synthesis of the glyceride backbone of TG. Conversely, under lipolytic conditions (isoproterenol) ~90 % of [^{14}C]-glucose carbons are used for the synthesis of glyceride backbone.

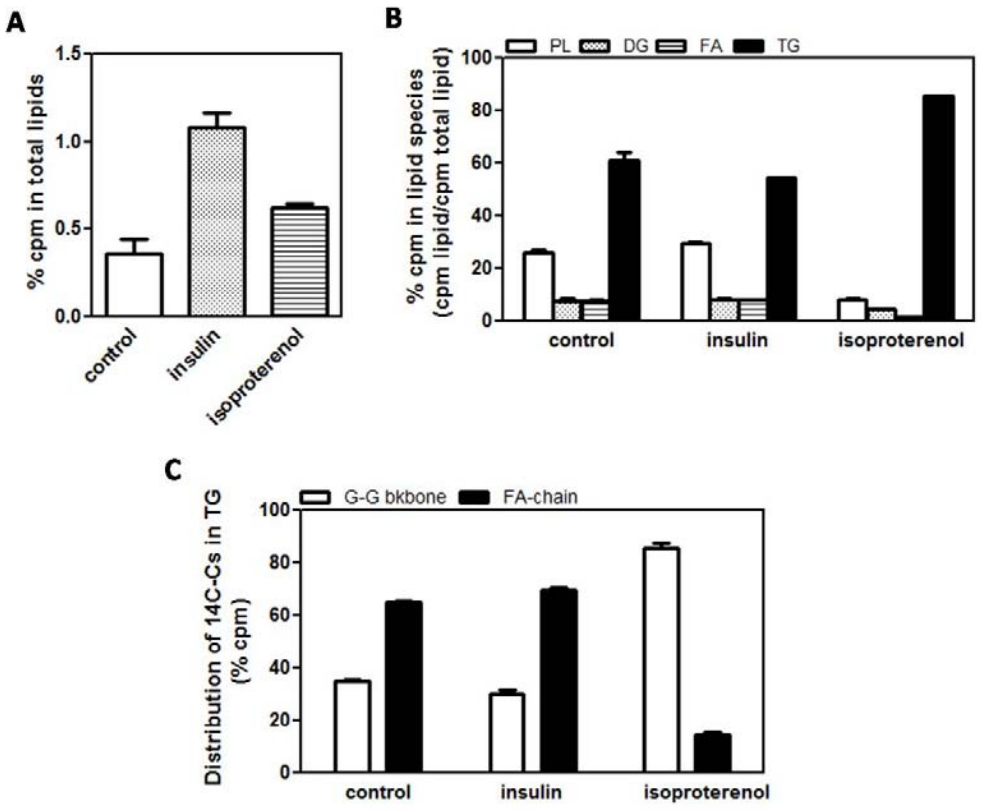


Figure 2: Incorporation of glucose carbons into the lipids of adipocytes. A) Percent of radiolabelled glucose incorporated into total lipids of adipocytes incubated with hormones and [U-^{14}C]-glucose; B) Relative distribution of glucose carbons among lipid classes (PL: phospholipids, DG: diacylglycerol, FA: fatty acids, and TG: triglyceride); C) Distribution of radiolabeled carbons between the FA chain and glycerol (G-G) backbone of TG. All data is expressed as mean ± SD (n=3)

Actual rates of glucose conversion into lipids. In the previous sections, we obtained percentages of radioactivity incorporated into the lipid fractions under different conditions. These numbers are useful but do not provide all the information needed to calculate the rate of utilization of glucose carbons for the synthesis of glyceride-glycerol or fatty acids. In order to determine these rates, it was necessary to estimate the specific activity of glucose inside adipocytes. Since adipocytes produce large amounts of lactate (Table I and Fig. 3A), which is expected to be derived mostly from the metabolism of glucose, an estimate of the glucose specific activity inside of cells was calculated by determining the specific activity of lactate released into medium. Due to the difference in the rates of glycogenolysis (Fig. 3B), the specific activity of glucose was dependent on the cell treatment and significantly different among the conditions of the incubation. The

specific activity of glucose in cells incubated with isoproterenol was lower compared to that of control and insulin treated cells.

The estimated rates of glucose conversion for the synthesis of lipids showed that, compared to control and insulin treated cells, adipocytes incubated with isoproterenol use more glucose to make both total lipids and TG (Fig. 3C and D). The rates of TG synthesis were estimated from the specific activity of glucose, the radioactivity in total lipid extracts, and the fraction of radioactivity in TG and in the glyceride backbone of TG (Fig. 3F).

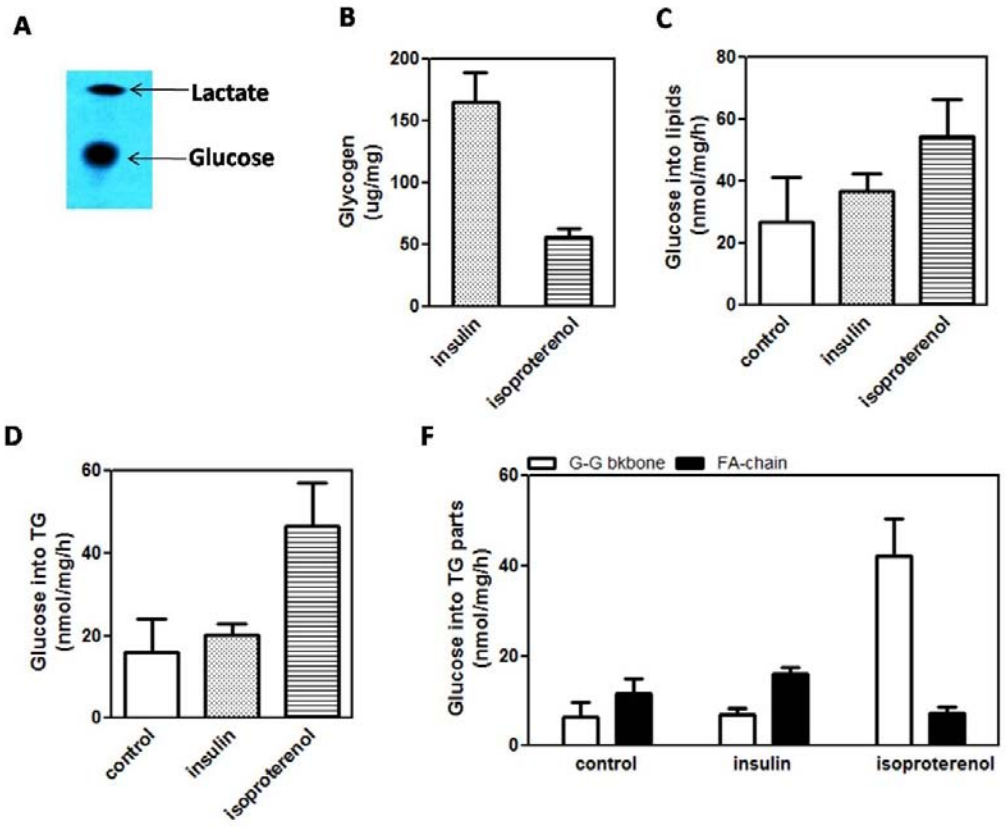


Figure 3: Rates of glucose utilization for lipid synthesis. A) Two major medium metabolites, lactate and glucose, were separated using aqueous-base TLC, and visualized by autoradiography. Radioactivity in each spot was counted to determine the specific activity of lactate (cpm/nmol) with the amount of lactate present in the medium; B) Intracellular glycogen content was estimated to determine the effects of hormones; C) Rate of incorporation of glucose carbons in total lipids; D) into TG and F) into the glycerol backbone and fatty acid moieties of TG.. These rates were calculated using the specific activity of glucose inside the cells. All data are shown as mean ± SD (n=3).

Summary

In this chapter, we have shown results, that as shown by previous studies in adipocytes, were expected. For instance we have shown that incubation with insulin promotes a low lipolytic state and accordingly adipocytes release less glycerol and NEFA. Similarly, we have shown that the cells release large amount of glycerol and NEFA in the presence of isoproterenol, which is a well known lipolytic agent (Table I). These results have been shown in several previously reported studies [78, 79].

We have also shown that the utilization of glucose increases significantly not only in the presence of insulin, but also in its absence when the cells are exposed to isoproterenol. The stimulation of glucose utilization when lipolysis is stimulated has been previously demonstrated [80]. However, there is a common belief that cells cannot use much glucose in the absence of insulin. In this sense reiterating old studies that are often forgotten was necessary. The studies included in this chapter were also necessary for us to find out how much we could do with the small amounts of material available from adipocytes grown in tissue culture plates.

The most important finding of the studies shown is that it is possible to estimate the intracellular specific activity of radiolabeled glucose. This is an original aspect of the studies and it is important because it allows estimating the rates of utilization of glucose carbons for the synthesis of fatty acids and glyceride-glycerol. Here, we showed that an increase in the lipolytic rate is accompanied by a concomitant increase in the incorporation of glucose carbons in the glycerol backbone of TG. This result shows that even in the absence of insulin, adipocytes re-esterify most of the fatty acids produced by the increased lipolytic rate, and this generates a demand for glucose carbons to produce glycerol-3-phosphate (G3P). In the presence of insulin, the incorporated glucose is utilized mostly for the synthesis of fatty acids.

The studies shown in this chapter constituted the basis of the studies directed to understand the effects of RGZ in the metabolism of glucose and TG in adipocytes that are presented in the following chapters.

CHAPTER III

CHANGES IN THE METABOLISM OF GLUCOSE AND TG IN ADIPOCTE TREATED WITH ROSIGLITAZONE

Introduction

RGZ improves insulin sensitivity of the body and decreases hyperglycermia and NEFA in T2D patients. The beneficial effects of RGZ translate in an enhanced glucose disposal in muscle and suppressed hepatic glucose production in liver. Since the target of RGZ, PPAR-γ, is mainly expressed in adipose tissue, the origin of anti-diabetic effects of this drug are thought arise from the metabolic changes induced in adipose tissue. The purposes of the studies presented in this chapter were to:

1. *Investigate the changes induced by RGZ on the metabolisms of glucose and triglycerides in adipocytes.* These studies were directed to learn about the role of adipocytes in the glucose and NEFA lowering effects of RGZ. We investigated the effects of RGZ on: a) the rate of utilization of glucose; b) the rate of synthesis and hydrolysis of TG.

2. *Investigate some of the possible biochemical causes of the metabolic changes induced by RGZ.* We investigated changes in the expression and/or activity of certain enzymes involved in the metabolism of glucose and triglycerides.

.

Experimental Procedures

Metabolism of glucose and lipids in adipocytes. Mature adipocytes were incubated in the DMEM/10% FBS medium containing either ethanol (control) or 500 nM RGZ for 24 hours. After 24 hours, cells were briefly washed by incubating them in KRBH medium containing 6mM glucose, 2mM lactate, and 0.12% FA-free BSA. The wash medium was replaced with the KRBH experimental medium containing either insulin (1μg/ml), isoproterenol (1μg/ml), or DMSO (control) in addition to 6.0mM glucose, 2mM lactate, and 0.12% albumin. The incubation in the presence of radiolabeled glucose proceeded for 1h. After this time, the medium was removed and placed on ice and the cells were homogenized in 150mM Tris buffer containing 50mM sodium chloride (pH 7.5).

Enzyme Activity Assay. Activities of hexokinase (HK) [81], glycerol 3-phosphate dehydrogenase (GPDH) [82], lactate dehydrogenase (LDH) [83], and glyceraldehydes 3-phosphate dehydrogenase (GAPDH) [84] were measured spectrophotometrically based on the oxidation/reduction of NAD^+/NADH as previously described. Glycerol kinase (GK) activity was determined spectrophotometrically using a colorimetric assay [85, 86].

Transcriptional changes in glycerol kinase expression: The transcriptional levels of GK were determined by reverse transcription PCR. Total RNA was extracted from 3T3L-1 adipocytes incubated in the medium containing either 500nM RGZ or ethanol (control) for 24 hours with Trizol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instruction. One step RT-PCR was performed to the total RNAs for GyK, and 18SrRNA (18R) with Qiagen one-step RT-PCR kit (Qiagen, Valencia, CA). Integrated Density Values (IDVs) for GK was normalized by IDV of 18R using AlphaEaseFC program (Alpha Innotech Corp, San Leandro, CA). Mouse primers used were following: GK sense 5'-GCTGTAATCCGCTGGCTAAG-3', and antisense 5'-GTGCAGCAAAAGCGATATGA-3'; 18R sense 5'-TACCACATCCAAGGAAGGCA-GCA-3', and antisense 5'-TGGAATTACCGCGGCTGCTGGCA-3'. All primers were designed and obtained from Integrated DNA Technologies, Inc (Coralville, IA).

Western Blot. Western blot analysis was performed using whole cell homogenate of the cells treated with or without RGZ and the hormones. Aliquots of the cell homogenates containing identical amounts of protein (15 µg) were separated by SDS-PAGE in 4-20% gels and blotted onto nitrocellulose membranes. Bound antibodies were detected using the enhanced chemiluminiscence assay system (Pierce Chemicals, TX). The photographic films were scanned and the intensity of the spots determined by densitometry using the AlphaEaseFC program (Alpha Innotech Corp, San Leandro, CA).

Primary antibodies against mouse adipose triglyceride lipase (ATGL) and hormone sensitive lipase (HSL) were purchased from Cell Signaling Technology (Danvers, MA). Goat anti-mouse GAPDH primary antibody was obtained from Chemicon International, CA.

Statistics. The statistical significance between untreated and RGZ treated samples was determined with the Student's t-test using Instat software (GraphPad Software Inc, La Jolla, CA)

Other Methods. Cell culture, glycogen assay, and analysis of lipid and metabolites were performed using the procedures described in Chapter II. Same materials as in chapter II were used for the experiments (if not mentioned).

Results

Effect of RGZ treatment on fatty acid metabolism. We first tested the concentration of RGZ and time of incubation needed to observe metabolic changes in adipocytes. For this purpose we monitored the cellular output of NEFA. This study showed that, independently of the presence or absence of insulin, a maxium effect is reached at a concentration of RGZ of about 500nM (Fig. 4A). This concentration is similar to the average concentration found in plasma of patients treated with normal doses of RGZ. Several times of incubation were tested and it was found that after 16h of incubation the effects leveled off (Fig. 4B). Therefore, all the following experients were carried out with pre-incubation the cells with 500nM RGZ for 24h. After this treatment, the cells were

incubated in KRBH medium containing either insulin (1µg/ml), isoproterenol (1µg/ml), or DMSO (control) in addition to 6.0mM glucose, 2mM lactate, and 0.12% albumin (FA-free BSA).

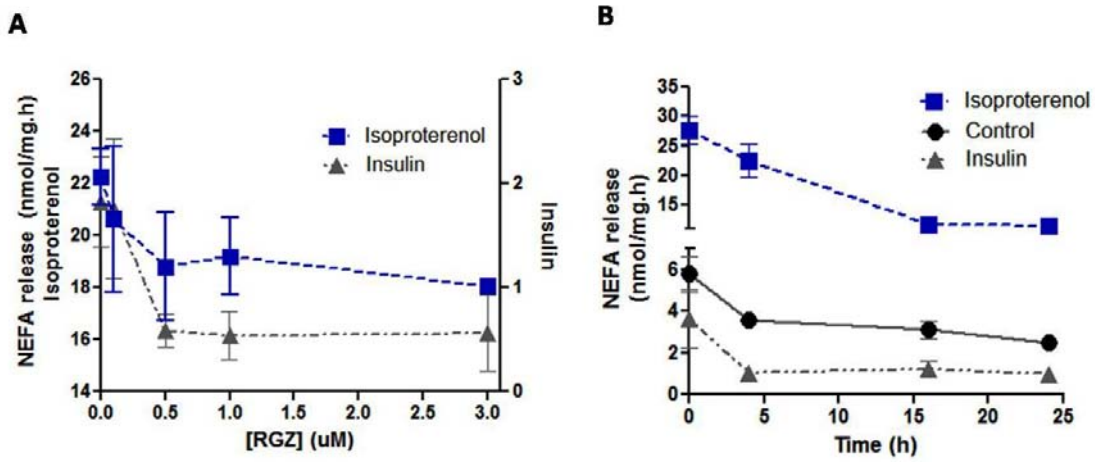


Figure 4. Effects of RGZ concentration and incubation time on NEFA release from adipocytes. Adipocytes were incubated with different concentration of RGZ for 24h, and NEFA release was measured (A). The effect of 500nM RGZ on NEFA release from adipocytes in the presence or absence of hormones was determined (B). All data represent mean ± SD (n=3, ●: control, ■:isoproterenol, ▲: insulin).

In order to investigate the effect of RGZ in TG metabolism we determined the cellular ouputs of NEFA and glycerol, as well as the cellular content of FFA and ability of the cells to metabolize externally added FFA. The effects of RGZ were determined under three lipolytic conditions, low (insulin), intermediate (no hormones added) and high (isoproterenol). Table II shows the average data of lipoylysis (glycerol release) and NEFA output from a single experiment. Figure 5A and 5B show the average changes (%) in NEFA and lipolysis from several experiments. Contrarily to the expected result, a decrease in lopolysis, treatment of adipocytes with RGZ promotes an increase in lipolysis. The results were somewhat surprising because simultaneously to the increase in lipolysis there is a decrease in the output of FA.

.

Table II. Glycerol and NEFA release in response to RGZ.

	Control		Insulin		Isorproterenol	
	−	+ RGZ	−	+ RGZ	−	+ RGZ
Glycerol Release (nmol/mg.h)	29.39 ± *5.2*	40.40 ± *6.7* **	26.34 ± *5.1*	37.25 ± *5.3* ***	197.48 ± *33.4*	284.58 ± *37.7* ***
NEFA Output (nmol/mg.h)	5.78 ± *1.1*	3.32 ± *0.4* **	3.19 ± *0.3*	2.46 ± *0.8*	47.59 ± *11.5*	28.84 ± *5.1* **

Adipocytes were incubated with 500nM RGZ (+RGZ) or DMSO (-) prior to the experiment for 24h. Glycerol and NEFA released into the incubation medium were using colorimetric assay after 1h incubation time as described in the method section. Initial components of the medium consisted of 6mM glucose, 2mM lactate, 0.12% FA-free BSA, and KRBH besides hormones (insulin and isoproterenol). Adipocytes All data is expressed as rate (nmol/mg.h) ± SD (n=5-9, **: $p<0.001$ and ***: $p<0.0001$ for +RGZ groups compared to non-treated (-) samples).

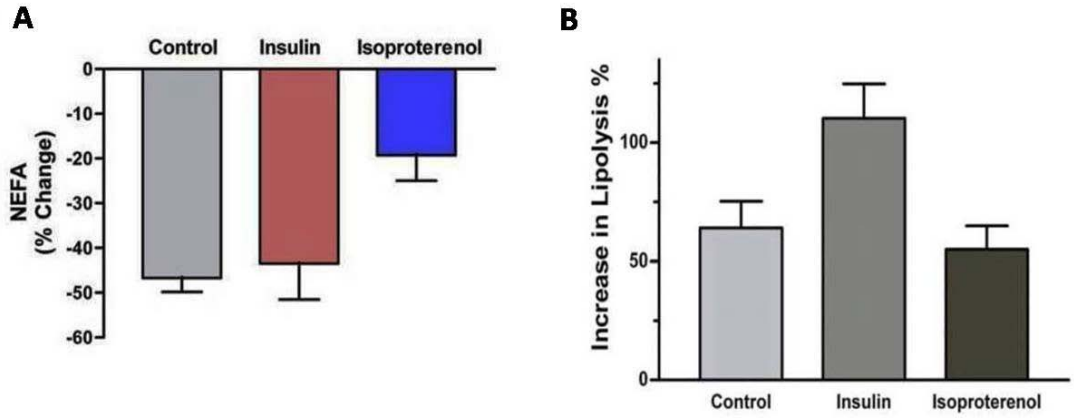


Figure 5. Average NEFA reduction and increase in lipolysis in RGZ-treated adipocytes. Average changes (%) of the rates of NEFA release (A) and lipolysis (B) were calculated using data from several experiments. All data is shown as mean ± SD (3-4 experiment, n=12).

In addition to these results, the analysis of lipid contents in adipocytes showed no increase in the levels of intracellular NEFA and TG (Fig. 6A and B). These results suggested that the lower cellular output of NEFA was due to a RGZ promoted increase in the synthesis of TG (FA re-esterification), which exceeds the increase in lipolysis. To confirm this interpretation the ability of adipocytes to uptake externally added NEFA was determined by incubating the cells in the presence of BSA containing 1.6mM FA. As shown in Figure 6C, under the three experimental lipolytic conditions tested, RGZ effectively increases the ability of adipocytes to uptake and esterify FA. Overall, the studies of lipolysis, NEFA release and uptake, intracellular TG and NEFA content indicate that RGZ promotes an asymmetric up-regulation of two opposite processes, lipolysis and NEFA re-esterification. Thus, RGZ promotes a synthesis biased increase in the rate of the futile cycle constituted by the processes of synthesis and hydrolysis of TG.

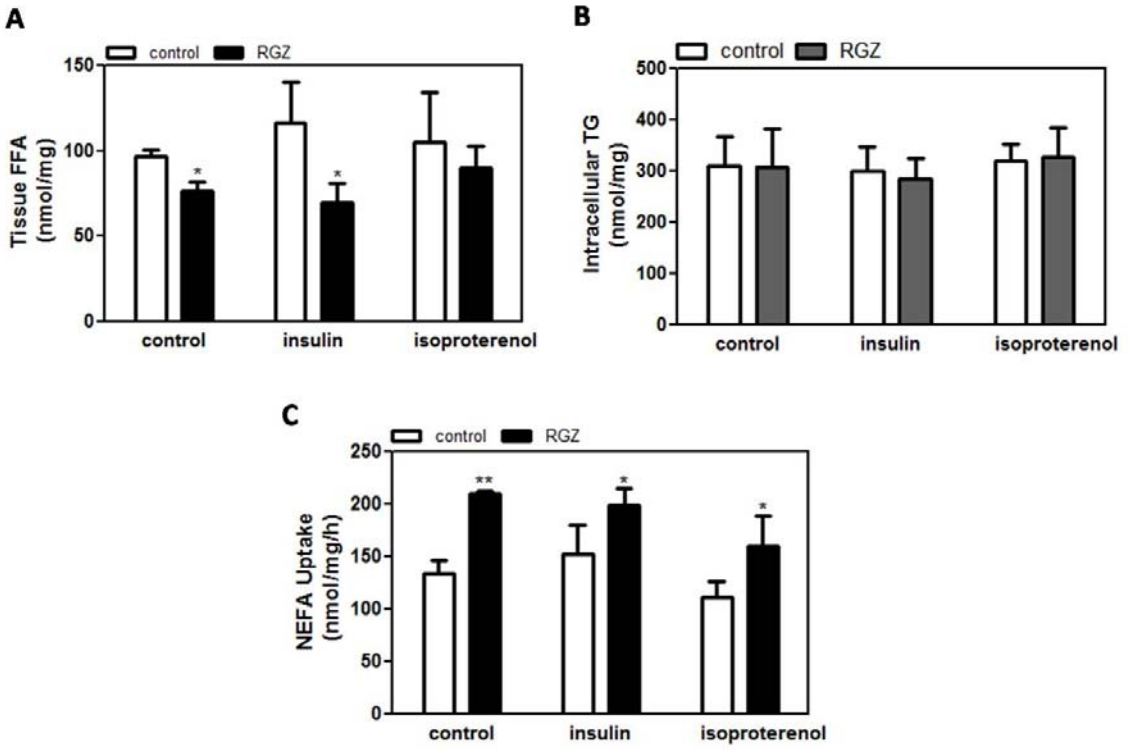


Figure 6. Effects of RGZ on intracellular FA, tissue TG, and NEFA absorption in adipocytes. Intracellular FA (A) and TG (B) were measured using cells incubated with different hormones for 1h after 24h RGZ treatment. NEFA uptake was studied in KRBH medium containing 1.6mM FA, 9mM glucose and 0.12% BSA after 1.5 hours of incubation time. The rate of NEFA absorption was calculated based on NEFA disappearance from the medium over time (C). Blank bars (□) indicate control (0h RGZ incubation) and filled bars (■) express RGZ-treated samples. All data show mean ± SD (n=3 for A, n=12 for B, n=9 for C. *: $p<0.05$ and **: $p<0.005$.)

Effect of RGZ treatment on glucose metabolism. To learn about possible effects of RGZ on the metabolism of glucose, the consumption of glucose, the levels of intracellular glycogen and the levels of lactate in the experimental medium were determined. These determinations were performed in untreated and RGZ-treated cells before and after a 1 h incubation period in the experimental medium. The data from one representative experiment are shown in Table III. These data indicate that RGZ promotes a large increase in glucose consumption and lactate production. Average increases in glucose consumption in RGZ-treated cells were similar in the absence of hormones –control cells- (~65%) or in the presence of isoproterenol (~61%), and lower in the presence of insulin (~38%). On the other hand, the increases in glucose consumption were accompanied by proportional increases in lactate production (Table III).

These results indicate that RGZ promotes an increase in the rate of glycolysis. In addition to the increase in uptake of extracellular glucose, RGZ treatment also accelerated the breakdown of cellular glycogen (Fig. 7A & B). Therefore, we can conclude that RGZ does not promote accumulation of glucose, but rather it promotes a significant increase in glycolysis. Overall, these studies have shown that RGZ increases the rate of glucose utilization to fuel the promoted increase in the rate of the futile cycle of TG synthesis and hydrolysis.

Table III. RGZ-induced changes in glycolysis

	Control		Insulin		Isorproterenol	
	–	+RGZ	–	+RGZ	–	+RGZ
Lactate Production (nmol/mg.h)	287.05± *155.2*	805.82 ± *264.4* ***	652.20 ± *133.2*	939.75 ± *186.4* ***	1304.15 ±*178.8*	2325.66 ±*568.4* ***
Glucose Consumption (nmol/mg.h)	325.28 ± *69.8*	536.21 ± *70.6* ***	498.98 ± *65.4*	689.62 ± *90.6* ***	539.82 ± *65.4*	869.12 ± *82.7* ***

Adipocytes were incubated with 500nM RGZ (+ RGZ) or DMSO (-) for 24 h prior to the *in vitro* experiment. Lactate released into and glucose consumed from the medium after 1 hour incubation were determined as described in methods. The initial medium contained 6mM glucose, 2mM lactate, 0.12% FA-free BSA, and KRBH besides hormones (insulin and isoproterenol). All rates are expressed as means ± SD (n=9-12, ***: $p<0.0001$ for +RGZ groups compared to non RGZ treated group in each lipolytic state).

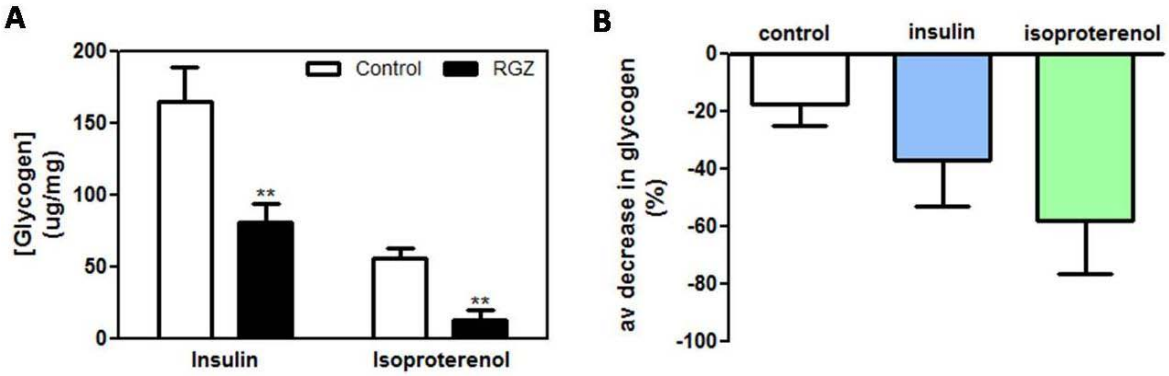


Figure 7. Intracellular glycogen content in adipocytesis is altered by RGZ. Intracellular glycogen concentration was measured in the cells treated with and without 500nM RGZ for 24 hours (A). Average decrease in glycogen concentration was calculated (B). After the incubation with/without 500nM RGZ for 24h the cells were maintained in the medium containing 9.5mM glucose and 0.12% BSA. The empty bar (□) indicates control (oh incubation with RGZ) and the black bar (■) represent incubation with RGZ for 24h. Data is expressed as mean ± SD (n=3 for the determination of glycogen concentration. 3-4 sets of experiments were used to estimate average reduction in response to RGZ. **: $p<0.005$).

Effect of RGZ on the activities and expression of some enzymes involved in TG and glucose metabolism. Since RGZ is an activator of the transcription factor PPARγ, it was reasonable to expect that changes in the expression of some enzymes were underlying the changes observed in TG and glucose metabolism. Therefore, to gain some understanding on the nature of the biochemical changes induced by RGZ, we determined the activity and/or expression of some key enzymes involved in glucose and TG metabolism.

The activities of glycerol 3-phosphate dehydrogenase (GPDH), hexokinase (HK), lactate dehydrogenase (LDH) and glyceraldehyde dehydrogenase (GAPDH) were determined in the cell homogenates. The activities among DMSO (control), insulin, and isoproterenol

treated groups were averaged because there was no significant difference in activity (data not shown). Consistently, with the increase in glucose consumption and lactate

production induced by RGZ treatment, this study showed that RGZ induced the expression, as inferred from the enzyme activities, of HK and LDH (Fig. 8). No significant changes were found in the activity of GAPDH. However, the study also showed that RGZ increases the activity of GPDH, an enzyme needed for the synthesis of G3P and, therefore, for the synthesis of TG.

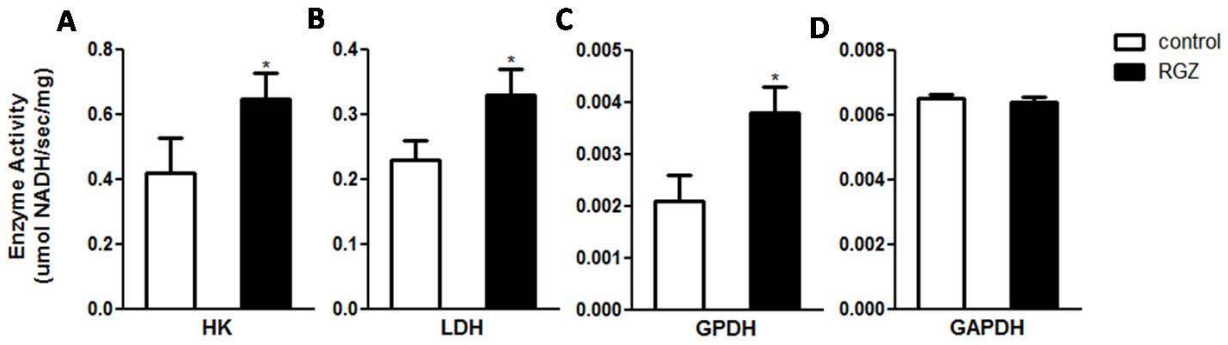


Figure 8. Effect of RGZ on the expression of glycolytic enzymes. Enzymatic activities of HK (A), LDH (B), GPDH (C), and GAPDH were determined in cell homogenates. Blank bars indicate control (□, no RGZ) and filled bars (■) show RGZ treated groups. All data are expressed as mean ±SD. (n=6 for HK, LDH, and GPDH, and n=12 for GAPDH. *$p<0.05$).

Given the fact that a previous study [71] suggested that RGZ would increase FA re-esterification due to its effect of the expression of glycerol kinase (GK), we also studied the effect of RGZ on the activity of GK and its expression, at the mRNA level. Analysis of GK mRNA expression, confirmed the reported study [71], and indicated that RGZ enhanced GK expression around 1.5 fold ($p<0.01$) in adipocytes (Fig. 9A). However, GK activity did not show any significant change in response to RGZ or hormone treatments (Fig. 9B).

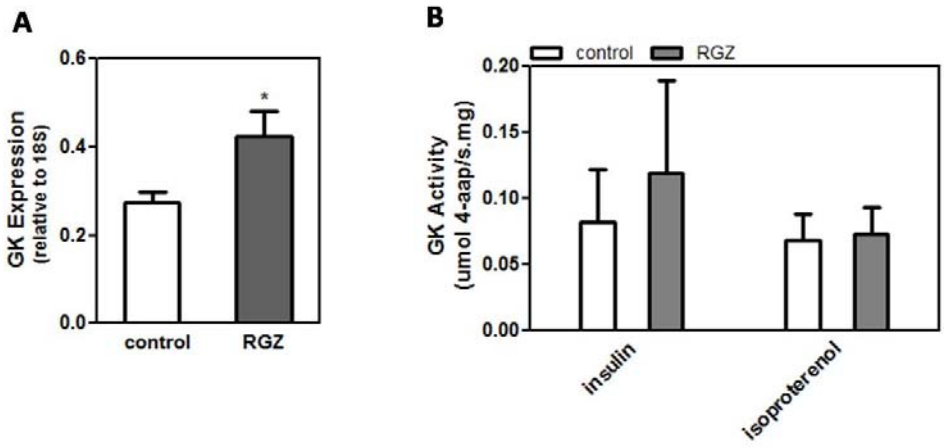


Figure 9. Rosiglitazone up-regulates GK expression but not activity. Expressions of GK mRNA were determined by RT-PCR (A). The enzyme activity of GK is determined using colorimetric assay (B). Blank bars (□) indicate 0h RGZ treatment whereas filled bars (■) show samples with 24h RGZ incubation. All data are expressed in mean ± SD (n=3, *: $p<0.01$).

Possible changes induced by RGZ in the expression of the two main lipases involved in the degradation of TG were investigated by western blotting. As shown in the Figure 10, adipocytes increased the expression of ATGL in response to RGZ treatment. Expression of ATGL was independent of the physiological condition. On the other hand, no changes in the expression of HSL were observed. These results suggest that the increase in the lipolytic rate observed in RGZ treated adipocytes is, at least in part, due to an increase in the expression of ATGL. Similar results on the expression of ATGL had been previously reported [87].

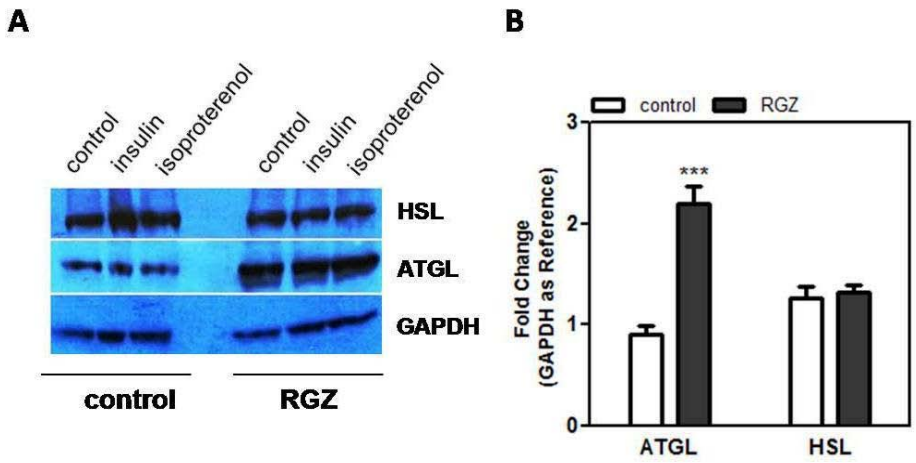


Figure 10. Effect of RGZ on the expression of lipases. Western blots against ATGL, HSL, and GAPDH (as reference) were performed in cell homogenates (A). The figure B shows the relative changes in expression. These were calculated from the average of the combined data (control, insulin and isoproterenol). Blank bars (□) indicate control (no RGZ) and filled bars (■) show RGZ treated groups. All data are expressed as mean ±SD (n=3).

Summary

Our studies have shown that RGZ increases the lipolytic activity of adipocytes and, concomitantly, it increases the rate of synthesis of TG. This increase in the rate of the futile TG cycle requires a major input of energy to re-esterify the excess of fatty acids produced [80, 88]. In agreement with this, RGZ treated adipocytes consume higher amounts of glucose and produce more lactate. The increase in the rate of the futile cycle is asymmetric and biased toward the synthesis of TG. This bias may be responsible for the lower output of NEFA observed in RGZ treated adipocytes (Fig.11).

In this study we have only determined the activity of a handful of enzymes. However, we observed the increase in the expression and activity of ATGL, HK, and LDH. These enzymes could explain most of the effects of RGZ on the metabolism of glucose and on the hydrolysis of TG. We have not studied most of the enzymes involved in the esterification of fatty acids and TG synthesis. However, the increase in GPDH activity suggest that part of the increase in the rate of TG synthesis induced by RGZ could be due to the increased production of G3P. The lack of increase in GK activity suggests that the direct phosphorylation of glycerol may not be responsible for the beneficial effects of RGZ.

Overall, the results of these studies are consistent with the effects exerted by RGZ in the levels of glucose and NEFA of T2D patients. The results suggest that adipose tissue could have a role in improving not only the concentration of NEFA but also in decreasing the concentration of glucose in blood.

Although the role of glyceroneogenesis in TG synthesis and RGZ action will be covered in the next chapter, the studies shown so far do not support a major role of glyceroneogenesis in RGZ action. This inference is supported by the fact that RGZ increases the utilization of glucose and the production of lactate. An increase in glyceroneogenesis would decrease the utilization of glucose and increase the utilization of lactate carbons.

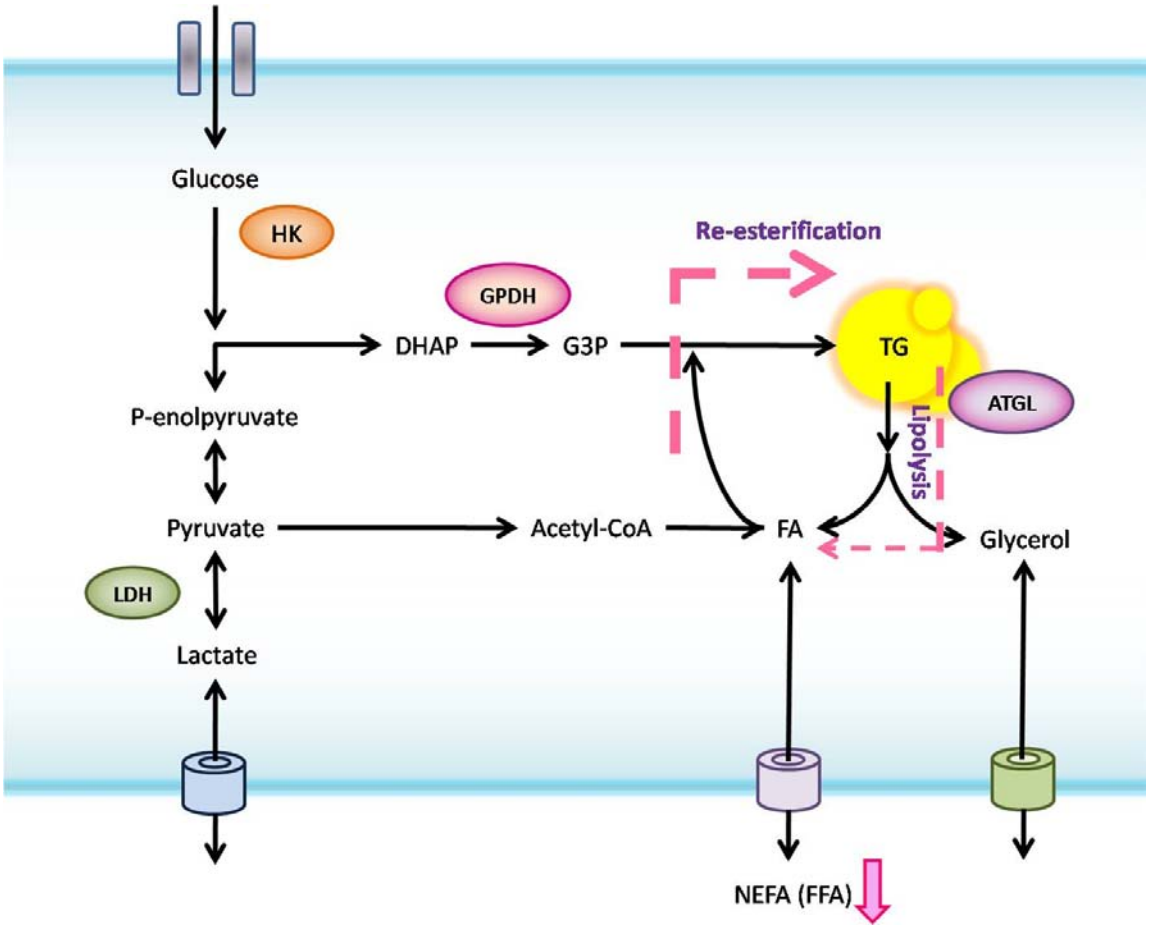


Figure 11. The overview of the RGZ-activated pathway for the synthesis of G-G from glucose in 3T3L-1 adipocytes. This figure shows an abbreviated version of the metabolic pathway activated by RGZ treatement. Enzymes upregulated in response to RGZ including hexokinase (HK), lactate dehydrogenase (LDH), glycerol 3-phosphate dehydrogenase (GPDH), and adipose triglyceride lipase (ATGL) are also shown. The futile cycle accompanied by an increase in lipolysis and re-esterification is emphasized in dotted allows.

CHAPTER IV

CONTRIBUTION OF GLYCOLYSIS AND GLYCERONEOGENESIS TO THE SYNTHESIS OF GLYCERIDE-GLYCEROL: EFFECT OF RGZ

Introduction

In the previous chapters, we have shown that alterations in glucose and TG metabolism in adipocytes could explain the anti-diabetic and NEFA-lowering effects of RGZ. So far, two major mechanisms have been proposed to explain the anti-diabetic actions of RGZ: glycerol kinase up-regulation [71] and glyceroneogenesis [50, 64]. Both pathways are thought to contribute the synthesis of glycerol 3-phosphate (G3P) that eventually serves as glyceride-glycerol (G-G) in TG. In addition to these pathways, a third source of carbons for the synthesis of G3P is glucose. Since the contribution of glycolysis for the synthesis of G3P/G-G has never been investigated, evaluation of the role of glycolysis in the synthesis of TG is a main aspect of this chapter.

GK up-regulation in terms of transcription and enzyme activity were observed in human and mouse adipose tissues in response to RGZ [71]. However, the degree of contribution of GK to the synthesis of G-G in adipocytes has not been investigated. In the previous chapter, we showed that the activity of GK does not change after RGZ treatment. On the other hand previous studies from other labs were not able to reproduce the reports indicating a role of GK in RGZ action [72]. Therefore, we decided not to carry out additional studies on the role of GK in RGZ action.

.

Glyceroneogenesis is the pathway involving non-glucose precursors (alanine, pyruvate, and lactate) for the synthesis of G3P. Normally this pathway is active in liver in fasting condition. Several studies suggested that this pathway is activated in adipocytes in response to RGZ treatment and could represent the main contributor of the anti-diabetic effects of the drug. However, the rate of utilization of pyruvate/lactate for the synthesis of G-G has not been determined.

We have hypothesized that it is possible to determine in a quantitative fashion the contributions of both glycolysis and glyceroneogenesis to the synthesis of glyceride-glycerol. Accordingly, in this chapter, we show the results of the evaluation of the contribution of glycolysis to the synthesis of TG under three lipolytic conditions. Moreover, we have examined the effects of RGZ on this contribution. We have also evaluated the contribution of glyceroneogenesis to the synthesis of glyceride-glycerol and compared it to the contribution provided by glucose metabolism.

Experimental Procedures

Metabolism of glucose, lactate/private, and lipids in adipocytes. Fully differentiated adipocytes were incubated in the DMEM/10% FBS medium containing either ethanol (control) or 500 nM RGZ for 24 hours. After 24 hours, cells were washed in KRBH medium containing 6mM glucose and 0.12% FA-free BSA. The wash medium was replaced with the KRBH incubation medium containing either insulin (1μg/ml), isoproterenol (1μg/ml), or DMSO (control) in addition to 6.0mM glucose, 2mM lactate, and 0.12% FA-free BSA. Upon the replacement of the wash medium with the incubation medium, [U-^{14}C]-glucose (1.5 to 6μCi/well) or [U-^{14}C]-lactate (1μCi/well) were added to the assigned wells. The incubation in the presence of radiolabeled substrates proceeded for 1 hour. After this time, the medium was removed and placed on ice, and the cells were homogenized in 150 mM Tris buffer containing 50 mM sodium chloride (pH 7.5).

Calculations for glycolysis. Rates of G-G backbone synthesis (total TG synthesis = de novo TG synthesis + TG re-esterification) from glucose were calculated from the radioactivity incorporated into lipids and the specific activity of glucose. The rates of incorporation of glucose carbons into TG, glyceride-glycerol and fatty acids, were calculated from the distribution of radioactivity (percentages) among lipid species, and between the fatty acyl chains (FA-chain) and glycerol backbone (G-G) of TG.

Calculations for glyceroneogenesis. The main problem in estimating the rate of synthesis of glyceride-glycerol (G-G) using radiolabeled lactate or pyruvate consists in determining the specific activity of any of these compounds in the cells.

In the following paragraphs we describe an approach that was used to obtain estimates of the rate of glyceroneogenesis in adipocytes. The partition of pyruvate between the pathways of FA synthesis and lactate production can be estimated under any cellular condition by incubating the cells with [U-^{14}C]-glucose and determining the radioactivity associated with cellular fatty acids and lactate. FAs are isolated from cellular lipids, whereas lactate is recovered from the cell culture medium. If, in addition, we know the amount of lactate produced during a given time period, the amount of pyruvate used for fatty acid synthesis can be estimated from the equation 1:

Eq.1:

$$\text{Pyr used for FA Synthesis (mol)} = \frac{3}{2} \times \left(\frac{\text{cpm FA}}{\text{cpm Lactate}}\right)_{14C-Glu} \times \text{Lac Produced (mol)}$$

To estimate the synthesis of glyceride-glycerol from pyruvate, we use the number of moles of pyruvate used for FA synthesis, as obtained from the previous equation, and the ratio of pyruvate carbons incorporated into FA and G-G that is determined from the incubation of the cells with ^{14}C-Lactate.

Eq.2:

$$\text{Pyr used for G} - \text{G (mol)} = \frac{2}{3} \times \left(\frac{\text{cpm G} - \text{G}}{\text{cpm FA}}\right)_{14C-Lac} \times \text{Pyr for FA Synthesis (mol)}$$

Combining equations 1 and 2:

Eq. 3:

$$\text{Pyr used for G} - \text{G synth} = \left(\frac{\text{cpm FA}}{\text{cpm Lactate}}\right)_{^{14}\text{C-Glu}} \times \text{Lac Produced} \times \left(\frac{\text{cpm G} - \text{G}}{\text{cpm FA}}\right)_{^{14}\text{C-Lac}}$$

The previous equations are based on the fact that the production of both lactate and FA has pyruvate as a common precursor and, therefore, the ratio of radioactivity associated to them is directly proportional to their molar ratio.

According to equation 3 the synthesis of glyceride-glycerol from pyruvate can be estimated on the basis of two parallel experiments, one using [U-^{14}C]-glucose and the other [U-^{14}C]-lactate. Aside from the difference of the radiotracer used, the cells will be incubated under identical experimental conditions, including the concentrations of glucose, lactate and hormones.

To estimate the relative contribution of glyceroneogenesis to the synthesis of TG we compared the rate of glyceride-glycerol synthesis from glyceroneogenesis with the total rate of glyceride-glycerol synthesis. This means, that we also need to determine the rates of incorporation of glucose and glycerol carbons into the glycerol backbone of TG. The synthesis of glyceride-glycerol from glucose and glycerol carbons can be estimated from the relative incorporation of glucose carbons into G-G and lactate and the amount of lactate produced from glucose (Eq. 4).

Eq. 4:

$$\text{G} - \text{G Synthesis from Glucose and Glycerol} = \left(\frac{\text{cpm G} - \text{G}}{\text{cpm Lactate}}\right)_{^{14}\text{C-Glu}} \times \text{Lactate Produced}$$

Overall the contribution of glyceroneogenesis to the synthesis of glyceride-glycerol is obtained from equations 3 and 4 as:

Eq. 5:

$$\text{Glyceroneogenesis (\%)} = \frac{\text{G}-\text{G Snythesis from Pyr}}{\text{Total G}-\text{G Synthesis}} \times 100$$

$$\text{Glyceroneogenesis(\%)} = \frac{\left(\frac{\text{cpm G}-\text{G}}{\text{cpm FA}}\right)_{14\text{C}-\text{Lac}}}{\left(\frac{\text{cpm G}-\text{G}}{\text{cpm FA}}\right)_{14\text{C}-\text{Glu}} + \left(\frac{\text{cpm G}-\text{G}}{\text{cpm FA chain}}\right)_{14\text{C}-\text{Lac}}} \times 100$$

Equation 5 shows that estimation of the role of glyceroneogenesis is quite simple and does not require determining the specific activity of lactate. The simplicity of equation 5 suggests that these measurements could be characterized by relatively low standard errors.

Statistics: A) First we calculated the mean value and the standard error of the estimate of pyruvate used for FA synthesis (Eq. 1). The experimental values of (cpm FA/cpm Lact), lactate produced, and protein mass are obtained for each well in which the cells were incubated with ^{14}C-glucose. Values from different wells were averaged and used to estimate the standard deviation.

B) The mean values and corresponding standard deviations for the ratio of ^{14}C-lactate carbons used for G-G and FA synthesis (cpm G-G/ cpm FA) were estimated from the ratio values obtained in individual wells.

C) The amount of pyruvate used for G-G synthesis will be calculated from equation 2, or 3, by multiplying the means obtained in A and B. The resulting standard deviation will be calculated as:

$SD(C)^2 = [B* SD(A)]^2 + [A* SD(B)]$

Student t-test was performed for the assessment of statistical significance using Instat software (GraphPad Software Inc, La Jolla, CA)

.

Other Methods. Cell culture, determination of intracellular SA*glu*, and analysis of lipid and metabolites were performed using the procedures described in Chapter II and III.

Results

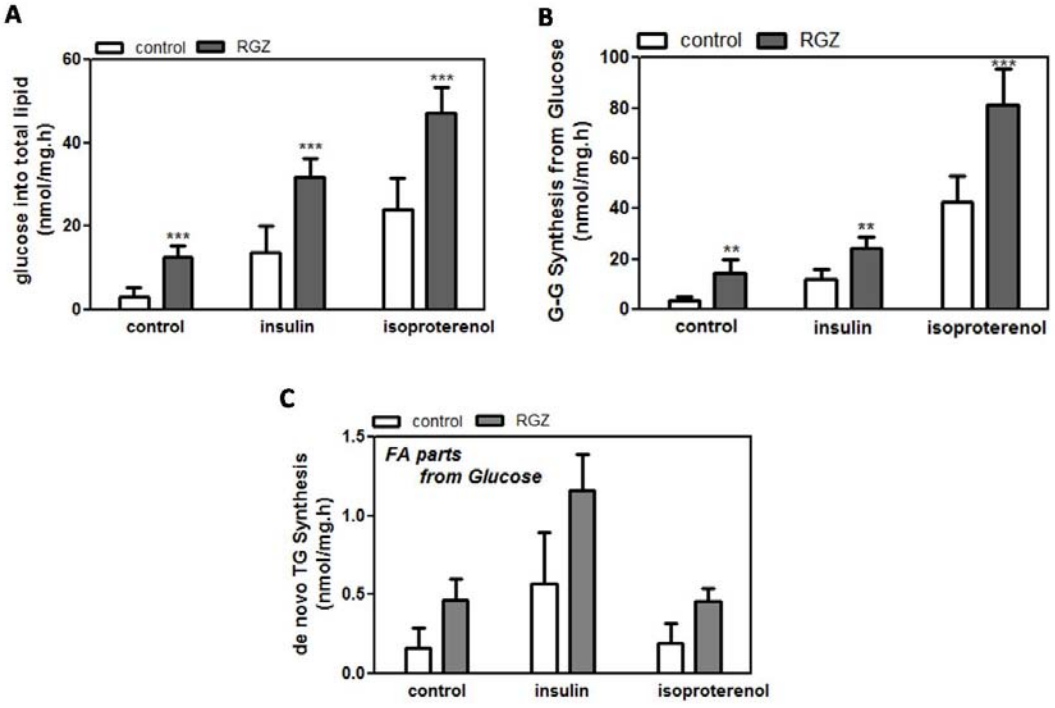
Utilization of glucose for the synthesis of glyceride-glycerol and fatty acids: effect of RGZ. To determine the contribution of glycolysis to the synthesis of glyceride-glycerol and possible changes induced by RGZ treatment, adipocytes treated with DMSO (control) or 500nM RGZ for 24 h were incubated in KRBH containing 6mM [U-^{14}C]-glucose and 2mM lactate. After 1h incubation with radiolabeled glucose, the medium was analyzed to determine the specific activity of lactate. The lipids of the cell homogenates were extracted to determine the radioactivity associated to them. The amount of glucose used for lipid synthesis was estimated from the specific activity of lactate and the radioactivity incorporated into lipids. Subsequent separation of the lipid classes allowed determining the fraction of radiolabel present in TG. This information and the distribution of radioactivity between the glycerol backbone and the fatty acid chains of TG were used to estimate the rates of glyceride-glycerol and fatty acid synthesis from glucose.

RGZ induced a significant increase in the amount of glucose incorporated into the total lipid fraction in all three conditions, control, insulin and isoproterenol (Fig. 12A). Cells incubated with isoproterenol, no insulin, showed the highest increase in glucose conversion to lipid.

Estimations of the synthesis of glyceride glycerol from glucose showed that under highly lipolytic conditions the cells use increasing amount of glucose to synthesize glyceride-

glycerol (Figure 12B). The figure also shows that RGZ stimulated the utilization of glucose for the synthesis of glyceride-glycerol under the three conditions studied. Average increases of G-G synthesis induced by RGZ were 2.7-fold for control ($p<0.005$), 2.2-fold for insulin ($p<0.005$), and 1.5-fold for isoproterenol treated cells ($p<0.0005$).

The rates of *de novo* TG synthesis from glucose were calculated from the radioactivity found in the fatty acids of TG. The results showed that de novo TG synthesis represents a small fraction of the glyceride-glycerol synthesized from glucose carbons (Fig. 12C). This means that, as expected from the active futile TG cycle that takes place in adipocytes, most TG are synthesized from FA previously present in TG. The data shown in Fig 12C also suggest that, in the three lipolytic conditions, RGZ promotes an increase in the rate of FA synthesis from glucose in adipocytes.



.

Figure 12. Utilization of glucose for glyceride-glycerol and fatty acids. A) Rates of incorporation of glucose carbons in total lipids; B) rates of synthesis of glyceride-glycerol from glucose (total TG synthesis); C), de novo TG synthesis (Calculated from the moles of stearic acid equivalents synthesized from glucose/3). Blank bars (□) show 0 h RGZ treatment and black bars (■) express 24h RGZ incubation. Data is shown as mean ± SD (n=6, **: $p<0.005$, ***: $p<0.0005$).

Utilization of pyruvate for the synthesis of glyceride-glycerol and fatty acids: effect of RGZ. To investigate the contribution of glyceroneogenesis for the synthesis of G-G in response to RGZ, fully differentiated adipocytes were incubated with 500nM RGZ for 24 hours. To radiolabel the cellular pool of pyruvate and estimate the utilization of pyruvate for fatty acid and glyceride-glycerol synthesis, the cells were incubated in KRBH medium containing 6mM glucose, 2mM lactate (cold + radioactive), and 0.12% BSA.

To calculate the synthesis of glyceride-glycerol from pyruvate we used the equations 2 and 3 that were described in methods. This calculation requires determining the ratio of radioactivity between G-G and FA parts of TG after incubation of the cells with [^{14}C]-lactate. The calculation also requires determining the production of lactate, and the partition of radiolabeled glucose carbons between FA and lactate. The estimates of the rate of G-G synthesis from pyruvate suggested that RGZ does not increase glyceroneogenesis. Moreover, the rates of glyceroneogenesis were low under all experimental condition tested. As shown in the Figure 13, the maximum rate of G-G synthesis from pyruvate was estimated to be around 1.0 nmol/mg.h in RGZ–treated cells. This rate is minimal compared to the estimated rates of G-G synthesis from glucose (Fig 12B).

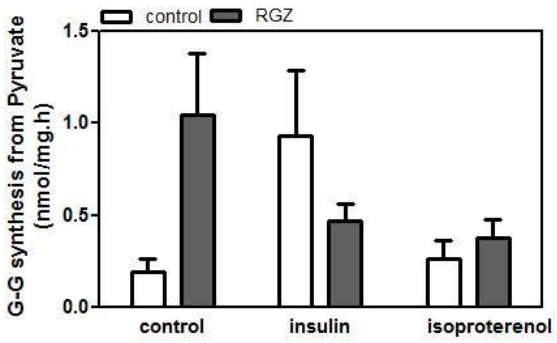


Figure 13. Rate of glyceroneogenesis. The rate of the synthesis of G-G from pyruvate was estimated using the method described above.). Blank bars (□) show 0 h RGZ treatment and black bars (■) express 24h RGZ incubation. Data is shown as mean ± SD (n=6).

To emphasize the validity of this conclusion the partition of glucose and lactate carbons between the FA and G-G of TG were compared and are shown in Figure 14. The study of

the distribution of lactate carbons (radioactivity) between the G-G and FA moieties of TG indicated that nearly 95% of the lactate carbons are used for the synthesis of FA (Fig. 14A). Moreover, this fraction did not show significant changes when the cells are incubated with RGZ. A comparison of this data with the partition of glucose carbons between the G-G and FA moieties of TG (Fig 14B), shows that the relative utilization of glucose carbons for the synthesis of G-G is several times higher than that from lactate. Using these data and the equation 5 described in methods, the actual contributions of glyceroneogenesis to the synthesis of glyceride-glycerol were estimated (Fig. 14C).

These results showed that under any of the experimental conditions studied, the contribution of glyceroneogenesis to the synthesis of glyceride-glycerol is less than 6%. Moreover, as also shown in the Figure 13C, the contribution of glyceroneogenesis is not increased by RGZ.

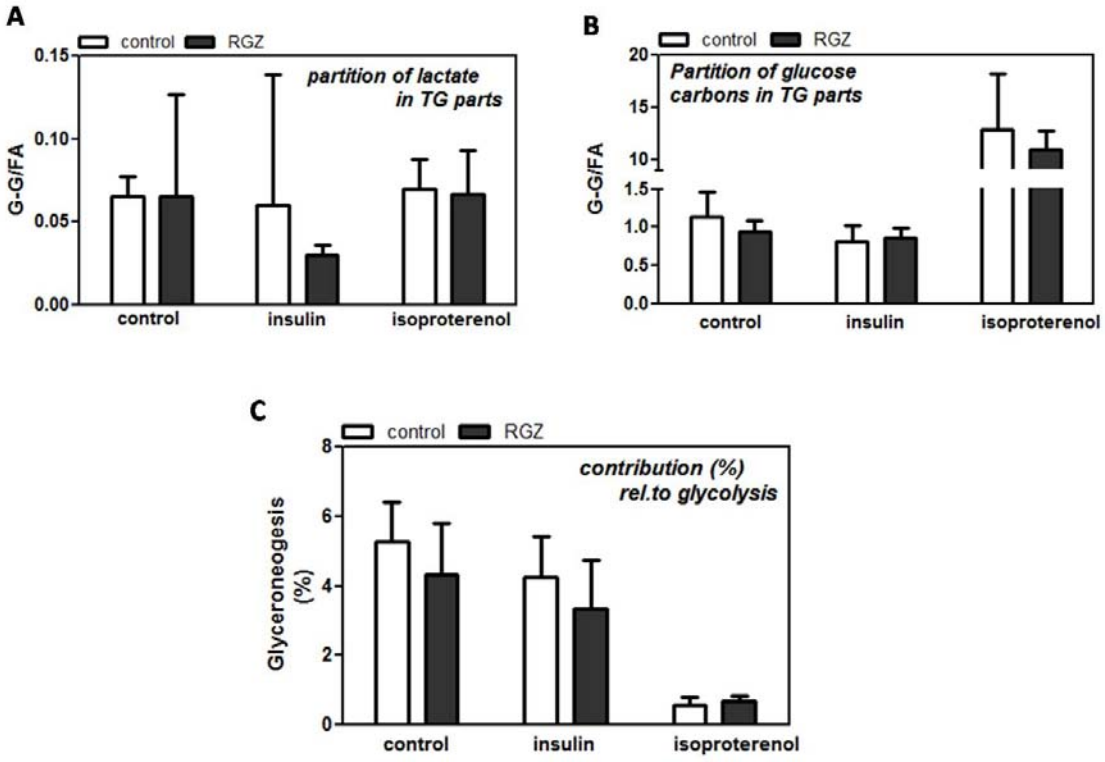


Figure 14. Contribution of glyceroneongenesis for the synthesis of G-G in adipocytes. Partition of [U-^{14}C]-glucose (A) and lactate (B) carbon into G-G and FA-chain were measured, and ratio is expressed. Contribution of glyceroneogenesis over glycolysis was also estimated (C). The empty bars (□) show 0h incubation with RGZ whereas the filled bars (■) indicate incubation with 500nM RGZ for 24 hours. All data is expressed in mean ± SD (n=6).

Effect of pyruvate on the metabolism of adipocytes. Previous studies from other labs suggested that glyceroneogenesis was important in human and rodent adipocytes. These studies used a high concentration of pyruvate (25mM) and as previously mentioned they did not determine rates of glyceroneogenesis [65, 67]. However, normal physiological concentration of pyruvate in human blood is around 0.02 to 0.15 mM [89, 90].

To determine the impact of pyruvate concentration on the metabolism of TG in adipocytes, the cells were incubated in KRBH medium containing different concentrations of pyruvate (0, 2, and 10mM) along with 6mM glucose, 0.12% BSA, and hormones (insulin or isoproterenol) for 1 hour. Analysis of the medium revealed that adipocytes in high lipolytic state (isoproterenol) produce more NEFA in response to the increase in pyruvate concentration (Fig. 15A). Moreover, suppression of lipolysis was observed in the cells incubated with pyruvate in both experimental conditions, isoproterenol or insulin (Fig. 15B). Although pyruvate did not significantly increase NEFA release in the presence of insulin, considering that it decreased the rate of lipolysis, it still promoted a relative increase in the production of NEFA (Fig. 15C).

These results indicate that pyruvate enhances NEFA production although it suppresses lipoylsis in adipocytes. In adipocytes most of NEFA usually originates from lipoylsis. Therefore these results show that excess pyruvate impairs normal metabolic activity in adipocytes. This action of pyruvate on adipocytes is pro-diabetic. The altered metabolic activity that enhances NEFA production even at low lipolytic rates would worsen the existing diabetic condition.

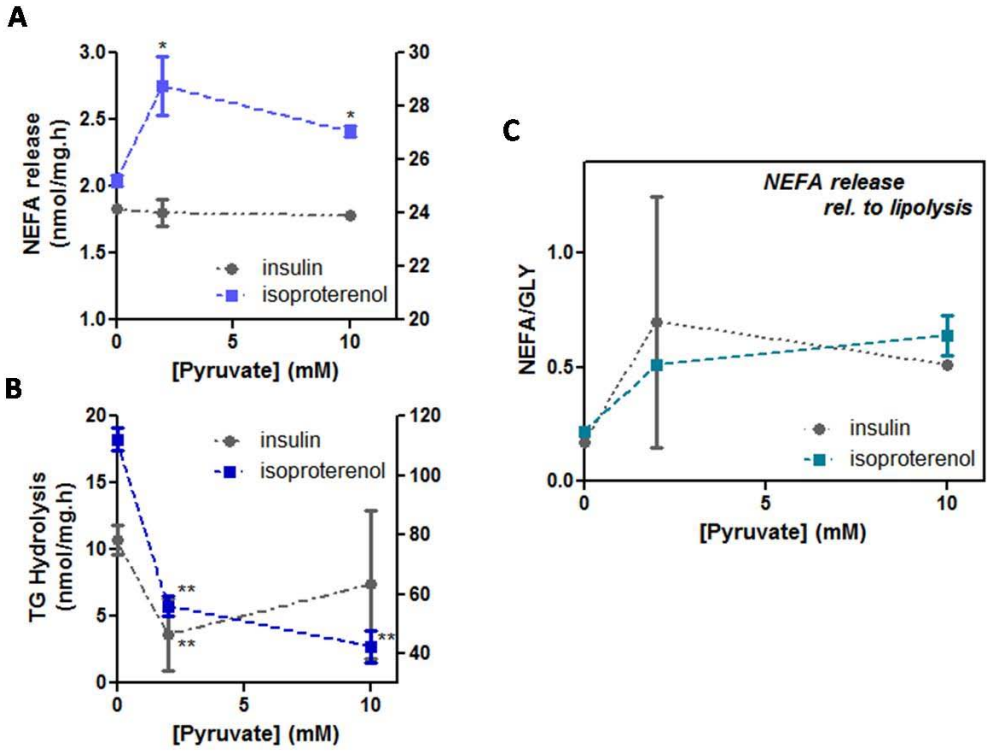


Figure 15. Relative NEFA production is increased in response to pyruvate. Adipocytes, incubated with different concentration of pyruvate along with 6mM glucose and 0.12% FA-free BSA for 1 hour were analyzed for NEFA production (A) and glycerol release (B) in the high lipolytic (isoproterenol) and low lipolytic (insulin) conditions. The degree of NEFA output relative to glycerol release (lipolysis) was also estimated (C). Data is shown in mean ± SD (n=2, *: $p<0.05$, **:$p<0.005$ compared to 0mM pyruvate).

Summary

Synthesis of G3P, ultimately serves as glyceride-glycerol (G-G) of TG, is one of the rate determining steps for FA re-esterfication that can contribute anti-diabetic effects exerted in adipocytes. In this chapter we investigated the roles of glycolysis and glyceroneogenesis in the synthesis of G-G and the effects of RGZ in the relative contributions of these pathways.

The estimated rates of glyceroneogenesis (G-G synthesis from pyruvate) suggest that the overall contribution of this pathway to the synthesis of G-G is minor (maximam ~5% in basal, non drug treated cells) as compared to the contribution of glycolysis. In addition, the treatment of adipocytes with RGZ did not have a major effect on the rates of the G-G synthesis from pyruvate in either lipolytic state. In the cells in high lipolytic state, in which glyceroneogesis was thought to have a major contribution to the synthesis of G-G, the amount of G-G synthesized from pyruvate was minimal (0.6 - 0.7% of the overall contribution). These results indicate that glyceroneogenesis may not have a significant role during fasting or in the anti-diabetic actions induced by RGZ in adipocytes.

Furthermore, our studies suggest that RGZ promotes a significant increase in the rate of glycolysis and, accordingly, an increase in the synthesis of G-G from glucose regardless the lipolytic states of adipocytes. Now we can speculate that the increase in glucose catabolism and use of glucose for the synthesis of G-G could be responsible of the higher rate FA re-esterification and, thus, of the lower output of NEFA.

CHAPTER V

DISCUSSION

Summary of the Study

Excess of circulating NEFA has been linked to toxic effects that contribute to the development of type II diabetes (T2D) [32, 35, 91, 92]. Being the largest FA storage and buffering site through lipolysis and esterification in the mammalian body, adipocytes have potential to contribute to both the prevention and development of T2D. The anti-diabetic agent, rosiglitazone (RGZ), is an agonist of the transcription factor PPAR-γ that is mainly expressed in adipocytes. The known effects of the drug include normalization of excess NEFA, thereby improving insulin sensitivity in muscle and liver, and glucose levels. This anti-diabetic action of the drug is thought to be induced by changes in adipocytes.

To understand the mechanism of action of RGZ, we investigated some aspects of the metabolism of glucose, pyruvate and TG using mouse 3T3L-1 adipocytes in this study.

The major findings of this study are the following:

- The rate of glucose and TG metabolism in 3T3L-1 adipocytes can be directly determined using specific activity (cpm/nmol) of lactate released into the medium.

- Adipocytes display various changes depending on the condition of incubation. Isoproterenol-treated adipocytes increase glucose consumption in the absence of insulin. Furthermore, the increased rate of lipolysis in these adipocytes facilitates the synthesis of G-G of TG using glucose carbons.

- RGZ increases the rate of two opposite pathways, lipolysis and FA uptake and re-esterification, in adipocytes regardless of the conditions of incubation (basal, low lipolytic, and high lipolytic states).

- RGZ increases the rate of the futile cycle of TG in a synthesis biased fashion. This could explain why RGZ treated adipocytes have a reduced output of NEFA.

- RGZ-treated adipocytes utilize larger amounts of glucose for the synthesis of G-G and this may be the cause of the enhanced rate of FA re-esterification.

- The greater utilization of glucose for G-G synthesis would be possible, at least in part, because RGZ up-regulates the expression of HK, LDH, GPDH, and ATGL.

- These studies suggest that neither glyceroneogenesis nor GK play significant roles in NEFA lowering effect of RGZ.

Overall the findings in this study provide new insights to the lipid metabolism of adipocytes and NEFA lowering effects of RGZ.

Rosiglitazone and Metabolism of Adipocytes

In this study it was found that upon incubation with RGZ adipocytes simultaneously up-regulate several metabolic pathways regardless the conditions of incubation (basal, low lipolysis and high lipolysis). These include lipolysis, glucose uptake, G-G synthesis from glucose, and FA re-esterification. Furthermore, the results of the rate estimations of glucose and TG metabolism in this study indicate that RGZ activates cycling of lipolysis and FA re-esterification through enhancement of G-G synthesis from glucose.

RGZ, through binding on PPAR-γ, either directly or indirectly regulates the transcription of genes to exert its anti-diabetic effect in adipocytes. We observed increases in activities of HK, LDH, and GPDH. These proteins relate to the synthesis of G3P that eventually ends up in G-G of TG. Up-regulation of these enzymes is positively correlated to the rate of the lipid metabolism estimated in this study. Also, analysis of protein expression suggested that ATGL, but not HSL, is up-regulated by RGZ incubation for 24 hours in adipocytes. According to the estimations of FA and G-G synthesis rates and the rate of re-esterification, RGZ-treated adipocytes use most of glucose for the synthesis of G-G instead of FA in all physiological condition.

Enhanced expression of ATGL supported the increased rate of lipolysis even in the presence of insulin. Although phosphorylation of HSL is inhibited by insulin, ATGL still exert its activity. Previous studies suggested that rate of re-esterification tend to be higher in lipolytic state, however, none of them directly estimated the rate of G-G synthesis. Enhancement of G-G synthesis pathway rather than FA synthesis may be the way of adipocytes to control the amount of FA release.

Lipolysis and FA Oxidation

Along with this study, several others have also confirmed that RGZ enhances lipolysis in adipocytes [87, 93-95]. Up-regulation of ATGL protein expression explains the increase rate of lipolysis in RGZ-treated adipocytes even in the presence of insulin. However, several studies showed the insulin reduces mRNA expression of ATGL and controls the rate of lipolysis [94-96]. Perhaps the down-regulation of ATGL protein expression was not seen in this study because insulin treatment lasted only 1 hour after the 24 hours incubation with RGZ. However, it seems that insulin did not acutely affect the activity of ATGL which was already expressed in response to RGZ. In other words, enhanced lipolysis in RGZ-treated adipopcytes in basal, low lipoyltic, and high lipolytic states suggest that ATGL, once expressed, is not regulated by hormones. T2D patients usually take RGZ in the presence of insulin. The effect of co-existing insulin and RGZ for long period of time in terms of ATGL expression and activity still remains to be investigated.

The results of this study showed that expression of HSL is not up-regulated by RGZ. However there are other studies showing a positive effect of RGZ on HSL expression

[97, 98]. Still, HSL is activated by phosphorylation via cAMP-activated protein kinase (PKA)[48], and this is suppressed in the presence of insulin. Therefore enhanced lipolysis in the cells treated with insulin is less likely due to the activity of HSL. This makes ATGL the major contributor for the higher rate of lipolysis in RGZ treated adipocytes.

In terms of lipid metabolism, it has been shown that the degree of lipolysis induced by isoproterenol is proportional to the rate of FA re-esterification in adipocytes [42, 50, 99]. Estimated rate of enhanced G-G synthesis from glucose in the presence of isoproterenol and RGZ is in agreement with those studies. Furthermore, although there have been implications of increased G-G synthesis in response to lipolysis in adipocytes, our study is the first to directly estimate the rate of G-G synthesis from glucose.

The major consequence of the increased rate of lipolysis in adipocytes that has been known for long time is reduction in ATP level [80, 100-103]. Since oxidation of one molecule of palmitate (16C FA) can generate up to ~129 ATPs, lower ATP levels indicate that adipocytes do not undergo efficient oxidative phosphorylation of FA for ATP production under lipolytic condition [80]. Probably this is because adipocytes' major role is to provide FA as energy to other tissues at the time of starvation. Another implication is the increased rate of FA re-esterification in adipocytes under lipolytic condition increases the need for ATP for FA re-esterification. Since this process requires a large input of ATP (7-9 ATP for re-esterification of 3FAs and production of G3P), under inefficient/slow FA oxidative phosphorylation, re-esterification of FA seems to contribute to a significant loss of ATP (increased AMP:ATP ratio) in adipocytes [80, 88]. This explains the larger amount of lactate production in isoproterenol and RGZ-treated adipocytes. Increased production of lactate from adipocytes in response to lipolytic condition indicates the cells undergo anaerobic glycolysis to generate ATP at a faster rate to meet ATP requirement for FA re-esterification.

Larger increases in glucose consumption and lactate production in RGZ-treated adipocytes in all conditions suggest that RGZ does not enhance efficient FA oxidation to produce ATP. In other words, rate of re-esterification is much faster than rate of FA oxidation in adipocytes. In this study, we observed a tendency of reduction in intracellular FA level even in the increase in lipolysis and reduction in NEFA output in RGZ-treated adipocytes. This might be solely due to increased re-esterification rate. However, up-regulation in mRNA expressions of uncoupling protein (UCP) and proteins relating to FA oxidation (carnitine acetyltransferase, acetyl-CoA acyltransferase,

cytochrome oxidase c) have been reported in adipocytes incubated with RGZ [62, 104]. Furthermore, adipocytes from RGZ-treated mouse showed a significant increase in O_2 consumption [105].Therefore there might be enhancement of FA oxidation in these cells for increased NADH production. In addition, RGZ may increase uncoupling of the electron transport chain and ATP synthase in mitochondria to prevent further ATP synthesis. These would contribute lowering intracellular FA level and increased glucose uptake for ATP synthesis.

However, according to the results from this study, we still expect that the contribution of FA oxidation in response to RGZ remains minimal compared to increased rate of FA re-esterification using glucose carbons because of increased glucose consumption and lactate output.

Glycolysis and G-G synthesis

Adipose glucose transporter protein, GLUT-4, has been shown to increase by RGZ treatment in mouse and human adipocytes, supporting increased glucose uptake by RGZ-treated adipocytes observed in this study. Levels of mRNA expressions of other glycolytic enzymes, including phosphofructokinase, pyruvate dehydrogenase subunit α and LDH, were also resported to be up-regulated in mouse adipose tissue by RGZ [106]. Several other studies have shown the increase in glucose uptake by adipocytes/adipose tissues.

Taken together with the increase in HK enzyme activity and increased glucose uptake regardless of condition of incubation, these data suggest that RGZ directly facilitates glucose consumption by adipocytes independent of the condition of incubation. In addition to facilitation of lipolysis, up-regulation of GPDH activity by RGZ supports enhanced rate of re-esterification via synthesis of G3P (G-G) from glucose. Moreover, the rate of GPDH was not affected by the hormones (data not shown), suggesting this enzyme is not acutely regulated by insulin or isoproterenol. Increased enzymatic activity of LDH is strong evidence of anaerobic glycolysis in RGZ-treated adipocytes. As mentioned above, this is most likely driven from ATP requirement imposed by enhanced lipolysis and re-esterification in the cells incubated with this drug.

FA Synthesis and Esterification

As mentioned above, the rate of lipolysis has a positive correlation with rate of re-esterification. Obviously, lipolysis alone does not explain RGZ's NEFA lowering action since cells incubated with isoproterenol increase NEFA release compared to adipocytes treated in insulin or DMSO (control). The rate estimation in this study revealed that RGZ-treated adipocytes undergo faster re-esterification over increase in lipolysis rate. This indicates that RGZ itself also increases FA re-esterification and NEFA uptake in adipocytes using processes that is not regulated by lipolysis.

Although we did not measure activity or expression of proteins relating to FA esterification and FA transport, several studies have resported the positive effect of RGZ on mRNA expressions of enzymes relating to those actions in adipocytes. These include acyl-CoA synthase (ACS) [62, 105], glycerol 3-phosphate acyltransferase (GPAT) [106], diacylglycerol acyl transferase (DGAT) [107, 108], and FA transporter protein (FAT/CD36) [62]. Along with increased GPDH activity in reponse to RGZ, these findings further support that RGZ facilitates FA re-esterification by up-regulating several proteins independent of condition of incubation.

The results from the present study showed that adipocytes treated with RGZ increases FA synthesis in some extent, but the amount of FA synthesized from glucose remains relatively small. Similar to the expression of proteins relating to FA esterification, studies of expression of enzymes relating to FA synthesis have also shown several proteins that are up-regulated by RGZ. Increased mRNA expression of adipose fatty acid synthase (FAS) in response to RGZ was reported [107]. Elevated level of protein expression of acetyl-CoA carboxylase (ACC), an enzyme catalyzing initial step of FA synthesis, was also found in adipose tissue in rat treated with RGZ and high fat diet along with decrease in AMP activated protein kinase (AMPK) level [109]. These results suggest that RGZ enhances FA synthesis in adipocytes. However, since ATP level in adipocytes incubated with RGZ is expected to be low, AMPK should be active and inhibit ACC in the experimental setting of this study. Therefore, ACC activity might be enhanced to some extent in RGZ-treated adipocytes, but this would not result in production of a large amount of FA.

Glyceroneogenesis and Glycerol Kinase

GK enzyme assay and the estimation of G-G synthesis from lactate/pyruvate indicated that GK up-regulation and glyceroneogenesis do not contribute enhancement of G-G synthesis to lower NEFA in plasma in the adipocytes treated with RGZ in any physiological states.

Rates or expressions of phosphoenol pyruvate carboxylase-C (PEPCK-C) and pyruvate carboxylase (PC) in adipocytes were not investigated in this study. Several studies have shown there are increases in gene expression or activity of those enzymes in response to RGZ in adipocytes [68, 104, 105, 110]. Also, as mentioned in Chapter I, the studies supporting up-regulation of glyceroneogenesis have shown increased incorporation of pyruvate carbons in lipid and TG fractions in adipocytes under high pyruvate concentration [65, 67, 68].

The results in the present study suggest high concentration of pyruvate induces pro-diabetic effects in adipocytes by increasing NEFA release even in the absence of lipolysis. Moreover, the estimated rate of G-G synthesis from pyruvate indicates that contribution of glyceroneogenesis for the synthesis of G-G is minimal compared to G-G synthesized from glucose.

.

REFERENCES

1. *Centers for Disease Control and Prevention. National diabetes fact sheet: general information and national estimates on diabetes in the United States.* [cited 2010 March 16]; Available from: http://apps.nccd.cdc.gov/DDTSTRS/FactSheet.aspx.

2. Hogan, P., T. Dall, and P. Nikolov, *Economic costs of diabetes in the US in 2002.* Diabetes Care, 2003. **26**(3): p. 917-32.

3. Guilherme, A., et al., *Adipocyte dysfunctions linking obesity to insulin resistance and type 2 diabetes.* Nat Rev Mol Cell Biol, 2008. **9**(5): p. 367-77.

4. Alberti, K.G., *The costs of non-insulin-dependent diabetes mellitus.* Diabet Med, 1997. **14**(1): p. 7-9.

5. *Economic costs of diabetes in the U.S. In 2007.* Diabetes Care, 2008. **31**(3): p. 596-615.

6. Atkinson, M.A. and G.S. Eisenbarth, *Type 1 diabetes: new perspectives on disease pathogenesis and treatment.* Lancet, 2001. **358**(9277): p. 221-9.

7. Kukreja, A. and N.K. Maclaren, *Autoimmunity and diabetes.* J Clin Endocrinol Metab, 1999. **84**(12): p. 4371-8.

8. DeFronzo, R.A., *Pathogenesis of type 2 diabetes mellitus.* Med Clin North Am, 2004. **88**(4): p. 787-835, ix.

9. Gillespie, K.M., *Type 1 diabetes: pathogenesis and prevention.* CMAJ, 2006. **175**(2): p. 165-70.

10. Castaneda, C., et al., *A randomized controlled trial of resistance exercise training to improve glycemic control in older adults with type 2 diabetes.* Diabetes Care, 2002. **25**(12): p. 2335-41.

11. DeFronzo, R.A., *Lilly lecture 1987. The triumvirate: beta-cell, muscle, liver. A collusion responsible for NIDDM.* Diabetes, 1988. **37**(6): p. 667-87.

12. DeFronzo, R.A., et al., *Effects of insulin on peripheral and splanchnic glucose metabolism in noninsulin-dependent (type II) diabetes mellitus.* J Clin Invest, 1985. **76**(1): p. 149-55.

13. Haist, R.E., J. Campbell, and C.H. Best, *The preventation of diabetes.* N Engl J Med, 1940. **223**: p. 607-615.

14. Nathan, D.M., *Long-term complications of diabetes mellitus.* N Engl J Med, 1993. **328**(23): p. 1676-85.

15. *The effect of intensive treatment of diabetes on the development and progression of long-term complications in insulin-dependent diabetes mellitus. The Diabetes Control and Complications Trial Research Group.* N Engl J Med, 1993. **329**(14): p. 977-86.

16. *Intensive blood-glucose control with sulphonylureas or insulin compared with conventional treatment and risk of complications in patients with type 2 diabetes (UKPDS 33). UK Prospective Diabetes Study (UKPDS) Group.* Lancet, 1998. **352**(9131): p. 837-53.

17. Boulot, P., et al., *French multicentric survey of outcome of pregnancy in women with pregestational diabetes.* Diabetes Care, 2003. **26**(11): p. 2990-3.

18. Rayfield, E.J., et al., *Infection and Diabetes: the case for glucose control.* American Journal of Medicine, 1982. **72**: p. 438-450.

19. Reaven, G.M., et al., *Measurement of plasma glucose, free fatty acid, lactate, and insulin for 24 h in patients with NIDDM.* Diabetes, 1988. **37**(8): p. 1020-4.

20. DeFronzo, R.A. and E. Ferrannini, *Insulin resistance. A multifaceted syndrome responsible for NIDDM, obesity, hypertension, dyslipidemia, and atherosclerotic cardiovascular disease.* Diabetes Care, 1991. **14**(3): p. 173-94.

21. Sharma, A.M., *Adipose tissue: a mediator of cardiovascular risk.* Int J Obes Relat Metab Disord, 2002. **26 Suppl 4**: p. S5-7.

22. Mokdad, A.H., et al., *Diabetes trends in the U.S.: 1990-1998.* Diabetes Care, 2000. **23**(9): p. 1278-83.

23. Pajvani, U.B., et al., *Fat apoptosis through targeted activation of caspase 8: a new mouse model of inducible and reversible lipoatrophy.* Nat Med, 2005. **11**(7): p. 797-803.

24. Reitman, M.L., et al., *Lipoatrophy revisited.* Trends Endocrinol Metab, 2000. **11**(10): p. 410-6.

25. Engfeldt, P. and P. Arner, *Lipolysis in human adipocytes, effects of cell size, age and of regional differences.* Horm Metab Res Suppl, 1988. **19**: p. 26-9.

26. Baker, N., *In vivo tracer studies of perturbed fatty acid transport and metabolism in adipose tissue.* Int J Obes, 1985. **9 Suppl 1**: p. 155-67.

27. Unger, R.H., Y.T. Zhou, and L. Orci, *Regulation of fatty acid homeostasis in cells: novel role of leptin.* Proc Natl Acad Sci U S A, 1999. **96**(5): p. 2327-32.

28. Gustafson, B., et al., *Inflamed adipose tissue: a culprit underlying the metabolic syndrome and atherosclerosis.* Arterioscler Thromb Vasc Biol, 2007. **27**(11): p. 2276-83.

29. Kadowaki, T., et al., *Adiponectin and adiponectin receptors in insulin resistance, diabetes, and the metabolic syndrome.* J Clin Invest, 2006. **116**(7): p. 1784-92.

30. Steppan, C.M., et al., *The hormone resistin links obesity to diabetes.* Nature, 2001. **409**(6818): p. 307-12.

31. Garland, P.B., E.A. Newsholme, and P.J. Randle, *Effect of fatty acids, ketone bodies, diabetes and starvation on pyruvate metabolism in rat heart and diaphragm muscle.* Nature, 1962. **195**: p. 381-3.

32. Boden, G., *Role of fatty acids in the pathogenesis of insulin resistance and NIDDM.* Diabetes, 1997. **46**(1): p. 3-10.

33. Juhan-Vague, I. and M.C. Alessi, *PAI-1, obesity, insulin resistance and risk of cardiovascular events.* Thromb Haemost, 1997. **78**(1): p. 656-60.

34. Nolan, C.J., et al., *Fatty acid signaling in the beta-cell and insulin secretion.* Diabetes, 2006. **55 Suppl 2**: p. S16-23.

35. Bergman, R.N. and M. Ader, *Free fatty acids and pathogenesis of type 2 diabetes mellitus.* Trends Endocrinol Metab, 2000. **11**(9): p. 351-6.

36. Boden, G., et al., *Free fatty acids produce insulin resistance and activate the proinflammatory nuclear factor-kappaB pathway in rat liver.* Diabetes, 2005. **54**(12): p. 3458-65.

37. Shimabukuro, M., et al., *Lipoapoptosis in beta-cells of obese prediabetic fa/fa rats. Role of serine palmitoyltransferase overexpression.* J Biol Chem, 1998. **273**(49): p. 32487-90.

38. Oakes, N.D., et al., *Diet-induced muscle insulin resistance in rats is ameliorated by acute dietary lipid withdrawal or a single bout of exercise: parallel relationship between insulin stimulation of glucose uptake and suppression of long-chain fatty acyl-CoA.* Diabetes, 1997. **46**(12): p. 2022-8.

39. Oakes, N.D., et al., *Mechanisms of liver and muscle insulin resistance induced by chronic high-fat feeding.* Diabetes, 1997. **46**(11): p. 1768-74.

40. Schmitz-Peiffer, C., et al., *Alterations in the expression and cellular localization of protein kinase C isozymes epsilon and theta are associated with insulin resistance in skeletal muscle of the high-fat-fed rat.* Diabetes, 1997. **46**(2): p. 169-78.

41. Vaughan, M. and D. Steinberg, *Effect of Hormones on Lipolysis and Esterification of Free Fatty Acids during Incubation of Adipose Tissue in Vitro.* J Lipid Res, 1963. **4**: p. 193-9.

42. Brooks, B., J.R. Arch, and E.A. Newsholme, *Effects of hormones on the rate of the triacylglycerol/fatty acid substrate cycle in adipocytes and epididymal fat pads.* FEBS Lett, 1982. **146**(2): p. 327-30.

43. Naito, C. and K. Okada, *Effect of glucose on lipolysis and on release of lipolytic products in isolated adipocytes.* Am J Physiol, 1975. **228**(1): p. 92-7.

44. Laurencikiene, J., et al., *NF-kappaB is important for TNF-alpha-induced lipolysis in human adipocytes.* J Lipid Res, 2007. **48**(5): p. 1069-77.

45. Bullo, M., J. Salas-Salvado, and P. Garcia-Lorda, *Adiponectin expression and adipose tissue lipolytic activity in lean and obese women.* Obes Surg, 2005. **15**(3): p. 382-6.

46. Belfrage, P., et al., *Identification and some characteristics of the enzyme protein of the hormone-sensitive lipase from rat adipose tissue.* Adv Exp Med Biol, 1978. **101**: p. 113-26.

47. Zimmermann, R., et al., *Fat mobilization in adipose tissue is promoted by adipose triglyceride lipase.* Science, 2004. **306**(5700): p. 1383-6.

48. Stralfors, P., P. Bjorgell, and P. Belfrage, *Hormonal regulation of hormone-sensitive lipase in intact adipocytes: identification of phosphorylated sites and effects on the phosphorylation by lipolytic hormones and insulin.* Proc Natl Acad Sci U S A, 1984. **81**(11): p. 3317-21.

49. Lass, A., et al., *Adipose triglyceride lipase-mediated lipolysis of cellular fat stores is activated by CGI-58 and defective in Chanarin-Dorfman Syndrome.* Cell Metab, 2006. **3**(5): p. 309-19.

50. Reshef, L., R.W. Hanson, and F.J. Ballard, *A possible physiological role for glyceroneogenesis in rat adipose tissue.* J Biol Chem, 1970. **245**(22): p. 5979-84.

51. Steinberg, D., M. Vaughan, and S. Margolis, *Studies of Triglyceride Biosynthesis in Homogenates of Adipose Tissue.* The Journal of Biological Chemistry, 1961. **236**: p. 1631-1638.

52. Turner, R.C., et al., *Glycemic control with diet, sulfonylurea, metformin, or insulin in patients with type 2 diabetes mellitus: progressive requirement for multiple therapies (UKPDS 49). UK Prospective Diabetes Study (UKPDS) Group.* JAMA, 1999. **281**(21): p. 2005-12.

53. Meglasson, M.D. and F.M. Matschinsky, *Pancreatic islet glucose metablism and regulation of insulin secretion.* Diabetes Metabolism Review, 1986. **2**: p. 163-214.

54. Fuhlendorff, J., et al., *Stimulation of insulin release by repaglinide and glibenclamide involves both common and distinct processes.* Diabetes, 1998. **47**(3): p. 345-51.

55. Mudaliar, S. and R.R. Henry, *New oral therapies for type 2 diabetes mellitus: The glitazones or insulin sensitizers.* Annu Rev Med, 2001. **52**: p. 239-57.

56. Greene, D.A., *Rosiglitazone: a new therapy for Type 2 diabetes.* Expert Opin Investig Drugs, 1999. **8**(10): p. 1709-1719.

57. Lebovitz, H.E., *alpha-Glucosidase inhibitors.* Endocrinol Metab Clin North Am, 1997. **26**(3): p. 539-51.

58. Stumvoll, M., et al., *Metabolic effects of metformin in non-insulin-dependent diabetes mellitus.* N Engl J Med, 1995. **333**(9): p. 550-4.

59. Lehmann, J.M., et al., *An antidiabetic thiazolidinedione is a high affinity ligand for peroxisome proliferator-activated receptor gamma (PPAR gamma).* J Biol Chem, 1995. **270**(22): p. 12953-6.

60. Adams, M., et al., *Activators of peroxisome proliferator-activated receptor gamma have depot-specific effects on human preadipocyte differentiation.* J Clin Invest, 1997. **100**(12): p. 3149-53.

61. Savage, D.B., et al., *Human metabolic syndrome resulting from dominant-negative mutations in the nuclear receptor peroxisome proliferator-activated receptor-gamma.* Diabetes, 2003. **52**(4): p. 910-7.

62. Wang, P., et al., *Absence of an adipogenic effect of rosiglitazone on mature 3T3-L1 adipocytes: increase of lipid catabolism and reduction of adipokine expression.* Diabetologia, 2007. **50**(3): p. 654-65.

63. Lautamaki, R., et al., *Rosiglitazone improves myocardial glucose uptake in patients with type 2 diabetes and coronary artery disease: a 16-week randomized, double-blind, placebo-controlled study.* Diabetes, 2005. **54**(9): p. 2787-94.

64. Hanson, R.W. and L. Reshef, *Glyceroneogenesis revisited.* Biochimie, 2003. **85**(12): p. 1199-205.

65. Tordjman, J., et al., *Thiazolidinediones block fatty acid release by inducing glyceroneogenesis in fat cells.* J Biol Chem, 2003. **278**(21): p. 18785-90.

66. Forest, C., et al., *Fatty acid recycling in adipocytes: a role for glyceroneogenesis and phosphoenolpyruvate carboxykinase.* Biochem Soc Trans, 2003. **31**(Pt 6): p. 1125-9.

67. Leroyer, S.N., et al., *Rosiglitazone controls fatty acid cycling in human adipose tissue by means of glyceroneogenesis and glycerol phosphorylation.* J Biol Chem, 2006. **281**(19): p. 13141-9.

68. Cadoudal, T., et al., *Acute and selective regulation of glyceroneogenesis and cytosolic phosphoenolpyruvate carboxykinase in adipose tissue by thiazolidinediones in type 2 diabetes.* Diabetologia, 2007. **50**(3): p. 666-75.

69. Cadoudal, T., et al., *[Glyceroneogenesis and PEPCK-C: pharmacological targets in type 2 diabetes]*. Med Sci (Paris), 2008. **24**(4): p. 407-13.

70. Franckhauser, S., et al., *Adipose overexpression of phosphoenolpyruvate carboxykinase leads to high susceptibility to diet-induced insulin resistance and obesity.* Diabetes, 2006. **55**(2): p. 273-80.

71. Guan, H.P., et al., *A futile metabolic cycle activated in adipocytes by antidiabetic agents.* Nat Med, 2002. **8**(10): p. 1122-8.

72. Tan, G.D., et al., *A "futile cycle" induced by thiazolidinediones in human adipose tissue?* Nat Med, 2003. **9**(7): p. 811-2; author reply 812.

73. Rubin, C.S., et al., *Development of hormone receptors and hormonal responsiveness in vitro. Insulin receptors and insulin sensitivity in the preadipocyte and adipocyte forms of 3T3-L1 cells.* J Biol Chem, 1978. **253**(20): p. 7570-8.

74. Engel, P.C. and J.B. Jones, *Causes and elimination of erratic blanks in enzymatic metabolite assays involving the use of NAD+ in alkaline hydrazine buffers: improved conditions for the assay of L-glutamate, L-lactate, and other metabolites.* Anal Biochem, 1978. **88**(2): p. 475-84.

75. Folch, J., M. Lees, and G.H. Sloane Stanley, *A simple method for the isolation and purification of total lipides from animal tissues.* J Biol Chem, 1957. **226**(1): p. 497-509.

76. Arrese, E.L., et al., *Diacylglycerol transport in the insect fat body: evidence of involvement of lipid droplets and the cytosolic fraction.* J Lipid Res, 2001. **42**(2): p. 225-34.

77. Baudin, B., et al., *Spectroscopic studies on angiotensin I-converting enzyme.* Enzyme Protein, 1994. **48**(5-6): p. 265-74.

78. van de Venter, M., D. Litthauer, and W. Oelofsen, *Catecholamine stimulated lipolysis in differentiated human preadipocytes in a serum-free, defined medium.* J Cell Biochem, 1994. **54**(1): p. 1-10.

79. Fried, S.K., M. Lavau, and F.X. Pi-Sunyer, *Role of fatty acid synthesis in the control of insulin-stimulated glucose utilization by rat adipocytes.* J Lipid Res, 1981. **22**(5): p. 753-62.

80. Rognstad, R. and J. Katz, *The balance of pyridine nucleotides and ATP in adipose tissue.* Proc Natl Acad Sci U S A, 1966. **55**(5): p. 1148-56.

81. Scheer, W.D., H.P. Lehmann, and M.F. Beeler, *An improved assay for hexokinase activity in human tissue homogenates.* Anal Biochem, 1978. **91**(2): p. 451-63.

82. Breen, G.A. and J. de Vellis, *Regulation of glycerol phosphate dehydrogenase by hydrocortisone in dissociated rat cerebral cell cultures.* Dev Biol, 1974. **41**(2): p. 255-66.

83. Hakala, M.T., A.J. Glaid, and G.W. Schwert, *Lactic dehydrogenase. II. Variation of kinetic and equilibrium constants with temperature.* J Biol Chem, 1956. **221**(1): p. 191-209.

84. Alexander, M., et al., *Insulin regulation of protein biosynthesis in differentiated 3T3 adipocytes. Regulation of glyceraldehyde-3-phosphate dehydrogenase.* J Biol Chem, 1985. **260**(22): p. 11978-85.

85. Fossati, P. and L. Prencipe, *Serum triglycerides determined colorimetrically with an enzyme that produces hydrogen peroxide.* Clin Chem, 1982. **28**(10): p. 2077-80.

86. Sakasegawa, S., et al., *A novel glycerol kinase from Flavobacterium meningosepticum: characterization, gene cloning and primary structure.* Biosci Biotechnol Biochem, 1998. **62**(12): p. 2388-95.

87. Kershaw, E.E., et al., *PPARgamma regulates adipose triglyceride lipase in adipocytes in vitro and in vivo.* Am J Physiol Endocrinol Metab, 2007. **293**(6): p. E1736-45.

88. Gauthier, M.S., et al., *AMP-activated protein kinase is activated as a consequence of lipolysis in the adipocyte: potential mechanism and physiological relevance.* J Biol Chem, 2008. **283**(24): p. 16514-24.

89. Landon, J., J.K. Fawcett, and V. Wynn, *Blood pyruvate concentration measured by a specific method in control subjects.* J Clin Pathol, 1962. **15**: p. 579-84.

90. Maughan, R.J., *A simple, rapid method for the determination of glucose, lactate, pyruvate, alanine, 3-hydroxybutyrate and acetoacetate on a single 20-mul blood sample.* Clin Chim Acta, 1982. **122**(2): p. 231-40.

91. Randle, P.J., et al., *The glucose fatty-acid cycle. Its role in insulin sensitivity and the metabolic disturbances of diabetes mellitus.* Lancet, 1963. **1**: p. 785-9.

92. Randle, P.J., et al., *Interactions of metabolism and the physiological role of insulin.* Recent Prog Horm Res, 1966. **22**: p. 1-48.

93. Festuccia, W.T., et al., *PPARgamma agonism increases rat adipose tissue lipolysis, expression of glyceride lipases, and the response of lipolysis to hormonal control.* Diabetologia, 2006. **49**(10): p. 2427-36.

94. Kim, J.Y., et al., *The adipose tissue triglyceride lipase ATGL/PNPLA2 is downregulated by insulin and TNF-alpha in 3T3-L1 adipocytes and is a target for transactivation by PPARgamma.* Am J Physiol Endocrinol Metab, 2006. **291**(1): p. E115-27.

95. Kershaw, E.E., et al., *Adipose triglyceride lipase: function, regulation by insulin, and comparison with adiponutrin.* Diabetes, 2006. **55**(1): p. 148-57.

96. Kralisch, S., et al., *Isoproterenol, TNFalpha, and insulin downregulate adipose triglyceride lipase in 3T3-L1 adipocytes.* Mol Cell Endocrinol, 2005. **240**(1-2): p. 43-9.

97. McTernan, P.G., et al., *Insulin and rosiglitazone regulation of lipolysis and lipogenesis in human adipose tissue in vitro.* Diabetes, 2002. **51**(5): p. 1493-8.

98. Teruel, T., et al., *Rosiglitazone up-regulates lipoprotein lipase, hormone-sensitive lipase and uncoupling protein-1, and down-regulates insulin-induced fatty acid synthase gene expression in brown adipocytes of Wistar rats.* Diabetologia, 2005. **48**(6): p. 1180-8.

99. Vaughan, M., *The production and release of glycerol by adipose tissue incubated in vitro.* J Biol Chem, 1962. **237**: p. 3354-8.

100. Angel, A., K.S. Desai, and M.L. Halperin, *Reduction in adipocyte ATP by lipolytic agents: relation to intracellular free fatty acid accumulation.* J Lipid Res, 1971. **12**(2): p. 203-13.

101. Angel, A., K. Desai, and M.L. Halperin, *Free fatty acid and ATP levels in adipocytes during lipolysis.* Metabolism, 1971. **20**(1): p. 87-99.

102. Vallano, M.L., M.Y. Lee, and M. Sonenberg, *Hormones modulate adipocyte membrane potential ATP and lipolysis via free fatty acids.* Am J Physiol, 1983. **245**(3): p. E266-72.

103. Kather, H., *Beta-adrenergic stimulation of adenine nucleotide catabolism and purine release in human adipocytes.* J Clin Invest, 1990. **85**(1): p. 106-14.

104. Wilson-Fritch, L., et al., *Mitochondrial biogenesis and remodeling during adipogenesis and in response to the insulin sensitizer rosiglitazone.* Mol Cell Biol, 2003. **23**(3): p. 1085-94.

105. Wilson-Fritch, L., et al., *Mitochondrial remodeling in adipose tissue associated with obesity and treatment with rosiglitazone.* J Clin Invest, 2004. **114**(9): p. 1281-9.

106. Festuccia, W.T., et al., *Depot-specific effects of the PPARgamma agonist rosiglitazone on adipose tissue glucose uptake and metabolism.* J Lipid Res, 2009. **50**(6): p. 1185-94.

107. Ranganathan, G., et al., *The lipogenic enzymes DGAT1, FAS, and LPL in adipose tissue: effects of obesity, insulin resistance, and TZD treatment.* J Lipid Res, 2006. **47**(11): p. 2444-50.

108. Liu, L.F., et al., *Regulation of adipose triglyceride lipase by rosiglitazone.* Diabetes Obes Metab, 2009. **11**(2): p. 131-42.

109. Lessard, S.J., et al., *Tissue-specific effects of rosiglitazone and exercise in the treatment of lipid-induced insulin resistance.* Diabetes, 2007. **56**(7): p. 1856-64.

110. Jitrapakdee, S., et al., *The peroxisome proliferator-activated receptor-gamma regulates murine pyruvate carboxylase gene expression in vivo and in vitro.* J Biol Chem, 2005. **280**(29): p. 27466-76.

VITA

Soreiyu Umezu

Candidate for the Degree of

Doctor of philosophy

Dissertation: GLYCOLYSIS AND GLYCERONEOGENESIS IN ADIPOCYTES: EFFECTS OF ROSIGLITAZONE, AN ANTI-DIABETIC DRUG

Major Field: Biochemistry and Molecular Biology

Biographical: Born in Sendai, Japan.

Education:

Bachelor of Science in Biochemistry and Molecular Biology
Oklahoma State University 2005

Completed the requirements for the Doctor of Philosophy in Biochemistry and Molecular Biology at Oklahoma State University, Stillwater, Oklahoma in May, 2010.

Experience:

Graduate Student Research Assistant, Department of Biochemistry, Oklahoma State University. Mentor: Dr. Jose L. Soulages, Ph.D. 2005-2010

Professional Activities and Awards:

2005 BMBGSA Research Symposium Poster Session: 3rd Place
2008 Keystone Symposium: Metabolism and Cardiovascular Risks
Institute of Diabetes and Digestive and Kidney Diseases (NIDDK) Scholarship
2008 OSU Mass Spectroscopy Workshop and Certification
2010 OSU Microarray Workshop and Certification

Professional Membership

2005-2010 Biochemistry and Molecular Biology Grad Student Association

Name: Soreiyu Umezu Date of Degree: July, 2010

Institution: Oklahoma State University Location: Stillwater, Oklahoma

Title of Study: GLYCOLYSIS AND GLYCERONEOGENESIS IN ADIPOCYTES: EFFECTS OF ROSIGLITAZONE, AN ANTI-DIABETIC DRUG

Pages in Study: 63 Candidate for the Degree of Doctor of Philosophy

Major Field: Biochemistry and Molecular Biology

Findings and Conclusions:

To gain better understanding of the action of the anti-diabetic drug, rosiglitazone (RGZ), we investigated some aspects of the metabolism of glucose, pyruvate and TG in mouse 3T3L-1 adipocytes.

The major findings of this study include: 1) The rate of glucose and TG metabolism in 3T3L-1 adipocytes can be directly determined using specific activity (cpm/nmol) of lactate released into the medium, 2) Adipocytes display various changes depending on the condition of incubation. Isoproterenol-treated adipocytes increase glucose consumption in the absence of insulin. Furthermore, the increased rate of lipolysis in these adipocytes facilitates the synthesis of G-G of TG using glucose carbons, 3) RGZ increases the rate of two opposite pathways, lipolysis and FA uptake and re-esterification, in adipocytes regardless of the conditions of incubation (basal, low lipolytic, and high lipolytic states, 4) RGZ increases the rate of the futile cycle of TG in synthesis biased fashion. This could explain why RGZ treated adipocytes have a reduced output of NEFA, 5) RGZ-treated adipocytes utilize larger amounts of glucose for the synthesis of G-G and this may be the cause of the enhanced rate of FA re-esterification. The greater utilization of glucose for G-G synthesis would be possible, at least in part, because RGZ up-regulates the expression of HK, LDH, GPDH, and ATGL, and 6) Neither glyceroneogenesis nor GK play significant roles in NEFA lowering effect of RGZ.

Overall the findings in this study provide new insights to the lipid metabolism of adipocytes and NEFA lowering effects of RGZ.

ADVISER'S APPROVAL: Dr. Jose L Soulages