NOTE TO USERS

The Characterization of the Glucose Tolerance Factor (GTF) in L6 Myotubes and 3T3-L1 Adipocytes

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Abstract

The Characterization of the Glucose Tolerance Factor (GTF) in L6 Myotubes and 3T3-L1 Adipocytes

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This thesis reports the effects of the Glucose Tolerance Factor (GTF) on the stimulation of 2-deoxyglucose (2-DG) transport in 3T3-L1 adipocytes and in L6 myotubes. The maximal effects on 2-DG transport in adipocytes were observed at concentrations of 133 nM insulin. The maximal effect of GTF was seen at 20 mg/ml in 3T3-L1 fat cells while maximal stimulation of GTF was seen in L6 myotubes at 50 mg/ml. Both insulin and GTF exhibited concentration dependent stimulation of 2-DG transport. In 3T3-L1 adipocytes combination of both insulin plus GTF gave higher sugar transport than either treatment alone. Also, we examined the effect of insulin, GTF and the combination of insulin and GTF on isoproterenol induced lipolysis. Lipolysis was decreased 28% by the addition of 1 mg/ml GTF. Addition of 0.04 nM insulin decreased lipolysis by 48%. In combination, GTF and insulin at the concentrations employed above decreased the lipolysis rate by 68%. Cells that were exposed to insulin, GTF, or a combination of both did not change ^{125}I-insulin binding to its receptor on 3T3-L1 adipocytes and therefore GTF does not bind to the insulin receptor. We observed no tyrosine phosphorylation of the insulin receptor β chain in the presence of GTF. When GTF was added in combination with insulin, we observed a decrease in tyrosine phosphorylation of the insulin receptor β chain compared to insulin alone. We observed phosphorylation of the insulin receptor substrate-1 (IRS-1) in the presence of insulin, GTF and a combination of insulin plus GTF at an exposure time of 15 minutes. When

GTF was added to insulin, we observed a decrease in IRS-1 phosphorylation compared to insulin alone. Also, we observed that GTF had an effect on GLUT 1 and GLUT 4 recruitment from endosomes to the plasma membrane. In the presence of insulin alone, effects on GLUT 4 translocation were greater than GTF alone, or in combination of insulin and GTF. There was a difference in GLUT 1 translocation compared to GLUT 4 translocation. GTF alone had the same effect on GLUT 1 translocation as insulin alone. The combination of insulin and GTF had higher effects of GLUT 1 translocation than either alone.

These observations indicated that GTF had similar effects to insulin in stimulating glucose uptake in 3T3-L1 adipocytes and in L6 myotubes. Also, GTF works in an additive manner with insulin giving a higher stimulation in glucose transport without interfering with insulin binding to its receptor. GTF's mode of action is not through the phosphorylation of the insulin receptor β chain. However, GTF seems to stimulate phosphorylation of downstream signals such as the IRS-1.

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TABLE OF CONTENTS

4. GLUCOSE TOLERANCE FACTOR (GTF)

MATERIALS AND METHODS

RESULTS

LIST of FIGURES

LIST of TABLES

ACRONYMS

1. Glucose Tolerance Factor (GTF)

2. 2-Deoxyglucose (2-DG)

3. Isoproterenol (IP)

4. Insulin Receptor Substrate-1 (IRS-1)

5. Phosphatidyl-inositol 3-kinase (PI3-Kinase)

6. Src Homology (SH)

7. Intracellular Membrane (IM)

8. Protein Kinase B (PKB)

9. Insulin Like Growth Factor (IGF)

10. Free Fatty Acids (FFA)

11. Body Mass Index (BMI)

12. Chinese Hamster Ovary (CHO)

13. Low Molecular Weight Chromium (LMWCr)

14. Dubelco's Minimal Eagles Medium (DMEM)

15. Phenol Red Free (PRF)

Introduction

1.1 Insulin and its Receptor

Insulin is a very important molecule due to its many functions primarily in glucose metabolism. Insulin's discovery by Banting and Best in 1925 led to great medical advancements in the treatment of diabetes. Diabetes can be fatal if untreated. People with type 2 diabetes are insulin resistant; therefore they need alternative medicines to replace or to potentiate insulin's effects. One such compound studied in this thesis is the Glucose Tolerance Factor or known as GTF. In order to characterize GTF, it is important to understand the insulin molecule and its mode of action.

Insulin is a small anabolic hormone with a molecular weight of 6 Kd (fig.1) (1). The insulin molecule has two amino acid chains held together by disulfide bonds (1). The A chain contains 21 amino acids and the B chain contains 30 amino acids (2). These amino acid sequences are similar throughout different species, but do have highly conserved regions such as both ends of the A chain and the C-terminus of the B chain (fig.2) (1, 2).

Insulin has many physiological effects. It can stimulate glucose, amino acid and uptake of ions such as zinc (3). Also, insulin can activate transcription factors and modulate differentiation and cell growth (fig.3) (4). These physiological effects all occur through insulin action on the insulin receptor. The insulin receptor contains two α-

subunits, which are totally extracellular to the plasma membrane. They are held together

by disulfide bonds. The α-subunits are attached to β-subunits by disulfide bonds. They

form a heterotetramer ($\alpha_2\beta_2$) (3-5). The β-subunits have three domains. They have an

extracellular domain, a transmembrane domain which span the plasma membrane, and a

cytosolic domain (3-5). The α-subunits contain the insulin binding site(s) and are

cysteine rich (3) and the β-subunits the autophosphorylation sites, such as the

juxtamembrane NPXY-Motif at position 960 and C-terminal (fig.4).
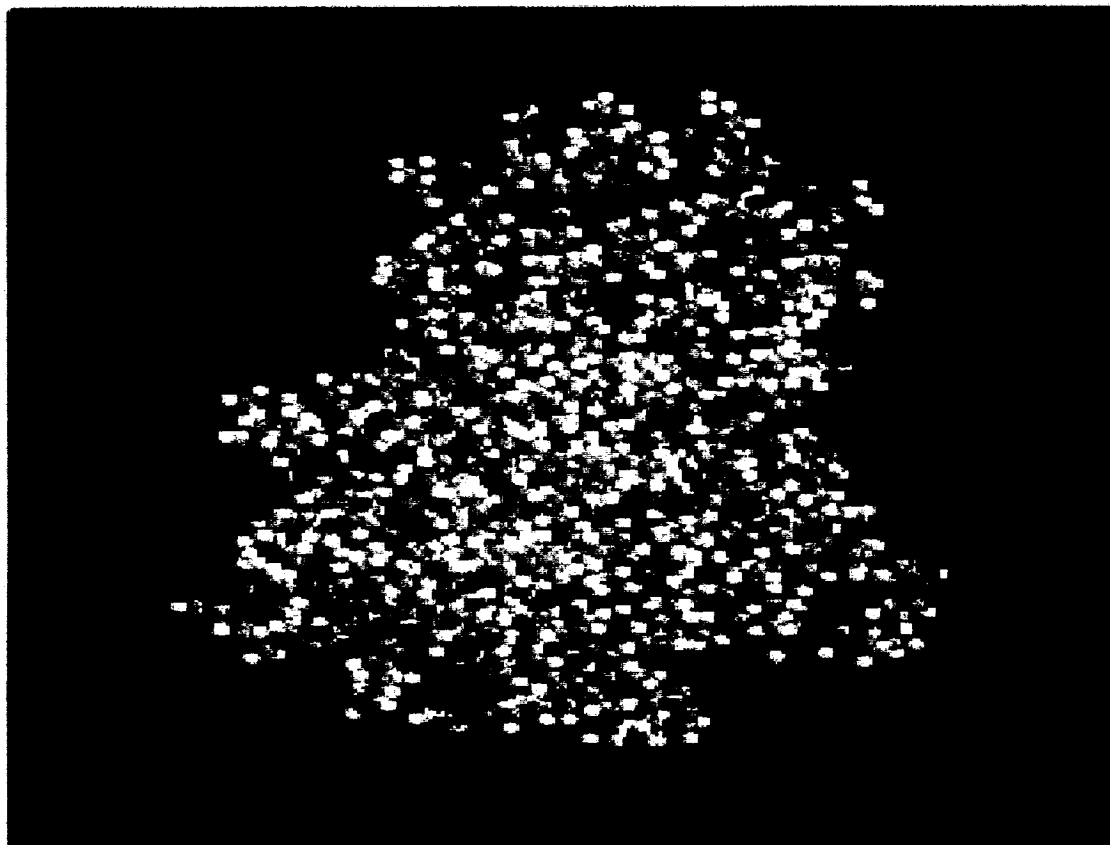
FIGURE 1. The structure of the insulin molecule.

The ball and stick presentation of the 3D structure of insulin depicts the A-chain and the B-chain which are held together by disulfide bonds represented in yellow. Insulin is an anabolic hormone that has a molecular weight of 6000 Daltons.

3
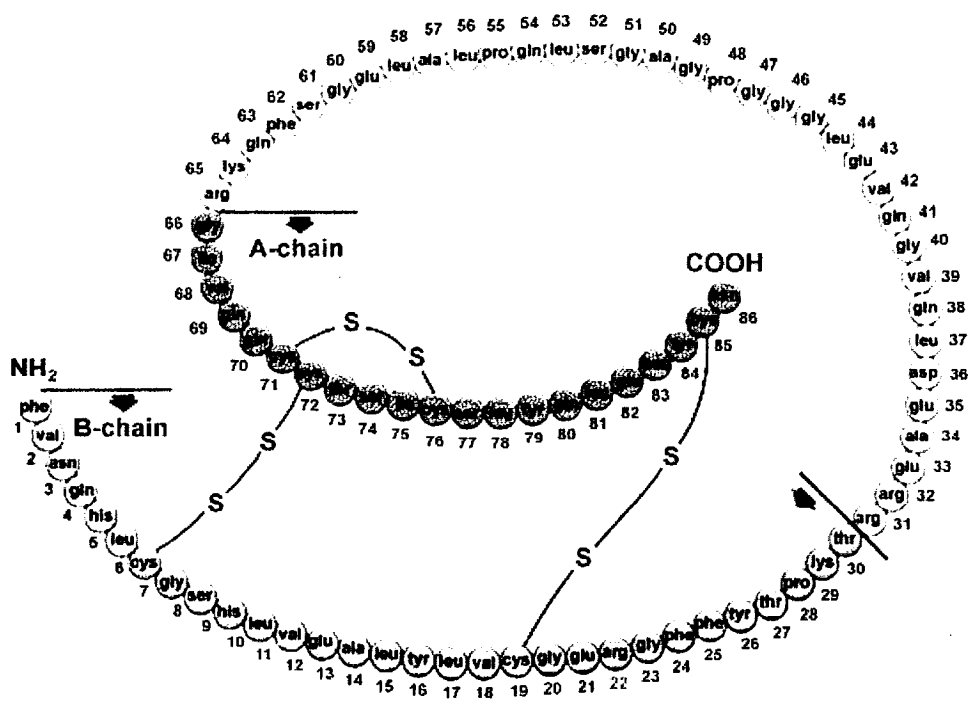
FIGURE 2. The amino acid sequence of insulin.

The diagram shows the amino acid sequence of the A-chain and B-chain of insulin. The red amino acid chain represents the A chain which is composed of 21 amino acids. The A chain has its own disulfide bond between amino acids 71 and 76. The green amino acid chain represents the B chain. The B chain has 30 amino acids and form two disulfide bridges with the A chain at amino acids 6 and 19. The amino acids outlined in yellow indicate the amino acids present in the pro-insulin state before being cleaved to insulin.

4
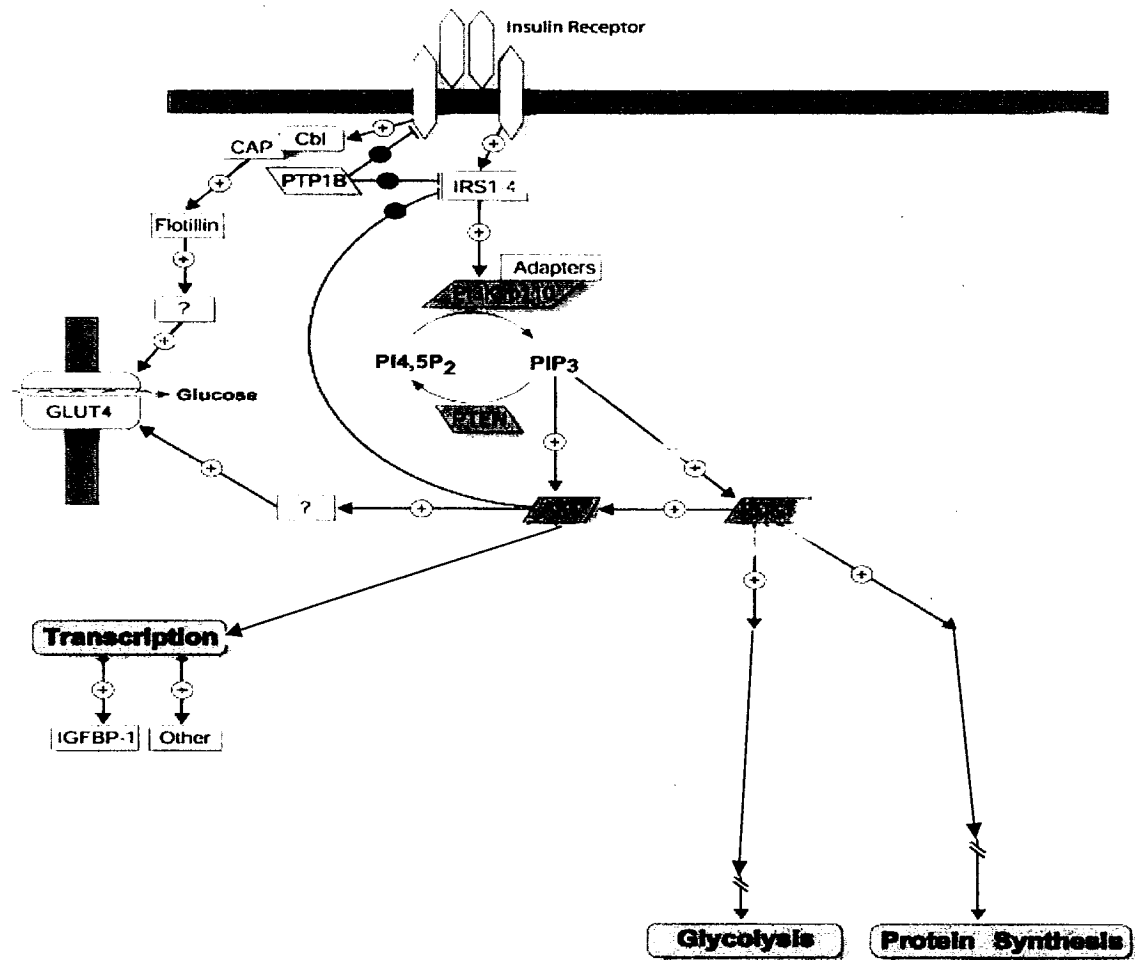
FIGURE 3. Overview of the insulin signaling pathway and its physiological functions.

This diagram shows the insulin receptor composed of two α chains that are located outside the cell membrane, and two β chains that span the membrane. When insulin binds to the receptor, it triggers a series of cascading events that allow numerous events to take place depending on physiological needs. The diagram shows different signaling molecules involved in the insulin pathway such as IRS-1, PI3-kinase, Ras, PKB/Akt, which are involved in glucose transport into cell via glucose transporter GLUT 4, and other signaling molecules involved in transcription, glycolysis, and protein synthesis.

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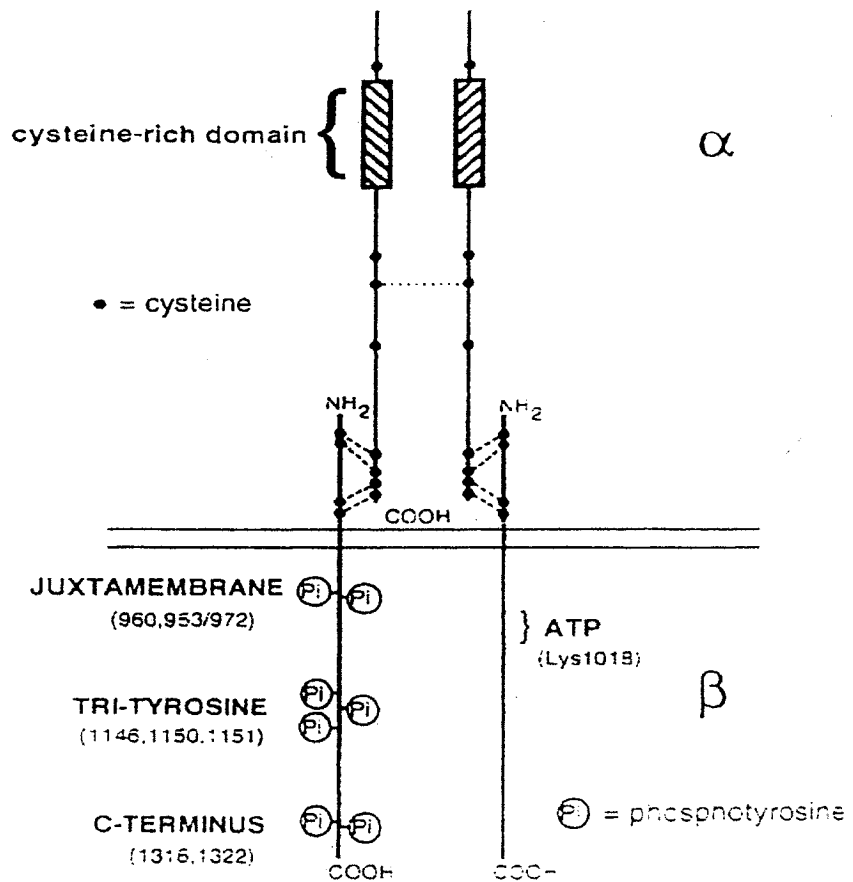
Ebina Y et al. The Human Insulin Receptor cDNA: the Structural Basis for Hormone-Activated Transmembrane Signalling. Cell 40: 747 (1985).

FIGURE 4. The structure of the insulin receptor.

The insulin receptor is composed of two α subunits held together by disulfide bridges. It is located extracellular to the plasma membrane and it is the insulin binding domain. The α subunits are cysteine rich. The β subunits have three domains. The first domain is located extracellelarly, which is bound to the α subunits by disulfide bridges. The second is a transmembrane domain, and third is the cytosolic domain. The cytosolic domain, which contains tyrosine residues at juaxtamembrane at positions 960, 953/972, at a tri-tyrosine cluster at positions 1146, 1150, 1151 and C-terminus at positions 1316, 1322. These tyrosine residues become phosphorylated when insulin binds to its receptor.

1.2 Insulin Binding to its Receptor

When insulin is secreted by the β cells of the pancreas, it circulates throughout the blood, and binds to its receptor on most cell surfaces (3). Upon insulin binding to the receptor, insulin and the receptor complex is internalized into the cell (6, 7). Once the complex is internalized, insulin is dissociated from its receptor by an acidic endosomal vesicle or lysosome (8). After the dissociation of insulin from its receptor, each segregate into separate vesicular structures (9, 10). Most of the insulin in the vesicular structures are degraded into lower molecular weight products and are used for energy through catabolism (11, 12). Most of the insulin receptors are recycled back to the plasma membrane (13, 14).

1.3 The Insulin Signaling Pathway

When insulin binds to the α-subunits, it mediates autophosphorolation of the β chain and trans-autophosphorylation between the β-subunits (fig.4) (3, 15-18). This allows the autophosphorylation of the tyrosine residues (19-22) and the phosphorylation of the Insulin Receptor Substrate -1 (IRS-1). IRS-1 protein has a molecular weight of 131 Kd and was detected in insulin-stimulated hepatoma cells by immunoprecipitation with anti-phosphotyrosine antibodies (23). It was shown by retarded migration on a SDS-PAGE gel that IRS-1 has many sites for phosphorylation (3). IRS-1 has 21 putative tyrosine phosphorylated sites (24, 25, 26) but phosphorylation occurs on eight tyrosine residues at positions 608, 628, 939 and 987 (3) when insulin activates the insulin

7

receptor. When insulin does not bind to its receptor, IRS-1 is serine phosphorylated (25). When insulin binds to the insulin receptor, the NH_2-terminal (pleckstrin homology) portion of IRS-1 binds to tyrosine at position 960 of the insulin receptor (27-29). Then the IRS-1 becomes tyrosine phosphorylated. This phosphorylation allows many enzymes and adaptor proteins to associate with IRS-1. Some of these proteins are phosphatidyl-inositol 3-kinase (PI3-kinase), phosphotyrosine phosphatase SHP2, which acts as an adapter molecule between growth factor PDGFr, and Grb 2-Sos complex when phosphorylated. Grb 2, which is also an adapter molecule, is a small protein that is located in the cytoplasm. The SRC homology domain 2 (SH2) of Grb 2 binds to IRS-1 when stimulated by insulin (3).

During glucose uptake in 3T3-L1 adipocytes, IRS-1 is tyrosine phosphorylated by the insulin receptor in the presence of insulin. The phosphorylated IRS-1 is located in the intracellular membrane (IM) compartment which is an aqueous insoluble multi-protein complex, which is the cytoskeleton portion underlying the plasma membrane (30). The phosphorylation of IRS-1 causes IRS-1 to associate with PI3-kinase. This association occurs in the intracellular membrane (IM) compartment (30). The phosphorylation of IRS-1 recruits PI3-kinase from the cytosol to the IM and then associates in the IM (31-34). IRS-1 is then translocated from the IM to the cytosol (31), and subsequently associates with the 85-Kd subunit of the PI3-kinase (3, 15, 31), which is a heterodimer made up of an 85-Kd (α-p85) and 110-Kd (p110) subunits (4, 5). The α p85 subunit has two SH2 domains and a SH3 domain also (35-37). The SH2 and SH3 domains are known as the Src homology domains. They bind to phosphotyrosine residues. The p110 subunit

contains the catalytic activity (38, 39). When IRS-1 is phosphorylated it comes together

with the p85 of the PI3-kinase and it becomes activated (40). The activated form of PI3-

kinase recruits protein kinase B (PKB) through its lipid products (41). PI3-kinase lipid

products phosphatidyl-inositol 3, 4, 5 triphosphate PI (3, 4, 5) P_3 and or phosphatidyl-

inositol 3, 4 biphosphate PI (3, 4) P_2 bind to the protein kinase B pleckstrin homology

(PH) domain containing serine/threonine kinase (42). This allows PKB to go to the

plasma membrane where it undergoes a conformational change. This conformational

change allows the serine and threonine to be accessible for phosphorylation (42-44). The

phosphorylation occurs at serine 473 and threonine 308 in PKBα. PKB has three

isoforms. PKBα, which is the major form, activated by insulin in muscle and adipocytes

(45). PKB β is activated in adipocytes and PKBγ is activated by insulin in cell culture

models (45). PKB is a cellular homologue of the transforming oncogene v-akt (46).The

activation of PKB by PI3-Kinase ultimately leads to glucose uptake by GLUT 4 the main

insulin-responsive glucose transporter in muscle and fat (fig.5) (47).
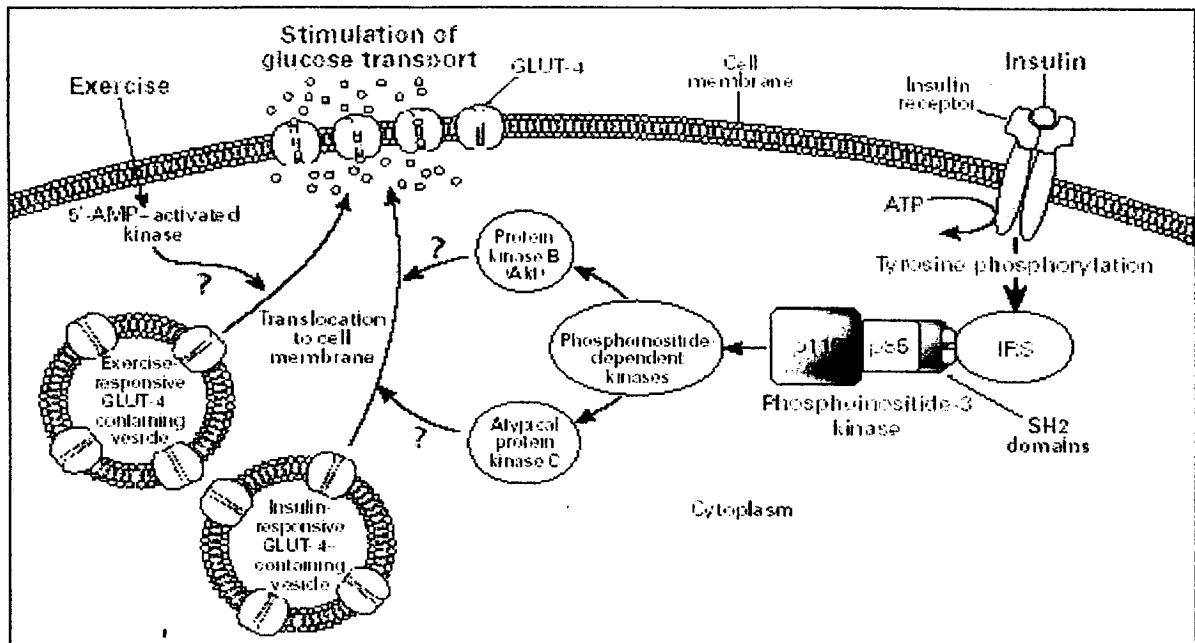
Shepherd PR, and Kahn BB. Glucose Transporters and Insulin Action: Implications for Insulin   Resistance and Diabetes Mellitus . New England of Medicine 341: 248-257, 1999

FIGURE 5. The insulin signaling pathway leading to glucose transport via GLUT 4 vesicles in muscle cells and adipocytes.

Insulin is secreted by β cells into the blood where it binds to the insulin receptor. Once insulin is bound to its receptor, autophosphorylation of the tyrosine residues on the insulin receptor β chain occurs. This leads to phosphorylation of IRS-1, which docks to other signaling molecules such as PI3-kinase through its p85 subunit. The catalytic subunit of PI3-kinase that is bound to p85 activates protein kinase B (Akt) and atypical protein kinase C. The activation of PKB and PKC allows translocation of GLUT 4 vesicles located intracellularly to the plasma membrane. The translocation of GLUT 4 transporter to the plasma membrane stimulates glucose transport into the cell.

Eight facilitated-diffusion glucose transporters have been identified (47-51). The first is GLUT 1 found in the brain, erythrocytes and endothelial cells. GLUT 2 was found in the kidneys, small intestines, in the pancreatic β cells, and the liver. GLUT 3 has been found in neurons, muscle cells and placenta. GLUT 4 has been found in skeletal muscle, cardiac muscle and adipose cells. GLUT 5 was found in the small intestine, sperm, kidney, brain, adipose cells and muscle (47). GLUT 6 is a glucose transporter pseudogene which was found using an erythrocyte/ GLUT 1 cDNA probe (48). The GLUT 6 cDNA was isolated from human small intestine and fetal skeletal muscle cDNA libraries (48). Part of the GLUT 6 mRNA is found in all human tissue (48). GLUT 7 was thought to be a liver plasma membrane transport protein with closest similarities to GLUT 2 (49). GLUT 7 cDNA was isolated from screening the rat liver library (49), but it was later found that GLUT 7 was a cloning artefact and it is not a liver plasma membrane transport protein (50). GLUT 8 was cloned from a murine embryonic teratocarcinoma cell line F9. It is 478 amino acids long, shortest of all the GLUTs (51). Northern Blots showed that GLUT 8 mRNA is expressed highly in the placenta. It was shown that GLUT 8 translocation occurred in the presence of insulin in mouse blastocyst (51). GLUT 8 is a glucose transporter in mammalian blastocyst (table1).

TRANSPORTER	TISSUE DISTRIBUTION
GLUT 1	BRAIN, ERYTHROCYTES,AND ENDOTHELIAL CELLS*
GLUT 2	KIDNEY, SMALL INTESTINES, EPITHELIA, LIVER, AND PANCREATIC β CELLS*
GLUT 3	NUERONS, AND PLACENTA*
GLUT 4	SKELETAL MUSCLE, CARDIAC MUSCLE, AND ADIPOSE CELLS*
GLUT 5	SMALL INTESTINES, SPERM, KIDNEY, BRAIN, ADIPOSE CELLS, AND MUSCLE*
GLUT 6	Pseudogene**
GLUT 7	LIVER (CLONING ARTIFACT)***
GLUT 8	PLACENTA, AND BLASTOCYST****

*New England Journal of Medicine 341: 248-257, 1999
**J.Biol.Chem. 265: 13276-13282, 1990
***Biochem J. 285: 173-177, 1992
****PNAS. 97: 7313-7318, 2000

TABLE 1. The location and characterization of 8 glucose transporters.

The table gives a summary of the eight glucose transporters. In this thesis, work was done on GLUT 1 and GLUT 4. GLUT 1 is found in the brain, erythrocytes, and endothelial cells, and is constitutive glucose transporter. GLUT 4 is found in the skeletal muscle, cardiac muscle, and adipose cells. GLUT 4 is responsive to insulin.

The main focus in this thesis is on the GLUT 4 transporters. The GLUT 4 transporter protein is part of a transport vesicle that contain a phospholipid membrane with other proteins such as the insulin-responsive amino peptidase, synaptobrevin (also known as V-SNARE) and the Rab-4 binding protein (fig.6) (47). When insulin is not present, about 90% of the GLUT 4 is present in intracellular vesicles (47-52,53). When insulin binds to its receptor it activates the tyrosine kinase phosphorylation of IRS-1 and SHC proteins (39). This leads to the phosphorylation of PI3-kinase by IRS-1 association with the p85 of PI3-kinase (39, 54). The activated PI3-kinase produces phophatidyl-inositol lipid products PI $(3, 4, 5)$ P_3 and or PI $(3, 4)$ P_2, which binds to the serine/threonine PH domain of the protein kinase B (40). This is associated with translocation of GLUT 4 to the plasma membrane from activation of PKB or atypical isoforms of protein kinase C (55-57). Once GLUT 4 is present at the plasma membrane, glucose is transported into the cell by facilitated diffusion.
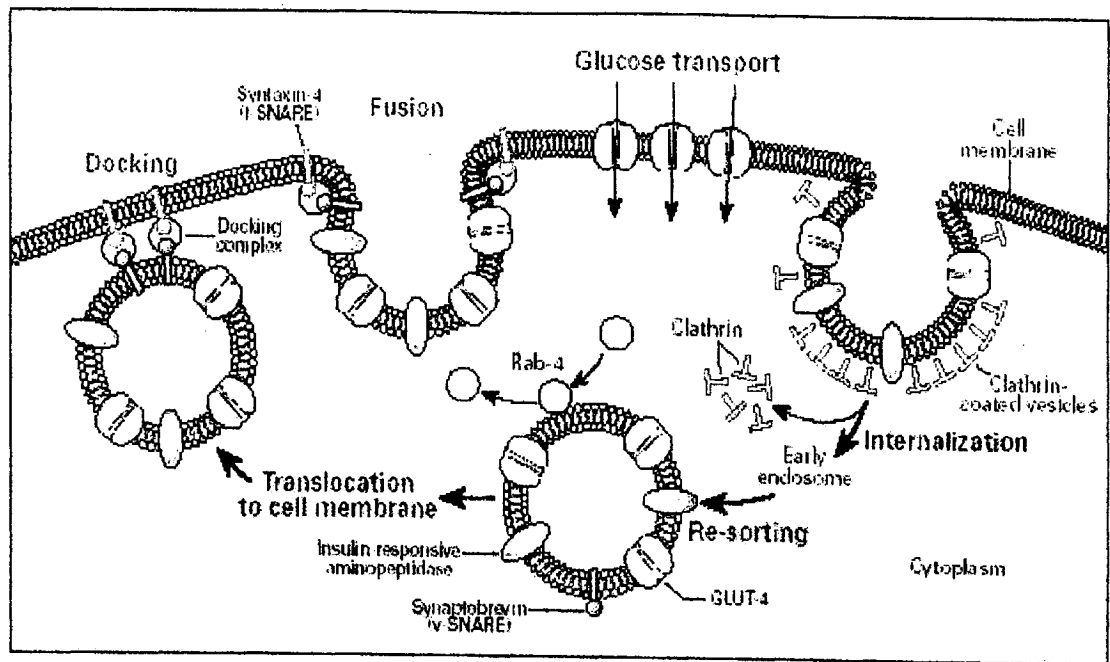
Shepherd PR, and Kahn BB. Glucose Transporters and Insulin Action:
Implications for Insulin   Resistance and Diabetes Mellitus .   New England
Journal of Medicine   341: 248-257, 1999

FIGURE 6. The mechanisms involved in the translocation of the GLUT 4
transporters in muscle cells and adipocytes.

When insulin binds to its receptor, the GLUT 4 transporter moves to the plasma
membrane. The GLUT 4 vesicle contains a vesicle-associated membrane protein-2 also
known as synaptobrevin (v-SNARE) and a small guanosine triphosphate-binding protein
called Rab-4. Here, synaptobrevin docks with a docking protein called syntaxin-4 (or also
known as t-SNARE). The GLUT 4 vesicle then fuses with the plasma membrane. Then
Rab-4 dissociates from the vesicle and moves to the cytosol. Once insulin is removed
from its receptor, budding of the clathrin-coated vesicle from the plasma membrane
causes internalization of the GLUT 4 back into the cytoplasm.

1.4 Regulation of the Insulin Signaling Pathway

Regulation of the insulin signaling pathway is done through a series of phosphorylation and dephosphorylation steps. Also, serine phosphorylations of certain signaling molecules such as IRS-1 can regulate the insulin signaling pathway (3). Other molecules such as PTPase 1B, which is a tyrosine phosphatase is able to dephosphorylate the insulin receptor in vitro and therefore can act as a potential negative regulator of insulin action (4).

2. Insulin's Role in Sugar Metabolism

2.1 Insulin and Glucose Transport

Glucose is a key source of energy and is constantly needed by the brain. After a meal, blood glucose levels rise and in response to the rise in blood glucose, insulin is secreted into the blood stream. Insulin then stimulates glucose transport, metabolism, and storage. Glucose which is used for energy during physical activity and for fuel is taken up by glucose transporters GLUT 1 and GLUT 4 in muscles and in adipocytes.

Cain et al. (58) made the important observation that vesicles - or - V-SNAREs of the synaptobrevin family are present on GLUT 4 vesicles (fig.6). Timmers et al. (59) have also found that these GLUT 4 V-SNAREs form a complex with recombinant NSF and α-SNAP and syntaxin-4. Insulin signaling may lead to an enhancement of vesicle V-SNARE participation to form complexes possibly via the activation of Rab protein GLUT 4 proteins (10). GLUT 4 proteins translocate from intracellular vesicular pools to the plasma membrane by a process of targeted exocytosis and then return to the intracellular environment via endocytotic events (60). GLUT 4 translocation occurs by docking to t-SNARES (Syntaxin-4) via its vesicle v-SNARE (synaptobrevin) (61-65). Other components involved in the docking of the vesicles to the plasma membrane are Synips which associates with Syntaxin-4. Rab-4 is also involved in GLUT 4 translocation (61). Disruption of the clatherin coat by acidification of the cytosol can cause endocytosis of

Glut-4 allowing internalization of the vesicle (fig .6) (61, 66, 67). Also, the GTPase

dynamin is involved in the endocytosis of GLUT 4 (68, 69). It was shown that insulin

induces phosphorylation of dynamin (70). This suggests that internalization of GLUT 4

occurs through a clatherin-coated pit-mediated dynamin-dependent endocytic pathway

(61).

Any disruptions in these steps in the insulin-mediated pathway can disrupt

glucose uptake by the glucose transporters. Inhibition of PI3-kinase with antibiotic

wortmannin can inhibit or reduce the translocation of the GLUT 4 transporter to the

plasma membrane (4, 41). Also, creating knockout mutants for the αp85 subunit of PI3-

kinase in mice inhibits GLUT 4 translocation (71). Other factors also can contribute to

the lack of glucose uptake by the cell.

2.2 Insulin and Type 1 Diabetes

A major problem arising from the lack of glucose uptake is a disease known as Diabetes mellitus. There are two types of Diabetes mellitus. Type 1 diabetes occurs due to a genetic or autoimmune disorder where the pancreatic beta cells are compromised (72). The β cells are targets of auto reactive T-lymphocytes (72). Also, the islets become inflamed where macrophages, dendritic cells and B-lymphocytes can be involved in the destruction of the pancreatic β cells (72). Other studies show that T cells (CD4$^+$ and CD8$^+$) are involved for Type 1 diabetes (73). Suppression of islet auto antigens such as GAD65 or GAD67 inhibits the development of insulitis and Type 1 diabetes (74).

There are other reasons for the occurrence of Type 1 diabetes. As mentioned previously, Type 1 diabetes can be inherited. One genetic factor can be the insulin like growth factor (IGF) gene. Specifically IGF-I and IGF-II are involved in the regulation of β cell replication and apoptosis (75, 76). If the equilibrium of growth and death of the β cells are disrupted by changes in IGF-I or IGF-II, glucose intolerance can result (77). Some experiments done by knocking out receptors specific for pancreatic β cells in mice showed that there are impairments or lack of glucose stimulated insulin secretion (78, 79). One study did not show any significance in the impairment of insulin secretion with gene variants of IGF-I and IGF-II (77). The researchers tested IGF-I gene polymorphism with (CA) $_n$ repeats located in its promoter region, and Apa I polymorphism in the IGF-II gene. The researchers observed no significant impairment of insulin secretion with the

IGF-I and IGF-II gene variants (77). People with Type 1 diabetes are dependent on insulin since the β cells cannot secrete sufficient amounts of insulin for proper glucose disposal. Therefore Type 1 diabetes is insulin dependent and regular injections of insulin are required. More modern approaches for treatment of Type 1 diabetes is by the use of insulin pumps. Injections and insulin pumps are usually inserted in the abdominal area. Insulin pumps usually secrete insulin in spurts throughout the day. People with Type 1 diabetes usually develop this disease as a child which Type 1 diabetes is also known as Juvenile onset diabetes. People with Type 1 diabetes are usually thin and are not obese.

2.3 Insulin and Type 2 Diabetes

The second form of diabetes, known as Type 2 diabetes, has complications due to the association with obesity. Type 2 diabetes occurs mainly in adults, but there are increases in the incidence of type 2 diabetes in children. About 80-85% of people with diabetes have Type 2 diabetes. Type 2 diabetes differs from Type 1 diabetes in the fact that people with Type 2 diabetes are insulin resistant to glucose stimulation (80-82). . This insulin resistance can be due to a number of factors such as a loss of β cell mass (83) due to β cell destruction and due to free fatty acids (83-86).

Elevated free fatty acids in the blood are predictors of decreases in glucose tolerance (87, 88). It was also shown in animals and in vivo that increases in free fatty acids can affect β cell decompensation by disrupting glucose metabolism. This disruption is caused by affecting the Randle cycle, which results in the inhibition of glucose-stimulated insulin secretion (74, 89- 92). The second way free fatty acids can affect β cell decompensation is by signal transduction (78). Insulin resistance due to free fatty acids affects protein kinase C and other stress-activated kinases (93, 94). A third way is through affecting transcription. Free fatty acids and acyl CoA derivatives are important ligands for several classes of nuclear receptors (95). Fatty acids regulate expression of hundreds of β cell genes (83, 96, 97). Also, free fatty acids are known to lower hepatic insulin clearing (87, 98). Free fatty acids in the systemic circulation may inhibit skeletal muscle glucose metabolism and contribute to peripheral insulin resistance (99). Also, increased triglyceride stores due to circulating free fatty acids can give higher rates of

lipolysis and thus influence both basal and stimulated insulin secretion (84). It is possible that the inhibition of glucose stimulated insulin secretion due to chronic free fatty acid presence is not due to an inhibitory effect but as a switch of β cell response to the free fatty acids and away from the glucose (85).

Type 2 diabetes is also associated with obesity, which is a major risk factor for non-insulin-dependent diabetes mellitus (84-86). There is increasing evidence that there are links between obesity and diabetes depending on the region where body fat is located (85, 87). Men that are obese (in the abdominal region) have a higher incidence of diabetes (81, 100). This rise in the incidence of disbetes is increased with the increasing of fat in the abdominal region (101). Obesity can cause many complications with diabetes, or be a health risk on its own. It was shown by Patterson et al. (102) that pre-pregnant woman with higher Body Mass Index (BMI) that had gestational diabetes had a greater risk of their child getting congenital malformations than pre-pregnant women with lower BMIs. Pre-pregnancy BMI was a major predictor of heart and renal/urinary complications (102). Obesity can cause other complications such as atheroscleroses and dyslipidemia (80). In recent studies researchers found that there are obesity-related loci on a chromosome region 12q23-24 (103). Li et al. (103) took DNA samples from parents of the families and were genotyped using microsatellite markers across the human genome. They used a program gene hunter 1.3 to find obesity related chromosomes with BMI's above 27. They showed strong evidence for linkage related phenotypes on chromosome region 12q23-24 (103). There is also evidence that there were some genes in certain chromosomes that were susceptible to Type 2 diabetes (104). Using linkage

analysis Silander et al. (104) found that genes on chromosomes 6, 11 and 14 are predisposed to Type 2 diabetes. The next step was to determine how genetic alterations of these genes can cause Type 2 diabetes. Since obesity is rapidly becoming a large health problem associated with hypertension, diabetes, cardiovascular disease and cancer, particular attention has been taken (103). Some simple changes in life-style such as dietary modifications, lowering glycemic intake can control the diabetes and help lose weight (105-107). Exercise can increase lipolysis of adipose tissue to release free fatty acids (108,109).

2.4 Disruption of the Insulin Signaling Cascade

Other factors that can cause diabetes cannot be modulated by nutritional improvements or exercise. One such factor is inhibition of the insulin signaling system. Incidences of diabetes have been associated with mutations in the insulin receptor (110). Disruption of the insulin receptor in humans or mice showed high levels of hyperglycaemia, with other developmental defects, which become lethal soon after birth (111-113). Knocking out IRS-I function in mice causes peripheral insulin resistance (114-116) and decreased insulin-stimulated peripheral glucose uptake. Disruptions of IRS-2 in mice develop progressive diabetes during the first 10 weeks of life (117). Mice homozygous for IRS-1 showed higher serum insulin levels before and after glucose intake (116). Mice that were homozygous for mutations in the IRS-1 locus showed abnormal glucose tolerance (115). Also, these mice showed growth retardation and lower birth weight (115,116). This is due to IRS-1 dependent and independent pathway of insulin and IGF-1 and IGF-2 signal transduction (115,116). Also it was shown that mice lacking IRS-1 did not activate PI3-kinase (115,116). Little or no PI3-kinase activity was co-immunoprecipitated in α-IRS-1 homozygous mouse for the mutation, but stimulation of PI3-kinase was seen (116). This can be due to the presence of IRS-2, which can be seen, in the anti-phosphotyrosine immunoblots of anti-p85 (115). Disruption of PI3-kinase with inhibitors such as wortmannin and LY294002 gave evidence that it is required for GLUT 4 recruitment (118). Also, experiments done by Hara et al. (71) showed that Chinese hamster ovary (CHO) cell lines with mutant p85α (Δp85) have

reduced glucose uptake. The formation of the IRS-1 and PI3-kinase complex is hindered but the tyrosine phosphorylation of PI3-kinase is unaffected (71). The lipid products of PI3-kinase PI (3, 4, 5) P_3 in Δp85 CHO cells were also reduced (71). This can lead to lower activation of Protein kinase B and other downstream mediators. GLUT 1 transporter in Δp85 CHO cells was impaired compared to wild type CHO cells (71). If further steps in the pathway are perturbed, then this can lead to impaired and/or reduced glucose uptake, which can lead to developing diabetes. The next step in the pathway is the activation of protein kinase B. Inhibition of Protein kinase B in L6 myotubes abolishes insulin-stimulated glucose transport (119). Using specific inhibitors of PKB/Akt completely inhibited translocation of the GLUT 4 transporters but had no effect on PI3-kinase (120,121). Finally, some mutations in the glucose transporters will result in inhibition of glucose uptake and other deleterious effects. For example, mutations in GLUT 1 cause seizures due to low glucose uptake across the blood brain barrier (122). Mutations in GLUT 2 cause the Fanconi-Bickle syndrome, which is an autosomal recessive metabolic disorder (123). People with Fanconi-Bickle tend to have impaired utilization of glucose and galactose, nephropathy, and hepatic and renal glycogen accumulation (123). Most important for Type 2 diabetes, mutations in GLUT 4 could cause insulin resistance (47).

Much work has been done to understand the mechanisms involved to regulate these defects in the insulin-mediated pathways. Many drugs have been developed to bypass or correct these defects.

3. Treatments for Type 2 Diabetes

3.1 Thiazolidinediones

Certain drugs like thiazolidinediones are used to treat Type 2 diabetes. Thiazolidinediones are a class of anti-hyperglycaemic agents which are taken orally (fig.12) (125,129). They are used as insulin sensitizing agents (124-130). Thiazolidinediones are known to be able to stimulate glucose transport (124,128), but the exact actions are still not fully understood (124,127,128,129). Experiments have been done to try to elucidate the molecular mechanisms of thiazolidinediones activation in the insulin pathway to stimulate glucose transport into cells (124,128,131). A thiazolidinedione named rosiglitazone (fig.7) was administered to non-diabetic and Type 2 diabetic rats in vivo and an increase in basal and insulin stimulated phosphorylation and activation of PKC-ε/λ was seen (124). The stimulated phosphorylation of PKC was not due to an increase of IRS-1 activated PI3-k or PKB (124). It was shown that rosiglitazone did increase basal glucose transport which was shown to be dependent on PI3-kinase, 3-phosphoinositide-dependent protein kinase-1 (PDK-1) and PKC-λ (124). Also, rosiglitazone showed an increase in tyrosine phosphorylation of Cbl, which is an adapter protein that has numerous functions (128). It functions as an ubiquitin ligase for protein breakdown. Also, it functions as a substrate for protein tyrosine kinases at positions 371 and 731 (128). Cbl increases the activity of p85 by PI3-kinase and PKC-λ (124). This was shown in 3T3-LI adipocytes.
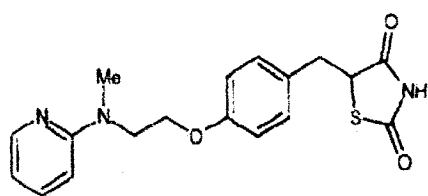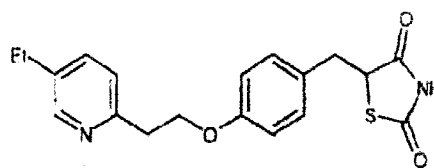
Rosiglitazone (BRL-49653)　　　　　Pioglitazone
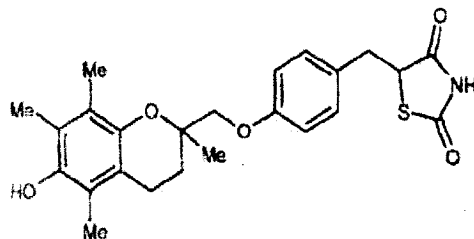
Troglitazone

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FIGURE 7. The structures of thiazolidinediones

Figure 7 shows the structure of three thiazolidinediones, Rosiglitazone, Pioglitazone, and Troglitazone.

26

wortmannin, an inhibitor of PI3-kinase also inhibited rosiglitazone action on 2-deoxyglucose uptake (124). Substitution mutations at positions 371 and 731 to phenylalanine in the pYXXM8 motif of Cbl decreased its interaction with the p85 subunit of PI3-kinase (128). This was shown to inhibit activity of PI3-kinase, PKC-λ and glucose transport (128). This also occurred with the presence of rosiglitazone. These results show that the pYXXM motifs of Cbl are required for TZD-induced activation of PI3-kinase, PKC-λ and glucose transport (128).

Thiazolidinediones are known to increase expression and translocation of the glucose transporters (125,127). Kramer et al. (125) measured protein expression of GLUT 1 and GLUT 4 in both muscle and adipose tissue. Adipose tissue of diabetic rats treated with rosiglitazone for 7 days showed a normalization of GLUT 4 and no change in normal rats (125). Protein levels of GLUT 4 were also measured. Kramer et al. (125) showed that protein content was increased in both skeletal muscle and adipose tissue. They performed a glucose uptake experiment to see if an increase in GLUT 1 resulted in an increase in glucose uptake. Kramer et al. (125) measured 3-0-methyl-D-glucose transport in epitroclearis muscles from obese Zucker rats, with or without treatment with rosiglitazone. It was shown that rosiglitazone did increase basal glucose transport corresponding to increased glucose transport of only GLUT 1 and not GLUT 4. Hernandez et al. (127) observed increased GLUT 4 translocation to the plasma membrane but without changes in mRNA or protein expression (127). Also, there was an increase in tyrosine phosphorylation of the insulin receptor β chain, IRS-1 and IRS-2 without increasing expression of IRS-1 and IRS-2 with pre-treatment of rosiglitazone. An

increase in PI3-kinase was observed with pre-treatment of rosiglitazone (127). An increase in PI3-kinase with rosiglitazone led to increased tyrosine phosphorylation while it decreased serine phosphorylation (127).

Thiazolidinediones are known to bind with high affinity for peroxisome proliferation activated receptors (PPARs) (125-127,130). Further PPARγ was found in adipocytes, which are nuclear hormone receptors involved in regulating terminal adipocyte differentiation (131-134). To identify the specific interaction of the thiazolidinedione with the PPAR receptor isoforms Young et al. (126) used a radioiodinated ligand [^{125}I] SB-236636. They observed specific binding of [^{125}I] SB-236636 with human PPARγ. This binding was PPARγ specific (126). No specific binding was detected in non-confluent non-differentiated 3T3-LI Fibroblasts, but seen in differentiated 3T3-L1 adipocytes (126). Severe insulin resistance has been shown to be caused by a loss-of-function in the human PPARγ gene (135). This mouse lacking in white adipose tissue (WAT) was severely diabetic (136,137). WAT has a function in glucose homeostasis.

3.2 vanadium

Other compounds like vanadium are thought to be potential anti-diabetic agents (138). The trace element vanadium has been shown to lower glucose levels in diabetic rats and on plasma and adipose tissues but its mechanism of function is still unclear (138, 139). Experiments done on streptozotocin (STZ) diabetic rats showed that the use of vanadium improved glucose uptake but does not increase the level of GLUT 4 translocation (139). Also the effect of vanadium is insulin-enhancing and not insulin-mimetic as vanadium depends on endogenous insulin (138), though vanadium does not increase plasma insulin levels (140, 141). A study done (142) showed that vanadium enhances insulin's inhibitory effect on lipolysis. In vitro studies done on rat adipocytes, show vanadium induce GLUT 4 translocation (143). This can be an indication that vanadium affects the insulin mediated pathway because the effect of vanadium is blocked by wortmannin, the inhibitor of PI3-kinase (144). In L6 myotubes vanadium induces GLUT 1 and GLUT 4 translocation and is independent of PI3-kinase (145). Vanadium was shown also to regulate food intake and lower body weight in fatty Zucker rats (146). A proposed mechanism of vanadium is based on inhibition of tyrosil-protein phosphatase activity (146).

3.3 Chromium

Chromium is another compound shown to be an important trace element for animals and humans (139-140). A chromium deficient diet led to development of glucose intolerance (150-155). Chromium has been studied as chromium salts (156) or organic chromium compounds like chromium picolinate (157,158) and as low molecular weight Chromium Substance (LMWCr) (159-161). Animals deficient in chromium have high blood glucose levels, but once fed food containing chromium their blood glucose levels decreased (150).

4. The Glucose Tolerance Factor (GTF)

4.1 Isolation and Purification of GTF

A yeast extract that was thought to be a chromium containing material was first isolated by Schwartz and Mertz (162). This extract was called the Glucose Tolerance Factor (GTF) since GTF can lower blood glucose levels in animals (163,164) and humans (165,166). Other similar GTF extracts were isolated from various sources such as brewers yeast and pork kidney extracts (164,167). It has been known that GTF extract is of low molecular weight with a cut off of 3500 and is stable in neutral or acidic conditions indicating it is cationic (163,167,168,172-174). Great effort has gone into purifying the active ingredient of GTF as a pure, biologically active compound, but it has not been identified yet (163,169-171).

Mirsky et al. (163) used 10g of Merck yeast extract powder to attempt to fully purify the GTF extract. Most of the chromium in the yeast was isolated in the concentrated dialyzate, which was passed through a DEAE-II cellulose column. The column was washed twice once with water and then with 0-2M NH_4Cl gradient. Fractions of 5 ml were collected and optical absorbance of 262 nm was used to determine which fraction contained chromium. The dialyzate was passed through a DEAE-cellulose column and was further purified in a DOWEX 50W-x8 column and eluted as mentioned above (163).Initial preparation of GTF contained chromium and thought to be important

for biological activity. However other studies showed that chromium free fractions can be obtained that have activity (168,174,175).

Haylock et al. (176) used similar methods to purifying GTF as Mirsky et al. (163), but further purified it by separating fractions through a DOWEX 50-x12 cation-exchange column using a pH gradient (176). All the chromium containing fractions from the ion-exchange columns were bulked and freeze-dried (176). The freeze-dried extracts were put in a sephadex G-10 desalting column using minimum volume of distilled water. The fractions collected were analyzed for chromium. It should be noted that the freeze-drying process did not cause any loss of chromium. Haylock et al. (176) separated the chromium from the GTF fraction and performed a yeast fermentation assay, which monitors CO_2 production manometrically to determine if chromium is important for biological activity. It is known that GTF lowers blood glucose and causes CO_2 to be produced. More studies showed that the biological activity does not contain chromium (171,177,178-182).

O' Donoghue et al. (171) further purified the chromium fraction using previous methods (176) and found that GTF contains basic peptides and free amino acids. Further purification separated the chromium and the biological activity of GTF, indicating that chromium is not involved in the biological activity (171). Amino acid analysis of GTF showed that 2% of free arginine and lysine are present, but no other significant number of other amino acids. The amino acid content accounted for 81% of the total weight of the sample mass (171). Amino acid stoichiometry ratios showed that there may be di-and tri-peptides present in the compound (171). Using a yeast bioassay system to determine

which peptide fraction contains activity. It was shown that fractions containing only arginine peptides (di-and tri-peptides) exhibited highest activity (171). There was no relationship between peptide and biological activity. To understand if the peptides and free amino acids work synergistically to give GTF its biological activity, hydrolysis of the peptide chains into constituent amino acids showed that GTF still had biological activity (171). Elucidation of the actual biological activity of GTF has still to be determined. Since 20% of the total weight of the sample mass has not been elucidated, it is postulated that this 20% of GTF contains the biological activity (171). Elucidation of the actual biological activity of GTF has still to be determined.

4.2 GTF's Function in Vivo and in Vitro

What is known is that GTF does lower blood glucose levels, and free fatty acids (ffa) in diabetic rats (183) and increase glucose uptake in yeast and animal cells (170). Experiments done by Mirsky on diabetic rats show that diabetic rats injected with GTF with no addition of exogenous insulin cause a significant decrease in blood glucose and free fatty acids (183). The effect of GTF on rats with lower blood glucose levels (250 mg/dl) was instant and lasted up to 24 hours (183). Rats with higher blood glucose levels (>350 mg/dl) showed a decrease with a single dose of GTF, but the decrease was gradual and lasted up to 24 hours. GTF decreased free fatty acids of diabetic rats within 30 minutes and the effects lasted up to 24 hours (183). GTF effects are well known, but its mode(s) of action are still unclear. Davis et al. (160) proposed that a natural occurring

biologically active chromium containing oligopeptide found mainly in mammalian liver

known as low molecular weight chromium (LMWCr) activates membrane

phosphotyrosine phosphatase (160). This was shown by kinetic studies (160). LMWCr

activation was due to insulin binding to its receptor, but LMWCr does not interfere with

insulin binding to its receptor (184). Insulin stimulation of LMWCr allows mobilization

of Cr, which in turn it can potentate, insulin's effects (173). The objective of the work

described herein was to define sugar transport and intracellular signaling in fat cells in

vitro. It was shown that chromium oligopeptides can activate insulin receptor tyrosine

kinase activity (172). This can occur only in the presence of insulin, as kinetic studies

showed no activation of tyrosine kinase activity without insulin (172). By use of

polyclonal antibodies, it was shown that LMWCr significantly potentiated a membrane

kinase activity only in the presence of insulin, since any disruption of insulin binding to

its receptor eliminates LMWCr to potentate kinase activity (172). Experiments done by

Davis and Vincent showed that LMWCr functions at the insulin receptor (172).

4.3 Characterization of GTF and its Mode of Action

In the present study we determinined GTF's characteristics and mode of action.

We observed that GTF increased glucose transport in 3T3-L1 adipocytes and L6

myotubes and inhibited isoproterenol induced lipolysis in fat cells. An additive effect

was observed for the combination of insulin and GTF for both glucose transport and

inhibition of lipolysis. No difference in specific [125]I -insulin binding was seen after 1 hour

exposure to GTF, nor displacement of [125]I -insulin from its receptor. Also, GTF did not

stimulate phosphorylation of the insulin receptor β chain. GTF stimulated

phosphorylation of the insulin receptor substrate-1 (IRS-1) and in combination with

insulin. We observed that GTF stimulated translocation of both GLUT 1 and GLUT 4

transporters from the intracellular membrane to the plasma membrane.

Materials and Methods

1. Preparation of GTF

Semi-purified GTF was kindly obtained from Dr. N. Mirsky's laboratory in Haifa Tivon, Israel and was mainly prepared as previously described (163). The semi-purified GTF prep used in this thesis did not contain chromium. The experiments described in this thesis were performed with partially purified material that was kindly given to us by Dr. Mirsky at the University of Haifa, Israel.

2. Cell Culture

Cell cultures of 3T3-L1 pre-adipocytes were maintained in 10% DMEM (v/v) fetal calf serum and 100U/ml penicillin and streptomycin. The cells were kept at 37°C in a 5% CO_2 humidified incubator. Once cells reached 80% confluence, they were plated in 35 mm diameter plastic petri plates (Falcon) at 1×10^5 cells/plate. When the cells reached confluence (~ 4 days), they were treated with induction medium containing 167nM insulin, 0.5nM isobutylmethyl xanthine (IBMX) and 0.25μM dexamethasone in 10% DMEM for 4 days and then in medium containing 10% DMEM with 167nM insulin for another 3 days (187). The medium was then changed to 10% DMEM for 3 additional days when the cells differentiate to fat cells (>90% adipocytes).

3. 2-Deoxyglucose Uptake

Differentiated 3T3-L1 adipocytes were serum starved for two hours before exposed to various concentrations of insulin, GTF, and a combination of insulin plus GTF for 1 hour. Then zero time absorption controls were washed twice with 4°C PBS (pH 7.4) and the 5 minute absorption times were washed twice with 37°C PBS (pH 7.4). To each plate 0.8mls of 0.05 mM [^{3}H]-2-DG (specific activity 50 μCi/μmole) was added and removed immediately for the zero time absorption and kept for 5 minutes at 37°C for the 5 minute absorption times. Cells were then washed 4 times with 4°C PBS (pH 7.4). Zero time absorption controls were subtracted. The monolayers were dissolved in 1N NaOH, and aliquots were taken for liquid scintillation counting and protein determination (188).

4. Insulin Binding

After differentiation of 3T3-L1 pre-adipocytes to adipocytes, the cells were washed twice with 25°C Hank's HEPES balanced salt solution (HHBSS) containing 0.1% BSA, pH 7.4. Then, ^{125}I-insulin (1ng/ml) alone (specific binding), or in combination with a large excess of unlabeled insulin (non-specific binding), was added to cultured adipocytes for 2 hours at 25°C on a rotary shaker and binding as described elsewhere (189). Protein was determined using the Lowry protein assay (188).

5. Insulin Displacement

The procedure was described above except the adipocytes were incubated with increasing concentrations of unlabeled insulin (10ng-200ng) and GTF (0.5mg-10mg) which represent concentrations used in other experiments that were physiologically significant to show any displacement of the ^{125}I- insulin bound. Protein was determined using the Lowry protein assay (188).

6. Preparation of Whole Cell Extracts

3T3-L1 adipocytes were washed twice with 4% PBS and solubilized with RIPA buffer (1xPBS 1% nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 1mM AEBSF, 1µg/ml aprotinin, 1µg/ml leupeptin, 1µg/ml pepstatin, 1mM vanadate and 1mM sodium fluoride); the cells were rocked in the cold for 15 minutes for further solubilization. The lysates were centrifuged at 11,000 g for 15 minutes at 4°C to clarify the supernatant. A modified Lowry assay (188) was used to determine the amount of protein the supernatant, which contains the whole cell lysate.

7. Ultra Centrifugation

After differentiation of 3T3-L1 pre-adipocytes to adipocytes, the cells were incubated with insulin, GTF, and a combination of the two for one hour. Then, cells were washed twice with PBS (4°C), and then cells were scraped with buffer I (Sucrose, Sodium Azide, EGTA, HEPES, 1mM AEBSF, 1 µl/ml leupeptin, and 1µg/ml pepstatin). The samples were pelleted by centrifugation at 700 g for 10 minutes at 4°C. The pellet was resuspended in Buffer I and then the cells were homoginized using a Dounce homogenizer. The samples were pelleted by centrifugation at 550 rpm for 5 minutes at 4°C to remove cell debris. The supernatants were placed in SW-41 Beckman tubes and pelleted by centrifugation at 14,800 rpm for 1 hour in the ultracentrifuge using a SW-41 swinging bucket rotor. The pellets were resuspended in Buffer I which contains the crude plasma membrane extracts. After this spin, the supernatants were placed in SW-41 tubes and pelleted by centrifugation at 34,860 rpms for 90 minutes. The pellets were collected with Buffer I which contains the High density microsomal extract.

8. Immunoprecipitation

Approximately 250 µg protein of whole cell lysate was used to form a complex with 2 µg of anti insulin β chain in (rabbit polyclonal) or 2 µg of Anti IRS-1 (rabbit polyclonal) in a microfuge tube. The complex was left to rock overnight at 4°C. The complex was captured by the protein agarose bead slurry and rocked for 2 hours and centrifuged at 1,500 g for 5 minutes. The bead slurry were pelleted by centrifugation and the supernatant was used to determine the β chain tyrosine phosphorylation using an anti phosphotyrosine antibody (4G10) and for IRS-1 phosphorylation using an anti-IRS-1 antibody to detect any phosphorylation of the insulin β chain receptor and/or IRS-1.

9. Western Analysis

40 µg of cell lysate proteins were mixed with 4x Laemmli buffer (8% SDS, 400nM DTT, 0.24M Tris (pH 6.8), 0.04% bromophenol blue, 40% glycerol, 4mM EDTA) in a 1:4 ratio. The proteins were separated by SDS-PAGE at 200v for 30 minutes on an 8% gel. Then the proteins were transferred at 100v for 1 hour onto a nitrocellulose membrane. The blots were washed with PBS-T (0.1% tween), probed with rabbit anti-mouse anti-phosphotyrosine, and/or anti-IRS-1 monoclonal antibodies in blocking buffer (with 3% BSA) and left rocking overnight at 4°C. Next, the membranes were washed again with PBS-T and incubated with horseradish peroxidase (HRP) secondary antibody. The membranes were incubated with ECL a chemiluminescence detection kit (Amersham) and exposed to Kodak x-Omat blue film. The intensities of the bands were quantified using UN-SCAN-IT computer software (silk scientific).

10. Lipolysis Assay

Epidymal fat pads were removed from Spraque Dawley male rats weighing 150-200 g. The tissue was placed in a plastic vial in Krebs bicarbonate buffer with 4% BSA and 2.5 mM glucose. Digestion was carried out in the same buffer with addition of 0.1% collagenase (Worthington) at 37°C water bath shaker at 40 rpm for 7 minutes. The digested tissue was passed through nylon gauze. The cells were washed three times in a fresh buffer and allowed to float. The washed adipocytes were diluted 1:8 in Krebs buffer and were used for lipolysis assays (190,191). All assays were made in triplicates. A suspension (0.25ml), containing between 20 and 50 mg adipocytes was incubated in plastic vials in the presence of isoproterenol (IP) (Sigma) and either insulin or GTF. The vials were incubated for an hour at 37 °C with continuous shaking of 40 rpm. At the end of the incubation period an aliquot of the supernatant was removed from each reaction mixture and measured for the free fatty acid (ffa) content as described (190,191). This was done by using a colorimetric enzymatic reaction using a stable copper reagent and a sensitive copper indicator (2-(2-thiazolylazo)-4-methoxyphenol). Photometric determination was used to measure the amount of free fatty acids produced.

11. Toxicity Assay

To determine the cell viability of 3T3-L1 in the presence of GTF a Cell Titer 96 Aqueous Non-Radioactive Cell Proliferation Assay (Promega) was used. 3T3-L1 adipocytes were cultures and differentiated in 96-well plates. After 2 days of incubation at 37°C in a 5% CO_2 humidified incubator, to each well 15 μl of phenazine methosulfate/ (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner-salt, (PMS/MST) was added. After every hour for 3 hours the media optical density was determined using an ELISA plate reader at 492 nm. This was done for 3 consecutive days. This assay was used as a colometric method to determine the number of viable cells in proliferation. PMS is used as a coupling reagent, where MTS is bioreduced by the cells to produce formazan which is soluble in tissue culture medium. This reaction is accomplished by the dehydrogenase enzymes that convert MTS into soluble formazan that can be measured at 490 nm, which is directly proportional to the number of living cells (192).

12. Materials

Dulbecco's Modified Eagles Medium obtained from Gibco (Grand Island Biologicals). Isoproterenol, Insulin from Bovine Pancreas and Anti-receptor Beta chain from Sigma. Anti-IRS-1 obtained from Interstate Biosignals. Anti-phosphotyrosine antibody (4G10) was kindly obtained from Dr. S. Richard's laboratory at the Lady Davis Institute and ^{3}H-2 Deoxyglucose obtained from ICN/Amersham. Also, ^{125}I-NaI was obtained from ICN/Amersham. Monocomponent insulin was a gift from Lilly Labs (Indianapolis). Semi-purified GTF was kindly obtained from Dr. N. Mirsky's laboratory in Haifa Tivon Israel.

13. Statistics

All experiments were done in triplicate and the statistical analysis used a one way ANOVA where the levels of significance was set at $p < 0.05$. All the data +/- SEM.

Results

1. The Effect of Insulin on 2-DG Transport in 3T3-L1 Adipocytes in Vitro

In order to determine if GTF had an effect on 2-deoxyglucose transport in 3T3-L1 adipocytes, we first showed 2-deoxyglucose transport by insulin on 3T3-L1 adipocytes. 3T3-L1 pre-adipocytes were differentiated into adipocytes, under proper culturing conditions. Once the cells were differentiated into adipocytes (~90% induced), the cells were exposed to various concentrations of insulin ranging from 0 nM to 133 nM for one hour.

Figure 8 shows the results of insulin's effect on 2-deoxyglucose transport in 3T3-L1 adipocytes. The data showed that increasing concentrations of insulin (0-133 nM) caused an increase in 2-deoxyglucose transport (nmoles/mg p/5 minutes) in the 3T3-L1 adipocytes in a dose dependent manner. The highest stimulated 2-deoxyglucose transport occurred at the highest concentration used in these experiments which was 133 nM compared to the control (0 nM), but 2-deoxyglucose transport still increased at higher concentrations. The asterix indicates that these are statistically significant for a One-Way ANOVA ($p < 0.05$) where n=3 with triplicate plates in all experiments.
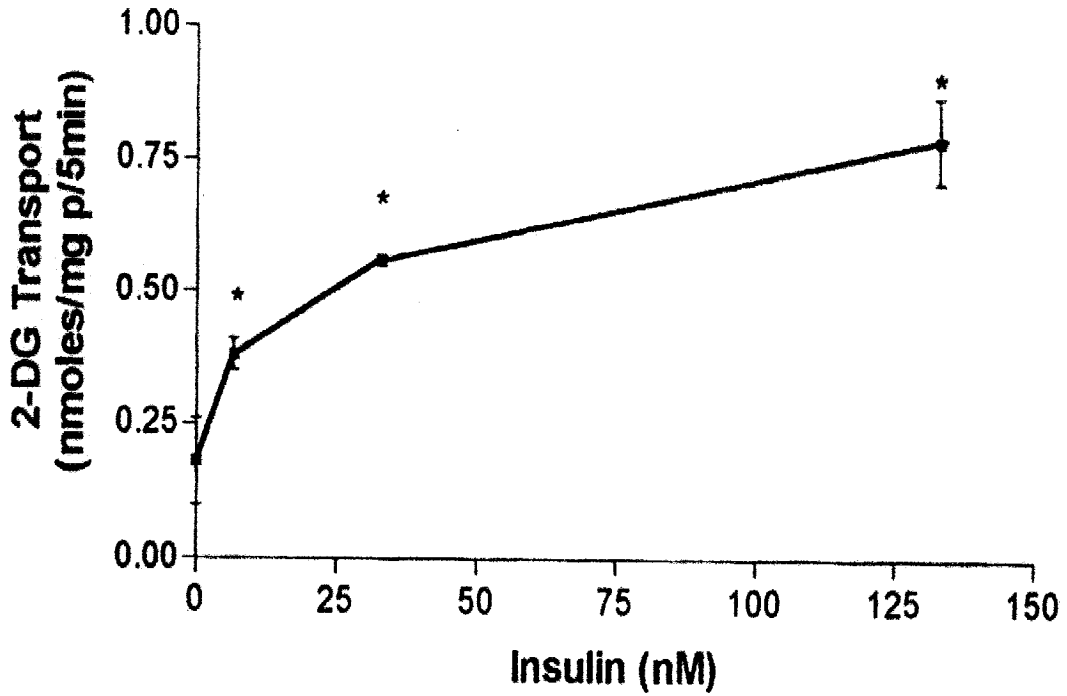
FIGURE 8. The effect of insulin on 2-DG transport in 3T3-L1 adipocytes.

3T3-L1 adipocytes were treated for one hour with different concentrations of insulin ranging from 0 nM to 133 nM and sugar uptake was measured. The data showed 2-DG transport (nmoles/mg P/5min) increased with increasing concentrations of insulin in a dose dependent manner. The highest 2-DG transport (nmoles/mg P/5min) in the experiment was observed at 133 nM, but is still increasing. The data represented the mean +/- SEM*. The application of the data to One Way ANOVA indicated a significant elevation for all levels of insulin employed (P<0.05)* where n=3 with triplicate plates in all experiments.

2. The Effect of GTF on 2-DG Transport in 3T3-L1 Adipocytes in Vitro

The next step was to observe if GTF has an effect on stimulating 2-DG transport in 3T3-L1 adipocytes as insulin did. The 3T3-L1 pre-adipocytes were induced and differentiated using various hormones into adipocytes. After differentiation of 3T3-L1 adipocytes, the cells were incubated with different concentrations of GTF ranging from 0 mg/ml to 20 mg/ml for one hour.

Figure 9 shows the effect of GTF on stimulating 2-DG transport. Like insulin, with increasing concentrations of GTF (0 mg/ml-20 mg/ml) there was an increase in 2-DG transport (nmoles/ mg P/5minutes) in 3T3-L1 adipocytes. GTF increased 2-DG transport in a dose dependent manner. Other evidence to support our results was shown by Mirsky and Berdicevsky (194). They showed that GTF increased 2-DG uptake in yeast (194). Maximal stimulation of 2-DG by GTF compared to the control occurred 10- between 10-20 mg/ml. The asterix indicates that these results are statistically significant for a One-Way ANOVA ($p < 0.05$) where n=3 with triplicate plates in all experiments.
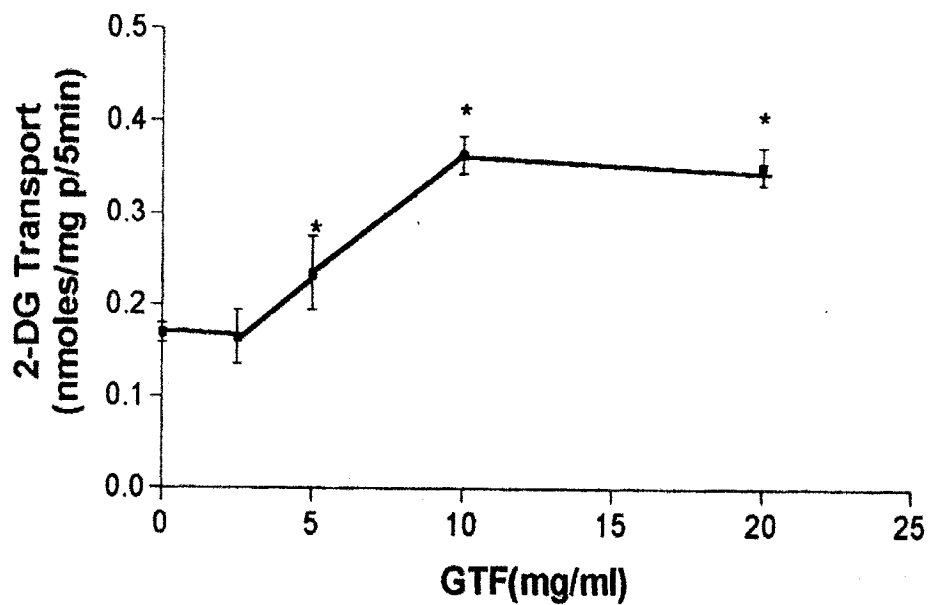
FIGURE 9. The effect of GTF on 2-DG transport in 3T3-L1 adipocytes.

The 3T3-L1 adipocytes were treated with varying concentrations of GTF from 0 mg/ml to 20 mg/ml for one hour. Then sugar transport (nmoles/mg p/5min) was determined. The data showed increasing concentrations of GTF, there was an increase in sugar transport. This occurred in a dose dependent manner, where 2-DG transport (nmole/mg P/5min) was maximal between 10- 20 mg/ml. The data shows that sugar transport starts to level off at 10 mg/ml GTF. For the GTF data One Way ANOVA indicated that significance was observed at 5-20 mg/ml (P<0.05)* where n=3-4 with triplicate plates in all experiments.

3. The Effect of Insulin +/- GTF on 2-DG Transport in 3T3-L1 Adipocytes in Vitro

Both insulin and GTF alone stimulated 2-DG transport in 3T3-L1 adipocytes in a dose dependent manner. The following experiments were done to determine if a combination of insulin and GTF had greater effect(s) on 2-DG transport in 3T3-L1 adipocytes. The 3T3-L1 pre-adipocytes were exposed to media containing drugs and hormones and subjected to adipocyte differentiation in 10% DMEM. The differentiated 3T3-L1 adipocytes were incubated for one hour with no drugs, 6.7 nM insulin, 2.5 mg/ml GTF, and a combination of insulin and GTF.

Figure 10 shows that treatment number one is the control, treatment number 2 is insulin alone (6.7 nM), treatment number 3 is GTF alone (2.5 mg/ml), and treatment number 4 is a combination of treatment number two and three (6.7 nM+2.5 mg/ml). The data shows that compared to the control, treatment two (6.7 nM insulin) increased 2-DG transport (nmoles/mg p/5minutes) by two-fold. Exposure to treatment three (2.5mg/ml GTF) increased 2-DG transport (nmoles/mg p/5minutes) about 1.6 fold. The combination of treatment number two and three had a greater increase of 2-DG transport that either treatment two alone or treatment three alone. The data indicated that insulin and GTF work in an additive manner. The asterix indicate that the results are statistically significant for a One-Way ANOVA ($p < 0.05$) where n=3 with triplicate plates in all experiments.
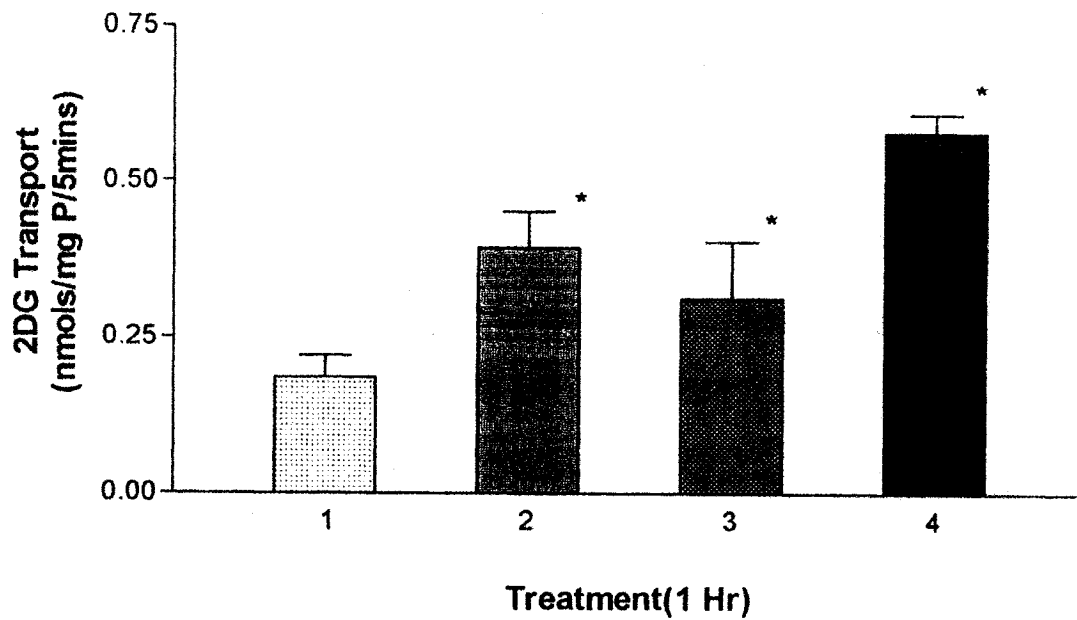
Treatment
1= Control
2= Insulin 6.7 nM
3= GTF 2.5 mg/ml
4= Insulin 6.7 nM+GTF 2.5 mg/ml

FIGURE 10. The effect of insulin +/- GTF on 2-DG transport in 3T3-L1 adipocytes.

The cells were treated for one hour with the various conditions and sugar transport (nmole/mgp/5min) was determined. The data represent three experiments with triplicate plates in each experiment. One Way ANOVA indicated that all treatment groups were significantly elevated ($P<0.05$)*where n=3 with triplicate plates in all experiments. Treatment (1) was the control, no insulin or GTF, treatment (2) represented + insulin (6.7 nM), treatment (3) was GTF (2.5 mg/ml) alone, and treatment (4) was a mixture of both insulin and GTF at the concentrations used in treatments (2) and (3).The data shows that 2-DG transport is increased with treatment (2), treatment (3), and treatment (4), compared to the control (1). Treatment (4) gave the highest sugar transport (nmole/mgp/5min) than any other treatment (1-3).

49

4. The Effect of Insulin and GTF on 2-DG Transport in 3T3-L1 Adipocytes in Vitro

Table 2 summarizes the 2-DG experiments done on 3T3-L1 adipocytes using various concentrations of insulin alone, GTF alone, and a combination of insulin and GTF. The results show that compared to the control, increasing concentrations of insulin alone (6.7 nM-133 nM) stimulated 2-DG transport (nmoles/mg p/ 5 minutes). Maximal insulin enhancement (6.63 times higher than uptake by untreated cells) was observed at the concentration of 133 nM. Similar results show that compared to the untreated cells (control), increasing GTF concentrations (0-50 mg/ml) increased 2-DG transport (nmoles/mg p/5 minutes). Maximal GTF enhancement (3.5 times higher than untreated cells) was detected at 50 mg/ml. When examining the 2-DG transport enhancement of the combination of insulin and GTF, the results in table 2 show that the combination exhibited a higher rate of 2-DG transport than either treatment of insulin or GTF alone. The data shows that the lowest concentrations of both insulin (6.7 nM) and GTF (2.5 mg/ml) in combination had higher 2-DG transport than either higher concentrations of insulin alone at 35.5 nM and GTF alone at 50 mg/ml. Maximal enhancement of the combination of insulin and GTF (5.84 times higher than untreated cells) was detected at 6.7 nM insulin plus 5 mg/ml GTF. The data indicates that insulin in combination with GTF (6.7 nM insulin + 2.5 mg/ml GTF) works in an additive manner.

Treatment	Concentration	2-DG Transport [nmoles/mg p/5 minutes]	Ratio: Treated/Control
Control		0.067 +/- 0.001	1
Insulin	6.7 nM	0.225 +/- 0.015	3.37 +/- 0.22
	35.5 nM	0.37 +/- 0.022	5.55 +/- 0.32
	133 nM	0.442 +/- 0.017	6.63 +/- 0.25
GTF	2.5 mg/ml	0.119 +/- 0.006	1.78 +/- 0.1
	5 mg/ml	0.165 +/- 0.006	2.47 +/- 0.09
	10 mg/ml	0.091 +/- 0.003	1.36 +/- 0.05
	20 mg/ml	0.151 +/- 0.008	2.26 +/- 0.09
	50 mg/ml	0.235 +/- 0.004	3.53 +/- 0.06
Insulin nM + GTF mg/ml	6.7 nM + 2.5 mg/ml	0.471 +/- 0.03	7.06 +/- 0.45
	6.7 nM + 5 mg/ml	0.597 +/- 0.047	8.96 +/- 0.71
	6.7 nM + 10 mg/ml	0.667 +/- 0.011	10.01 +/- 0.12

TABLE 2. The effect of insulin and GTF on 2-DG transport in 3T3-L1 adipocytes.

The data shows the effect of different concentrations of insulin, GTF, and a combination of insulin and GTF on 2-DG transport compared to the control. The adipocytes were exposed to either insulin alone, GTF alone, or in combination of insulin and GTF for one hour. Insulin had a maximal transport ratio of 6.63+/-0.25 at concentrations employed in this thesis at 133 nM, but still increases beyond 133nM. GTF has a maximal transport ratio of 3.53+/-0.06 at concentrations employed in this thesis is at 50 mg/ml. In combination, insulin and GTF gave a maximum transport ratio of 10.01+/-0.12 with 6.7 nM insulin and 10 mg/ml GTF. This indicates that in combination insulin and GTF work in an additive manner giving a higher transport ratio than either insulin alone, or GTF alone. The data represents three experiments (triplicate plates in each experiment) +/- SEM.

5. The Effect of Insulin and GTF on 2-DG Transport in L6-Myotubes in Vitro

From the previous experiments it was shown that GTF stimulated 2-DG transport in a dose dependent manner in 3T3-L1 adipocytes. The next sets of experiments were done to observe the effects of insulin, GTF, and a combination of insulin and GTF on 2-DG transport in L6 myotubes. L6-myoblasts were plated in 10% DMEM and induced using 2% α-MEM then serum starved with 0% α-MEM (187). The L6-myotubes were incubated with various concentrations of insulin, GTF, or no drugs present (control).

Skeletal muscle is another source for glucose storage and utilization and there for the following experiments were done to observe if GTF, and when in combination has an effect on stimulating 2-DG transport in L6 myotubes. Table 3 shows that after L6 myotubes were incubated with various concentrations of insulin (6.7-133 nM), there was an increase on 2-DG transport (nmoles/mg p/5 minutes) compared to untreated cells (control). The maximal stimulation of 2-DG transport by insulin (ratio 0.565+/- 0.13) occurred at 133 nM (the highest concentrations used in the experiments). The data in table 3 shows L6 incubated with various concentrations of GTF (0-50 mg/ml), had a slight increase on 2-DG transport (nmoles/mg p/ 5 minutes) compared to untreated cells (control). Maximal effect of GTF on 2-DG transport occurred at 50 mg/ml. The combination of insulin and GTF, exhibited a higher 2-DG transport than either insulin or GTF alone. Further experiments were not done using L6 myotubes because the results showed that only at 50 mg/ml of GTF had any significant effect on stimulating 2-DG transport in L6 myotubes, while lower concentrations of GTF had significant effects in

stimulating 2-DG transport in 3T3-L1 adipocytes. This may indicate that L6 myotubes

are less sensitive to GTF in stimulating 2-DG transport compared to 3T3-L1 adipocytes.

The asterix indicate they are statistically significant for a One-Way ANOVA where

$p<0.05$ and the data represents three experiments (triplicate plates in each experiment).

TREATMENT	2-DG TRANSPORT [nmoles/mg p/5 minutes]
Control	0.23 +/- 0.01
Insulin 6.7 nM	0.453 +/- 0.05*
Insulin 67 nM	0.497 +/- 0.02*
Insulin 133 nM	0.565 +/- 0.13*
GTF 10 mg/ml	0.227 +/- 0.01
GTF 20 mg/ml	0.259 +/- 0.007
GTF 50 mg/ml	0.29 +/- 0.012*

TABLE 3. The effect of insulin and GTF on 2-DG transport in L6-myotubes.

Insulin and GTF were employed as sugar transport stimulators in fully induced L6 myotubes. The data showed a dose dependent increase of sugar transport with increasing concentrations of insulin, and GTF compared to the control. The maximum sugar transport occurred at 133 nM for insulin, and 50 mg/ml GTF. The stimulation of GTF on sugar transport is lower in myotubes than in adipocytes. The data represent three experiments (triplicate plates in each experiment) +/- SEM *($P<0.05$) for a One Way ANOVA where n=3 with triplicate plates in all experiments.

6. The Effect of Insulin and GTF on ^{125}I-Insulin Binding to its Receptor in 3T3-L1 Adipocytes

Insulin's mode of action is through insulin binding to its receptor on most cell surfaces (3). Since GTF is able to stimulate 2-DG transport, the next sets of experiments were done to observe if GTF mode of action is through the insulin receptor. In order to perform the experiment, the 3T3-L1 adipocytes were treated with insulin, GTF, and a combination of insulin and GTF for one hour. Then the 3T3-L1 adipocytes were washed with Hanks Hepes Balanced Salt Solution (HHBSS)+ 0.1%BSA and incubated at room temperature for two hours on a rotary shaker with ^{125}I-insulin alone (total binding) or with a large excess of cold insulin (non-specific binding)

Figure 11 shows that compared to the control (treatment 1), there were no change in ^{125}I-insulin binding (fmoles/mg p) to its receptor on 3T3-L1 adipocytes in the presence of insulin (treatment 2), GTF (treatment 3) or in combination (treatment 4). All 4 treatments had similar specific ^{125}I-insulin bound (fmoles/mg p) to its receptor. These results indicate that GTF does not bind to the insulin receptor and suggests its mode of action is not through the insulin receptor.
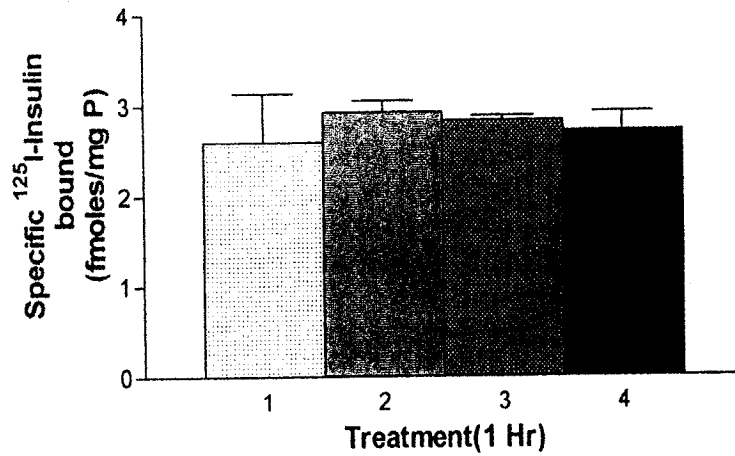
Treatment
1= control
2= insulin
3= GTF
4= Insulin + GTF

FIGURE 11. The effect of insulin +/-GTF on ^{125}I-insulin binding in 3T3-L1 adipocytes.

The 3T3-L1 adipocytes were treated with either, no insulin or GTF (treatment 1), 6.7 nM insulin alone (treatment 2), 2.5 mg/ml GTF alone (treatment 3), and a mixture of insulin (2) and GTF (3) for 1 hour. Then the cells were washed with HEPES+0.1%BSA and exposed to ^{125}I-insulin for two hours. The data represent three experiments, triplicate plates in all experiments +/- SEM. The data shows that there are no significant differences observed in ^{125}I-insulin binding (fmoles/mg P) among any of the treatments (1-4). One Way ANOVA indicated that no differences were observed between any of the treatments (P<0.05) where n=3 with triplicate plates in all experiments.

7. The Effect of Insulin and GTF on β Chain Phosphorylation in 3T3-L1 Adipocytes

Once insulin binds to the insulin receptor, autophosphorylation of the β chain of the receptor occurs. Insulin receptor β chain phosphorylation is an important early signal in the insulin cascade (186). Since GTF does not bind to the insulin receptor, does it stimulate phosphorylation of the insulin receptor β chain without binding to the insulin receptor? To answer this question, we examine the effects of insulin, GTF, and a combination of insulin and GTF on insulin receptor β chain phosphorylation in 3T3-L1 adipocytes. Differentiated adipocytes were incubated with insulin, GTF, in combination, or separately for 1 hour. The cells were extracted as described in the materials and methods. We examined the effects of GTF on tyrosine phosphorylation of the insulin receptor β chain. Immunoprecipitation of the insulin receptor β chain post treatment with insulin or a combination of both GTF and insulin was examined.

The Western blot data in figure 12 represents an immunoprecipitated insulin receptor β chain probed with anti-phosphotyrosine anti-serum (4G10). Figure 12 shows that compared to untreated cells (band in lane 1), insulin stimulated phosphorylation of the insulin receptor β chain (band in lane 2). GTF (band in lane 3) did not stimulate phosphorylation of the insulin receptor β chain compared to insulin (band in lane 2) or in combination with insulin (band in lane 4). The band in lane 4 shows that the combination of insulin and GTF stimulated phosphorylation of the insulin receptor β chain compared to untreated cells (band in lane 1) and GTF (band in lane 3), but lower than insulin alone

57

(band in lane 2). The reason for the apparent lowering of insulin receptor β chain phosphorylation by GTF in the presence of insulin is presently unknown. A reason may be due to the use of lower concentrations of insulin (1 mU/ml) and GTF (5 mg/ml) in combination, than in each treatment of insulin lone (20 mU/ml) and GTF alone (10 mg/ml). Future studies will be done to determine if GTF in combination with insulin lowers insulin receptor β chain phosphorylation, or is due to the lowering of concentrations of both insulin and GTF. The dark bands at the bottom of the western blot may be caused by un-precipitated insulin receptor β chain.

Figure 13 shows the densiometric analysis of the band intensities of the insulin receptor β chain western blot using the UN-SCAN-IT computer software. The results showed that insulin at 20 mU/ml (bar number 2) caused an increase in the phosphorylation insulin receptor β chain phosphorylation in comparison to the control (bar number 1), 10 mg/ml GTF (bar number 3), and in combination with insulin (1 mU/ml) and (5 mg/ml) GTF (bar number 4). GTF alone (10 mg/ml) caused very little increase in phosphorylation of the insulin receptor β chain phosphorylation. GTF in combination with insulin caused a decrease in the phosphorylation of the insulin receptor beta chain protein. The amount of phosphorylation is still higher than GTF alone, but much less than insulin alone. This may indicate that GTF has an inhibitory effect on the insulin receptor beta chain.
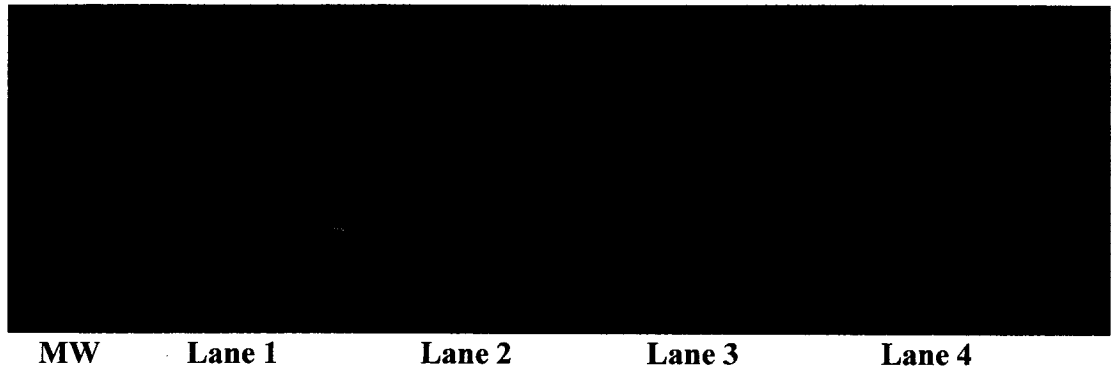
| MW | Lane 1 | Lane 2 | Lane 3 | Lane 4 |

Lane 1= Control
Lane 2= Insulin (20 mU/ml)
Lane 3= GTF (10 mg/ml)
Lane 4= Insulin (1 mU/ml) + GTF (5 mg/ml)

FIGURE 12. The effect of GTF on tyrosine phosphorylation of the insulin receptor β chain.

Figure 12 is a picture of a Western blot showing the immunoprecipitated insulin receptor β chain probed with an anti-phosphotyrosine (4G10) probe. The top band of the Western blot represents the phosphorylated insulin receptor β chain with a molecular weight of 95 Kd. Lane 1 is the control, lane 2 is plus insulin (20 mU/ml), lane 3 is plus GTF (10 mg/ml), and lane 4 is plus insulin (1 mU/ml) and GTF (5 mg/ml). The data is from three experiments +/- SEM. The exposure time was one hour for all the treatment groups on 3T3-L1 adipocytes. The pattern was consistent for each of the three experiments. The dark bands (that are not 95 Kd) at the bottom of the western blot represent proteins that are tyrosine phosphorylated that are not the insulin receptor beta chain.

The Effect of Insulin, GTF, and a Combination of Insulin and GTF on the Total Amount of Insulin
Receptor Beta Chain Protein
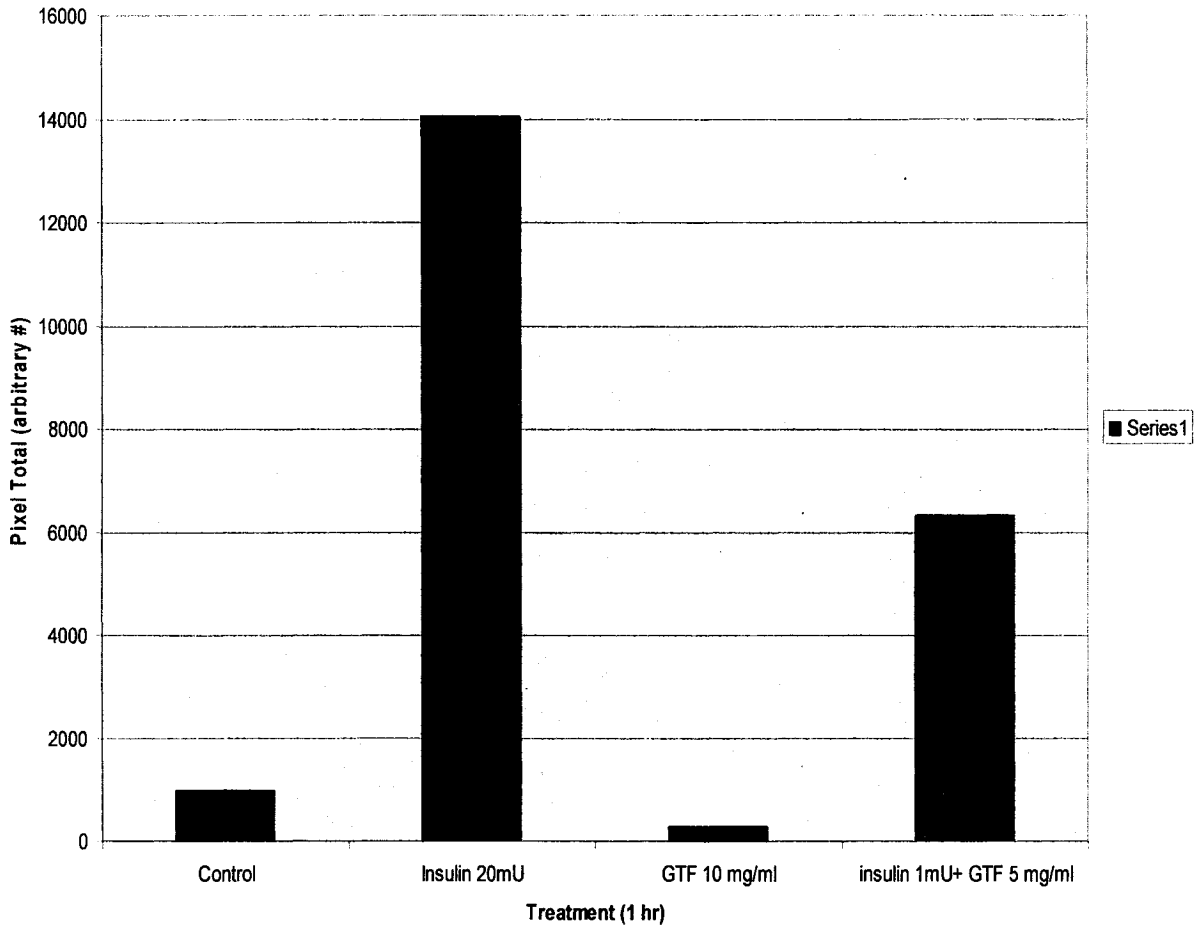
Figure 13. The effect of insulin, GTF, and a combination of insulin and GTF on total insulin receptor beta chain phosphorylation in 3T3-L1 adipocytes

The following is a chart indicating the differences in the amount of insulin receptor beta chain phosphorylation using anti-phosphotyrosine antibody (4G10) in the presence of insulin, GTF and a combination of insulin and GTF in 3T3-L1 adipocytes. The cells were exposed to each treatment for one hour. The samples are as follows; control (lane 1), insulin (20 mU/ml) (Lane 2), GTF (10 mg/ml) (lane 3), and insulin (1 mU/ml) + GTF (5 mg/ml) (lane 4). Using the UN-SCAN-IT computer program, this, analyzes the intensities of the bands present on the blot. The intensities of each band are expressed as intensities in pixel total (arbitrary numbers).

60

8. The Effect of GTF on Isoproterenol Induced Lipolysis

Insulin has other physiological roles other than stimulating glucose transport. Insulin is also able to inhibit lipolysis (183).We also wanted to observe if GTF has any similar role as insulin on inhibiting lipolysis. GTF was assayed for its ability to inhibit lipolysis. Adipocytes were incubated with isoproterenol (IP) (which induces free fatty acid release) in concentrations of $1 \times 10^{-7}M$ to $4 \times 10^{-5}M$ with or without the addition of GTF (5 mg/ml).

The data in Figure 14 shows that increasing concentrations of isoproterenol (IP) $(1 \times 10^{-7}-4 \times 10^{-5})$ on untreated cells (control) caused an increase in free fatty acids (μM). IP induced a dose dependent increase in free fatty acid production. This is represented in by the top line in figure 14. The addition of GTF (5 mg/ml) decreased the amount of free fatty acids produced in the presence of IP by 50%. This is represented by the lower line of the figure 14. The data in figure 14 shows that GTF like insulin has an inhibitory effect on lipolysis.
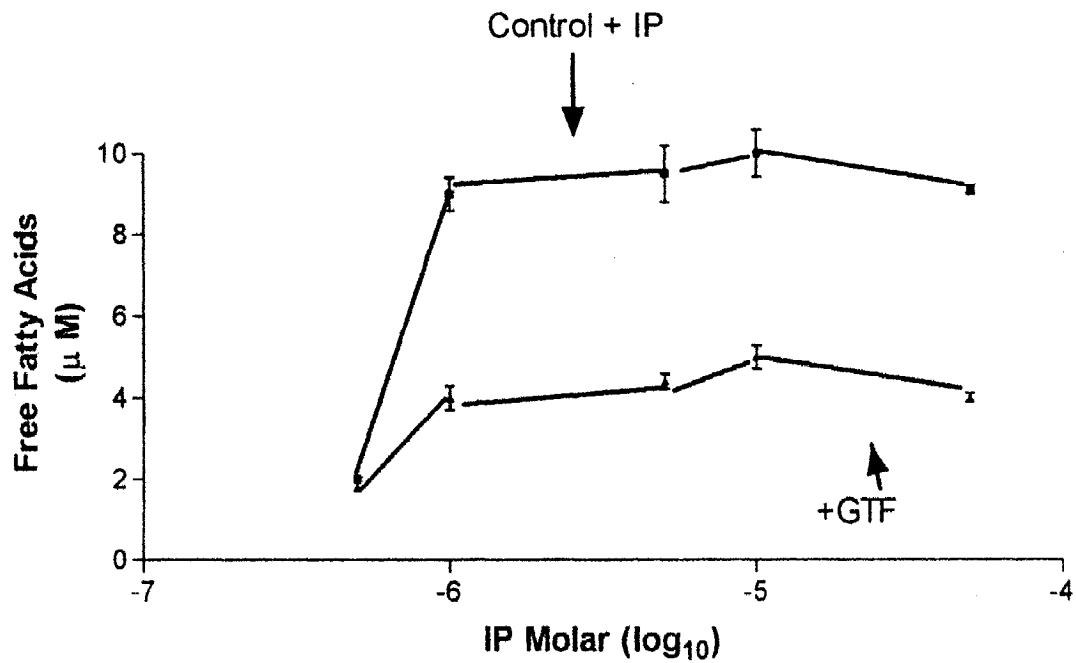
FIGURE 14. The effect of GTF on Isoproterenol (IP) induced lipolysis in 3T3-L1 adipocytes

3T3-L1 adipocytes were incubated for one hour with increasing doses of IP (from 5×10^{-7}M to 5×10^{-5} M) with or without GTF (5 mg/ml). Free fatty acids were determined as described under materials and methods. The data shows that addition of GTF (5 mg/ml) inhibits lipolysis in the presence of IP. The lower line represents the addition of GTF. The top line indicates higher levels of free fatty acids produced with control and IP, but upon addition of GTF (5 mg/ml) there is a decrease in levels of free fatty acids produced (lower line).

9. The Effect of GTF, Insulin or the combination of Insulin and GTF on lipolysis induced by isoproterenol (IP)

The effects of GTF, insulin, and the combination of insulin and GTF on IP induced lipolysis in rat adipocytes were also examined. Adipocytes were incubated with 5×10^{-6}M IP alone and with the addition of 0.004 or 0.04 nM insulin, 0.2, 1 or 5 mg/ml GTF in combination.

Figure 15 showed insulin concentrations of 0.004 or 0.4 nM, inhibited lipolysis in adipocytes by 16.8% and 45.4% respectively. GTF concentration of 0.2 mg/ml had no noticeable effects on lipolysis. A GTF concentration of 1 mg/ml alone inhibited ffa release by 28.4%. In combination 0.2 mg/ml GTF with 0.004 or 0.04 nM insulin potentiated the effect of inhibition of lipolysis by 34.8% and 66.3% respectively. A combination of 1 mg/ml GTF with 0.004 or 0.04 nM insulin inhibited lipolysis by 45.5% and 66.6% respectively. The results indicate, like the 2-DG transport ratios, GTF possesses similar properties as insulin in the inhibition of lipolysis. Higher concentrations of GTF have higher percentage of lipolysis inhibition. Low concentrations of GTF when combined with either low or high concentrations of insulin produce a greater rate inhibition of lipolysis than either GTF or insulin alone. The percentage of inhibition of lipolysis is even greater when a higher concentration of GTF is used (1 mg/ml) with low or high concentrations of insulin (0.004 or 0.04 nM).
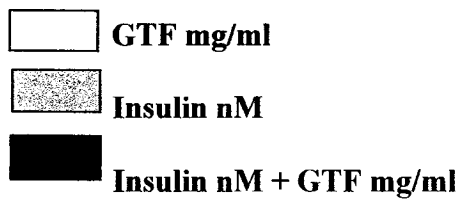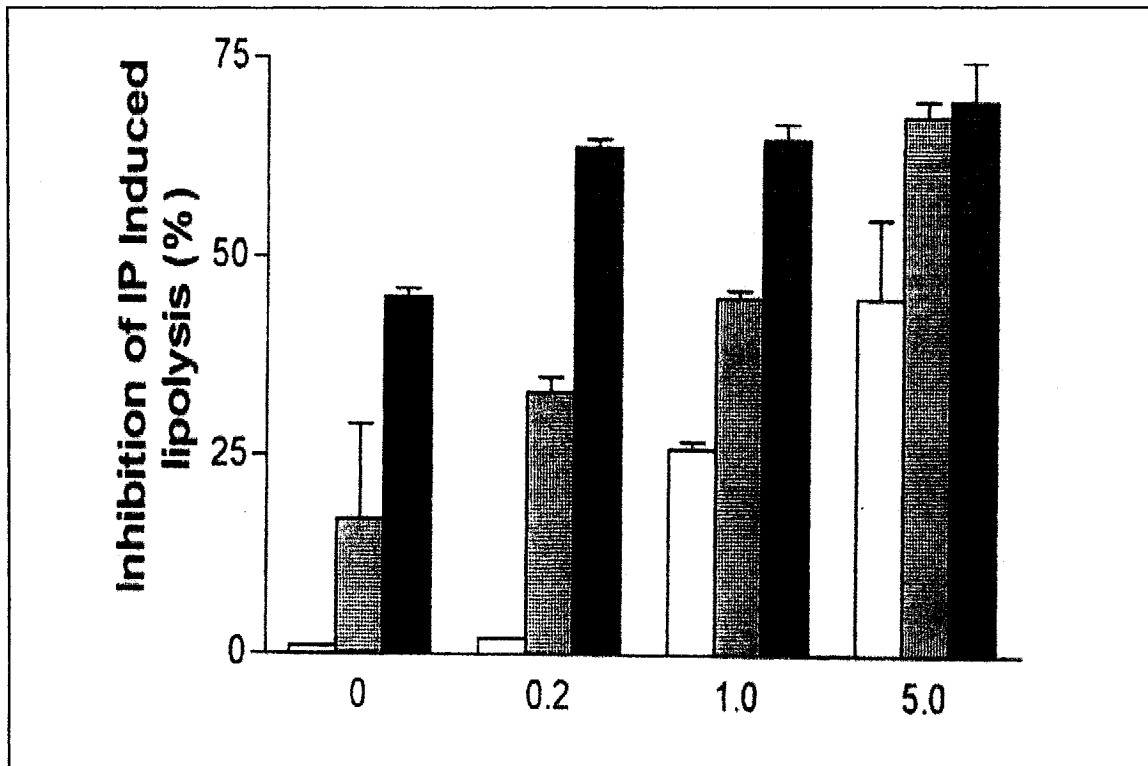
FIGURE 15. The effect of GTF, insulin, or the combination of insulin and GTF on lipolysis induced by isoproterenol (IP) in 3T3-L1 adipocytes.

3T3-L1 adipocytes were incubated for one hour in the presence of 5×10^{-6}M IP, with GTF (0, 0.2, 1, and 5 mg/ml), the white bar is GTF alone (no insulin), the gray dotted bar is plus 0.004 nM insulin, and the solid black bar is plus 0.04nM insulin. Free fatty acids were determined as described in the materials and methods. The data shows increased concentrations of GTF alone (white bars) increased the inhibition of IP induced lipolysis. Also, increased concentrations of GTF with increased concentrations of insulin in combination, gave a greater amount of inhibition of IP induced lipolysis (grey dotted bars and black bars). The highest inhibition on IP induced lipolysis was with the addition of GTF plus 0.04 nM insulin (black bars).

10. The Effect of GTF on Toxicity of 3T3-L1 Adipocytes

The next sets of experiments were done to determine if GTF has any effect on cell viability. If GTF is to be considered as an insulin substitute, it is very important to ascertain if GTF is toxic to the cell and reduces cell proliferation. In these experiments 3T3-L1 adipocytes were used as cell models. Cells were plated in 96 well plates at 10 000 cells/well in phenol red free DMEM (PRF-DMEM). Various concentrations of GTF (ranging from 0-5 mg/ml) were added to appropriate wells. After two days 15µL of PMS/MTS were added to each well. The addition of PMS/MTS causes an enzymatic reaction where it produces formazan which is soluble in the media. The optical density of formazan can be measured at 490 nm using an ELISA plate reader (192). The measurement of formazan is directly proportional to cell proliferation.

Table 4 shows the effect of exposure to various concentrations of GTF (0-5 mg/ml) for 4 days on 3T3-L1 adipocyte toxicity. Cell toxicity is measured by the amount of cell proliferation occurs. The data in table 4 shows that on day 3, increasing concentrations of GTF did not affect cell proliferation compared to untreated cells (control) which were 100% viable. Not only did GTF not affect cell proliferation, but increasing concentrations of GTF caused an increase in cell proliferation. Exposure to GTF (5 mg/ml) on day 3 caused a two-fold increase in cell proliferation compared to untreated cells. Similar results were observed on day 4. Exposure to GTF (5 mg/ml) on day 4 caused a 1.5 fold increase in cell proliferation. The reasons for this increase in cell proliferation is presently unknown but will be studied in future experiments. The results in table 4 show that GTF is not toxic to the 3T3-L1 adipocytes

	Control	0.5mg/ml	1mg/ml	2.5mg/ml	5mg/ml
Time					
Day 3	100	123 +/- 40	176 +/- 31	174 +/- 51	203 +/-14
Day 4	100	106 +/- 2	112 +/- 8	175 +/- 36	146 +/- 6

TABLE 4. The effect of GTF on cell toxicity in 3T3-L1 adipocytes

3T3-L1 adipocytes were plated in 10% phenol red free DMEM. After 2 days the cells were incubated with PMS/MST which is bioreduced by cells to form formazan. Formazan absorbance was measured at 490 nm in one hour time points for a period of 4 days to determine cell proliferation. The following data shows the % viability (i.e. cell proliferation).

11. The Effect of Insulin on ^{125}I-insulin Displacement from its Receptor in 3T3-L1 Adipocytes

First sets of experiments were done to show that insulin can displace ^{125}I-insulin from its receptor. Once the 3T3-L1 pre-adipocytes were differentiated to adipocytes, the cells were treated with ^{125}I-insulin alone (total binding) and with increasing concentration of insulin (10ng-200ng) with ^{125}I-insulin, or with a large excess of unlabeled insulin (40 μg) (non-specific binding).

Figure 16 shows the effect of insulin on ^{125}I-insulin displacement from its receptor. The results showed that increasing concentrations of insulin (0-200ng) displaced ^{125}I-insulin bound (fmoles/mg p) from its receptor. This occurred in a dose dependent manner, where 10 ng insulin slightly displaced ^{125}I-insulin from its receptor and 200 ng insulin had a large effect on displacing ^{125}I-insulin from its receptor. The data in figure 16 shows that there is a decrease in ^{125}I-insulin bound to the receptor due its displacement by the addition of unlabeled insulin (ng). This is shown by the decreasing line representing ^{125}I-insulin bound to its receptor with increasing unlabeled insulin (x-axis).

**DISPLACEMENT OF I^{125} -INSULIN WITH
INSULIN (UNLABELED)**

Insulin (unlabeled) ng added
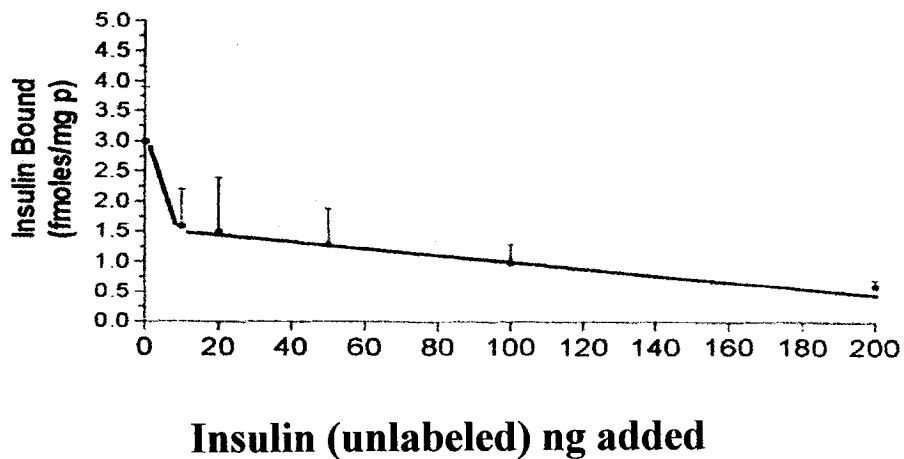
**FIGURE 16. The effect of insulin on the ^{125}I-insulin displacement of insulin from its
receptor in 3T3-L1 adipocytes**

Differentiated 3T3-L1 adipocytes were exposed for two hours with ^{125}I-insulin (total
binding), with increasing concentrations of unlabeled insulin (0-200 ng), or with a large
excess of unlabeled insulin (40 µg) (non-specific binding). The data shows increasing
concentrations of insulin gave a dose dependent displacement of insulin from its receptor.

12. The Effect of GTF on ^{125}I-insulin Displacement from its Receptor in 3T3-L1 Adipocytes

Our experiments showed that GTF did not bind to the insulin receptor. The next sets of experiments were done to determine if GTF displaces ^{125}I-insulin from its receptor. Once the 3T3-L1 pre-adipocytes were differentiated to adipocytes, the cells were treated with ^{125}I-insulin alone (total binding) and increasing concentration of GTF $(0.5 \times 10^{-10}\text{-}1 \times 10^{7}\text{ng})$ with ^{125}I-insulin, or with a large excess of cold insulin ($40 \mu g$) (non-specific).

Figure 17 shows the effect of GTF on ^{125}I-insulin displacement from its receptor. The results showed that increasing concentrations of GTF $(0.5 \times 10^{-10}\text{-}1 \times 10^{7}\text{ng})$ did not have any significant effect on ^{125}I-insulin displacement from its receptor. Figure 17 shows that GTF had no significant effect on decreasing ^{125}I-insulin bound (fmoles/mg p) from its receptor because there was no change with the addition of GTF $(0.5 \times 10^{-10}\text{-}1 \times 10^{7}\text{ng})$. This can be seen by a straight line in figure 17.

DISPLACEMENT OF 1^{125}-INSULIN BY GTF
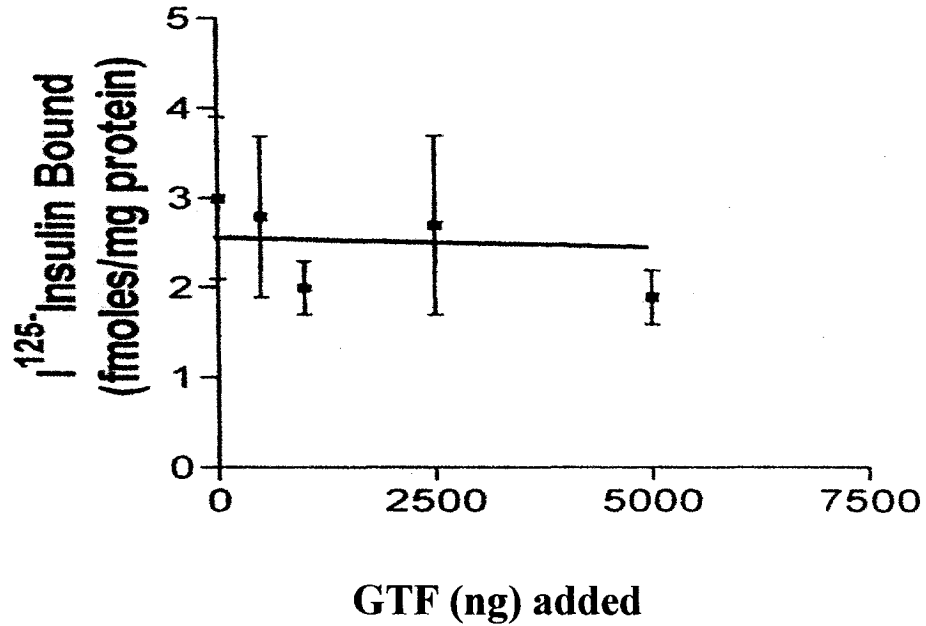
FIGURE 17. The effect of GTF on the ^{125}I-insulin displacement of insulin from its receptor in 3T3-L1 adipocytes

Differentiated 3T3-L1 adipocytes were exposed for two hours with ^{125}I-insulin (total binding), with increasing concentrations of GTF (0.5×10^{-10}-1×10^{7}ng), or with a large excess of cold insulin (40 µg) (non-specific binding) at room temperature.

13. The Effect of Insulin and GTF on GLUT 4 and GLUT 1 Translocation in 3T3-L1 Adipocytes

Our results showed that GTF stimulated 2-DG transport, but did not bind to the insulin receptor nor phosphorylated the insulin receptor β chain. Experiments were done to determine if GTF had an effect on both GLUT 4 and GLUT 1 translocation. To determine the effects of insulin, GTF, and a combination of insulin and GTF on GLUT 4 and GLUT 1 translocation, differentiated adipocytes were incubated with insulin alone, GTF alone, or in combination of insulin and GTF, or with no drugs for 1 hour. The cells were extracted and collected with buffer I (see materials and methods). The cells were pelleted by ultra centrifugation. The first spin at 14 800 rpms contained the crude plasma membrane extract. The second spin at 35 000 rpms collected the high density microsomal membrane. We examined the effects of insulin and GTF on GLUT 1 and GLUT 4 translocation of 3T3-L1 adipocytes by western blot analysis using anti-GLUT 4 and anti-GLUT 1 probe.

Figure 18 shows the effect insulin alone (20 mU/ml), GTF alone (10 mg/ml), and a combination of insulin (1 mU/ml) and GTF (5 mg/ml) on GLUT 4. The results showed that in the presence of insulin (20 mU/ml) there is more GLUT 4 present in the crude plasma membrane (band in lane 2) and high-density microsomal membrane (band in lane 6) compared to the control (band in lanes 1 and 5). GTF also showed the presence of GLUT 4 in both crude plasma membrane (band in lane 3) and high-density microsomal

membrane (band in lane 7) compared to the control (band in lanes 1 and 5), but lower

than insulin (20 mU/ml) alone (band in lanes 2and 6). The presence of GLUT 4 in the

crude plasma membrane (band in lane 4) and high density microsomal membrane (band

in lane 8) was observed to be slightly higher in combination with insulin (1 mU/ml) and

GTF (5 mg/ml), for both crude plasma membrane (band in lane 4) and high-density

microsomal membrane (band in lane 8) than GTF (10 mg/ml) alone (band in lanes 3 and

7) and the control (band in lanes 1 and 5). GLUT 4 was lower in combination for both

crude plasma membrane and high density microsomal membrane than insulin (20

mU/ml) alone (bands in lanes 2 and 6).

Figure 19 shows the densiometric analysis of the band intensities of the GLUT 4

western blot using the UN-SCAN-IT computer software. The results showed the effect of

insulin (20 mU/ml), GTF (10 mg/ml), and in combination of insulin (1 mU/ml) and GTF

(5 mg/ml) on GLUT 4 in the crude plasma membrane (blue bars) and high-density

microsomal membrane (red bars). The results showed that insulin treatment (20 mU/ml)

increased GLUT 4 content in both the crude plasma (blue bars) membrane and high-

density membrane (red bars) compared to either GTF alone (10 mg/ml), or in

combination with insulin. The combination of insulin (1mU) + GTF (5 mg/ml) has

slightly higher GLUT 4 content in the high density microsomal fraction (red bars), but

similar in the crude plasma membrane (blue bars) compared with GTF alone (10 mg/ml).

The high-density microsomal fractions in all treatments were higher than the control, but

lower in the crude plasma membrane with GTF and a combination with insulin.

Figure 20 shows the effects of insulin (20 mU/ml), GTF (10 mg/ml), and a

combination of insulin (1 mU/ml) and GTF (5mg/ml) on GLUT 1. The results showed

that in the presence of insulin (20 mU/ml) alone (band in lanes 2 and 6), GTF (10 mg/ml)

alone (band in lanes 3 and 7), and in combination of insulin (1 mU/ml) + GTF (5 mg/ml)

(band in lanes 4 and 8), there was higher GLUT 1 in both crude plasma membrane and

high-density microsomal membrane than the control (band in lanes 1 and 5). The results

showed that the combination of insulin (1 mU/ml) and GTF (5 mg/ml) had the greatest

increase in GLUT 1 in both crude plasma membrane (band in lane 4) and high-density

microsomal membrane (band in lane 8) compared to either insulin (20 mU/ml) alone

(band in lanes 2 and 6) or GTF (10 mg/ml) alone (band in lanes 3 and 7). Insulin (20

mU/ml) and GTF (10 mg/ml) alone both had similar increases in GLUT 1 in the crude

plasma membrane (bands in lanes 2 and 3) and high-density microsomal membrane (band

in lanes 6 and 7) compared to the control (band in lanes 1 and 5).

Figure 21 shows the graphical analysis of the band intensities of the GLUT 1

western blot using the UN-SCAN-IT computer software. The results showed that the

combination of insulin (1mU/ml) and GTF (5 mg/ml) had the highest increase in GLUT 1

for both crude plasma membrane (blue bars) and high-density microsomal membranes

(red bars) compared to insulin (20 mU/ml) alone, or GTF alone (10 mg/ml). Insulin (20

mU/ml) alone and GTF (10 mg/ml) alone had similar amounts of high-density

microsomal membrane, but GTF had slightly higher amounts crude plasma membrane.

These results showed that GTF has a greater effect on GLUT 1 transport and protein content than in GLUT 4. The reason for the difference is presently not known. This reason may explain GTF's ability to potentate insulin's effect.

| Lane 1 | Lane 2 | Lane 3 | Lane 4 | Lane 5 | Lane 6 | Lane 7 | Lane 8 |

CRUDE   **HDM**

Lane 1 and 5= Control
Lane 2 and 6= Insulin (20 mU/ml)
Lane 3 and 7= GTF (10 mg/ml)
Lane 4 and 8= Insulin (1 mU/ml) + GTF (5 mg/ml)

FIGURE 18. The effect of insulin, GTF, and a combination of insulin and GTF on GLUT 4 translocation in 3T3-L1 adipocytes

Figure 18 is a GLUT 4 Western blot using an anti-GLUT 4 antibody. 3T3-L1 adipocytes were treated with no drugs (control) (lanes 1 and 5), insulin (20 mU/ml) (lanes 2 and 6), GTF (10 mg/ml) (lanes 3and 7), and in combination of insulin (1 mU/ml) + GTF (5 mg/ml) (lanes 4 and 8) for one hour. Lanes 1-4 are measuring the effects of each treatment of GLUT 4 in the crude plasma membrane. Lanes 5-8 are measuring the effects of each treatment of GLUT 4 in the microsomal membrane. The data shows that all treatments have an effect on GLUT 4 translocation from the intracellular membranes to the plasma membrane compared to the control (lane 1and 5).

The Effect of Insulin, GTF, and in a Combination of Insulin and GTF on Total amount of GLUT 4 Protein
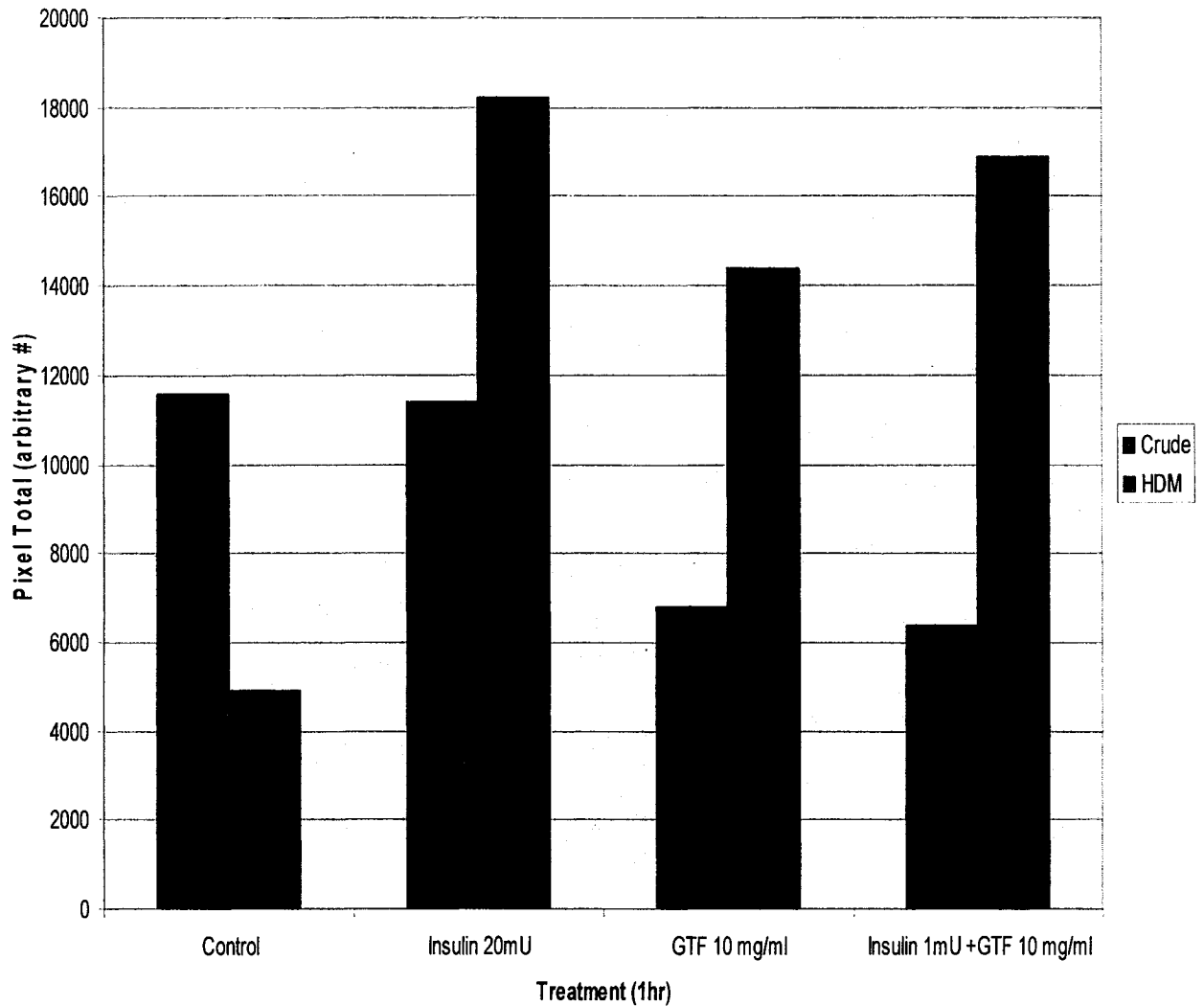
FIGURE 19. The effect of insulin, GTF, and in combination of insulin and GTF on GLUT 4 in 3T3-L1 adipocytes.

The following is a chart indicating the difference in GLUT 4 protein in the presence of insulin, GTF and a combination of insulin and GTF in 3T3-L1 adipocytes. The cells were exposed to each treatment for one hour. The samples are as follows; control, insulin (20 mU/ml), GTF (10 mg/ml), and insulin (1 mU/ml) + GTF (5 mg/ml). The values from each sample are expressed as intensities of the bands in pixel total (arbitrary numbers) by using a computer program called UN-SCAN-IT. The blue bars represent GLUT 4 present in the crude plasma membrane and the red bars represent GLUT 4 in the high-density microsomal membrane.

| Lane 1 | Lane 2 | Lane 3 | Lane 4 | Lane 5 | Lane 6 | Lane 7 | Lane 8 |

Lanes 1 and 5= control
Lanes 2 and 6= Insulin (20 mU/ml)
Lanes 3 and 7= GTF (10 mg/ml)
Lanes 4 and 8= Insulin (1 mU/ml) + GTF (5 mg/ml)

FIGURE 20. The effect of insulin, GTF, and a combination of insulin and GTF on GLUT 1 translocation on 3T3-L1 adipocytes

Figure 20 is a GLUT 4 Western blot using an anti-GLUT 1 antibody. 3T3-L1 adipocytes were treated with no drugs (control) (lanes 1 and 5), insulin (20 mU/ml) (lanes 2 and 6), GTF (10 mg/ml) (lanes 3and 7), and in combination of insulin (1 mU/ml) + GTF (5 mg/ml) (lanes 4 and 8) for one hour. Lanes 1-4 are measuring the effects of each treatment of GLUT 1 in the crude plasma membrane. Lanes 5-8 are measuring the effects of each treatment of GLUT 1 in the microsomal membranes. The data shows that all treatments have an effect on GLUT 1 translocation from the intracellular membranes to the plasma membrane compared to the control.

The effect of insulin, GTF, and in a combination of insulin and GTF on the total amount of GLUT 1 protein

FIGURE 21. The effect of insulin, GTF, and a combination of insulin and GTF on GLUT 1 in 3T3-L1 adipocytres.

The following is a chart indicating the difference in GLUT 1 protein in the presence of insulin, GTF and a combination of insulin and GTF in 3T3-L1 adipocytes. The cells were exposed to each treatment for one hour. The samples are as follows; control, insulin (20 mU/ml), GTF (10 mg/ml), and insulin (1 mU/ml) + GTF (5 mg/ml). The values from each sample are expressed as intensities of the bands in pixel total (arbitrary numbers) by using the UN-SCAN-IT computer program. The blue bars represent GLUT 4 present in the crude plasma membrane and the red bars represent GLUT 4 in the high-density microsomal membrane.

14. The Effects of Insulin and GTF on IRS-1 Phosphorylation in 3T3-L1 Adipocytes

GTF had an effect on both GLUT 4 and GLUT 1. Other signals in the insulin

pathway are involved in the translocation of the glucose transporters. If GTF had an

effect on GLUT 4 and GLUT1, then GTF might have an effect on those signals involved

in translocating the glucose transporters. We examined the effects of one of these signals

the insulin receptor substrate-1 (IRS-1). 3T3-L1 adipocytes were treated as previously

mentioned in materials and methods. We examined the effects of GTF on IRS-1

phosphorylation. Immunoprecipitation of the IRS-1 post-treatment with insulin (20

mU/ml), GTF (10 mg/ml) and in combination of insulin (1 mU/ml) and GTF (5 mg/ml

GTF) were also examined.

Figure 22 shows the effects of insulin, GTF, and a combination of insulin and

GTF on IRS-1 phosphorylation. The results in figure 22 indicated that insulin (20 mU/ml)

alone (band in lane 2), GTF (10 mg/ml) alone (band in lane 3) and the combination of

insulin (1 mU/ml) and GTF (5 mg/ml) (band in lane 4) exhibited phosphorylation of IRS-

1. The greatest amount of IRS-1 phosphorylation occurred with insulin (20 mU/ml) alone

(band in lane 2) compared to the control (band in lane 1) and GTF (10 mg/ml) alone

(band in lane 3). The combination of insulin and GTF had a higher effect on

phosphorylation of IRS-1 than control (band in lane 1) and GTF (band in lane 3). GTF

exhibited an effect on phosphorylation of IRS-1compared to the control (band in lane 1)

but lower than insulin (band in lane 2) or in combination with insulin (band in lane 4).

The results showed that GTF lowered the phosphorylation of IRS-1 in the presence of insulin. This may be due to GTF having an inhibitory effect on IRS-1or due to the low concentrations of insulin (1 mU/ml) and GTF (5 mg/ml) employed in combination compared to each treatment of insulin (20 mU/ml) alone or GTF (10 mg/ml) alone. Future studies will be done to determine what causes the apparent decrease of IRS-1 phosphorylation of GTF in combination with insulin.

Figure 23 shows the densiometric analysis of the band intensities of the IRS-1 Western blot using the UN-SCAN-IT computer software. The results showed that insulin (20 mU/ml) increased the amount of phosphorylated IRS-1 compared to the control, GTF (10 mg/ml) and in combination of insulin (1 mU/ml) and GTF (5 mg/ml). Also, GTF and in combination with insulin both increased the amount of phosphorylated IRS-1 protein compared to the control. GTF alone (10 mg/ml) exhibited a slightly higher increase in phosphorylation of IRS-1 compared with the combination treatment.

Lane 1   Lane 2   Lane 3   Lane 4

Lane 1= Control
Lane 2= Insulin (20 mU/ml)
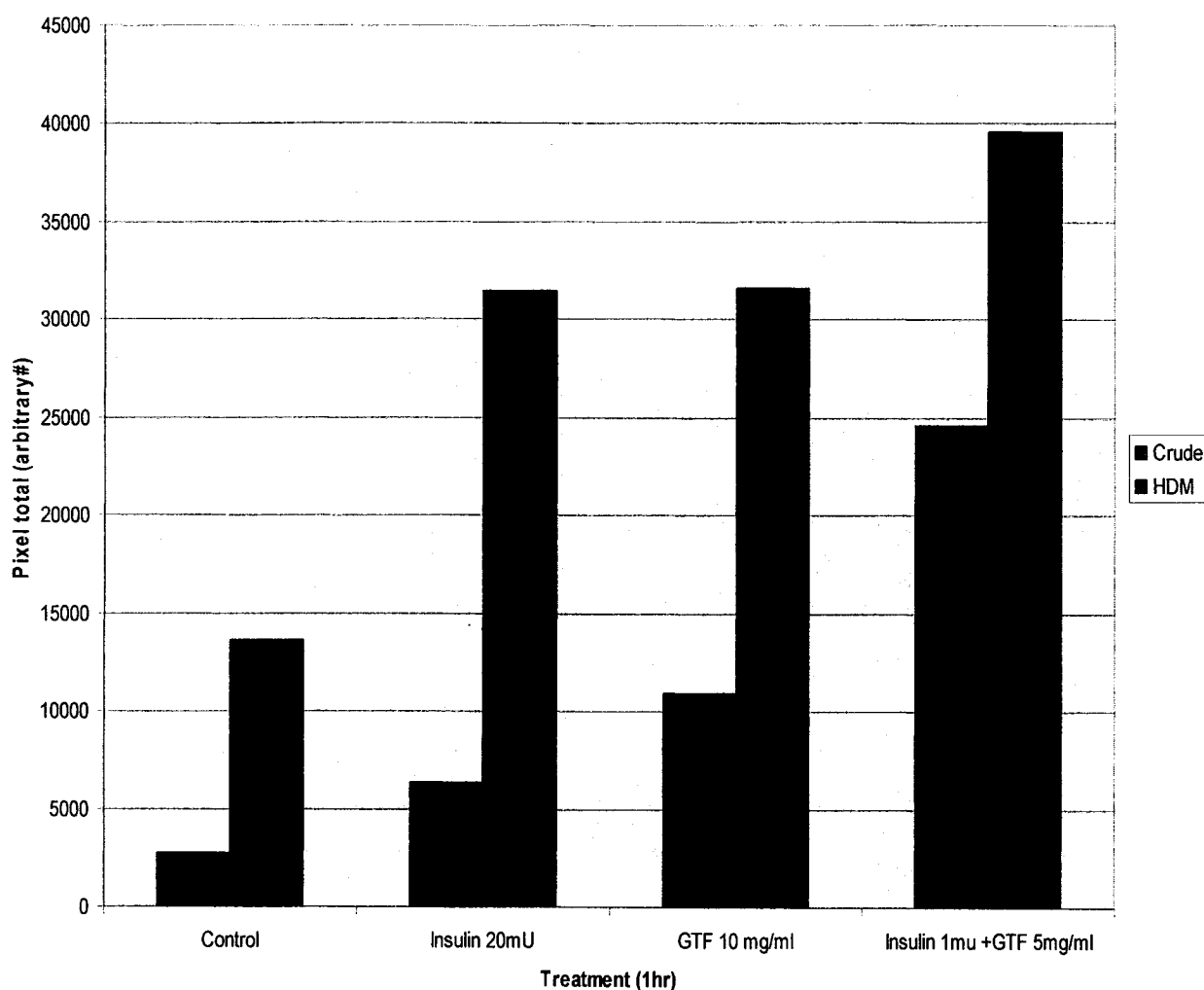Lane 3= GTF (10 mg/ml)
Lane 4= Insulin (1 mU/ml) + GTF (5 mg/ml)

FIGURE 22. The effect of insulin, GTF, and a combination of insulin and GTF on IRS-1 phosphorylation in 3T3-L1 adipocytes.

The Western blot shows IRS-1 phosphorylation using an anti-phosphotyrosine (4G10) probe.The figure shows the phosphorylation of IRS-1 at 131 Kd in the presence of insulin (20 mU/ml) alone (lane 2), GTF (10 mg/ml) alone (lane 3), and in combination of insulin (1 mU/ml) and GTF (5 mg/ml) (lane 4). Lane 1 is the control. The 3T3-L1 adipocytes were treated with each of the drugs for 15 minutes to give the maximal phosphorylation of the IRS-1

The Effect of Insulin, GTF, and a Combination of Insulin and GTF on the Total Amount of IRS-1 Protein
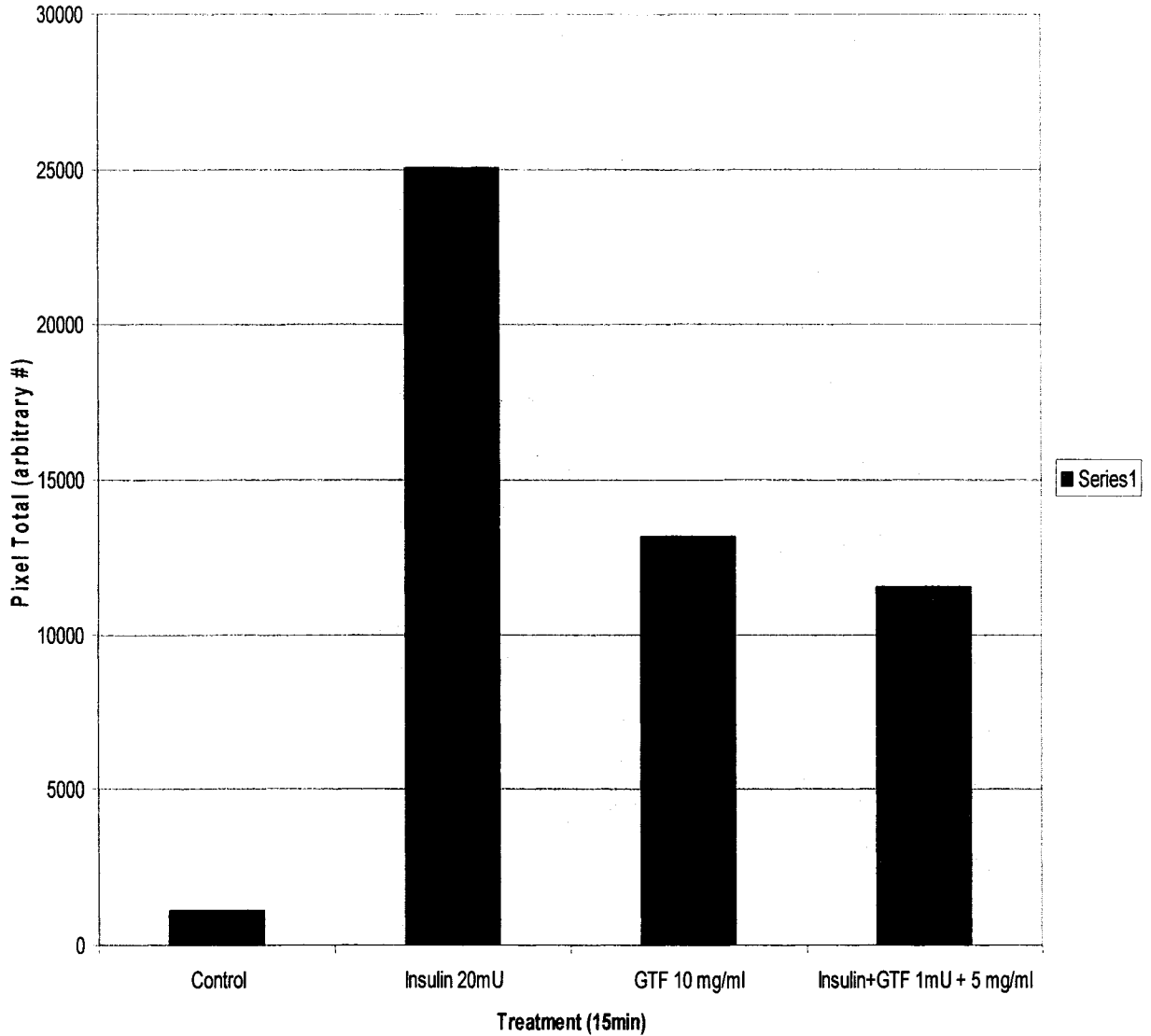
FIGURE 23. The effect of insulin, GTF, and in combination of insulin and GTF on the phosphorylated IRS-1 in 3T3-L1 adipocytes

The following is a chart indicating the difference in phosphorylation of IRS-1 in the presence of insulin alone (20 mU/ml), GTF alone (10mg/ml) and a combination of insulin (1 mU/ml) and GTF (5 mg/ml) in 3T3-L1 adipocytes. The cells were exposed to each treatment for 15 minutes. The samples are as follows; lane 1 is the control, lane 2 is insulin (20 mU/ml), lane 3 is GTF (10 mg/ml), and insulin (1 mU/ml) + GTF (5 mg/ml) is lane 4. The values from each sample are expressed as intensities of the bands in pixel total (arbitrary numbers) using the UN-SCAN-IT computer program.

82

Discussion

The incidence of diabetes, especially type 2 diabetes is on the rise. New alternative medicines are needed to replace or potentiate insulin's effects since many are insulin resistant. One possible drug is GTF. Since its discovery in 1957 by Schwartz and Mertz (162) much work has been done to purify it (163) and to understand its composition (171). Also, much work has been done to determine GTF's physiological effect(s) and mode of action (183). Most of the work done on GTF has been done in vivo. Mirsky et al. (183) have done work on diabetic rats to observe GTF's effect on lowering blood glucose levels. Mirsky et al. (183) also have done work on obese rats to observe the effect of GTF on lowering free fatty acids in the blood. Determining GTF's effect(s) in vitro and its mode of action have yet to be well characterized. In this thesis in vitro work on 3T3-L1 adipocytes and L6 myotubes were done to understand its mode of action and to study and interpret its effect(s).

If GTF will one day be used as an anti-diabetic drug, a major concern would be that GTF were safe. In this thesis, we examine GTF's effect on cell viability. 3T3-L1 adipocytes were plated with media containing various concentrations of GTF (0-5 mg/ml) for four days. Using a cell proliferation assay kit (Promega), we observed that GTF did not reduce cell proliferation (Table 4). The highest concentration of GTF (5 mg/ml) up to day 4 did not elicit any reduction on cell viability (Table 4). The reason for measuring cell proliferation up to day 4 is that by day 4 the cells become confluent and the cells do

not continue to divide. The cells are density dependent inhibited. Also, another reason that we measure cell proliferation up to day 4 is that during our experiments we exposed the cell for one hour with GTF; therefore exposure to GTF for 4 days was sufficient. Not only did GTF have no effect on reducing cell proliferation, but also, it had an effect on cell number (Table 4). Looking at the control we have 100% viability and with increasing concentrations of GTF there is an increase in cell proliferation, which was measured using a cell proliferation kit (Promega). This may be due to an increase in cell number or cell size. GTF may act as a growth factor or as a hormone but this has yet to be studied. Work done by Berdichevski and Mirsky (193) also observed yeast exposed to GTF grew in size and had different cell morphology than yeast without GTF.

Our first sets of experiments were done to determine if GTF has an effect on sugar transport in vitro. GTF is known to cause an increase in sugar uptake in rats without the addition of exogenous insulin (183). A single dose of GTF showed immediate effects on sugar uptake in vivo. The lowering of blood glucose levels by GTF lasted up to 24 hours (183). Evidence presented in this thesis shows that GTF does have an effect on glucose transport in 3T3-L1 adipocytes (figure 9) and on L6 myotubes (Table 3). Increasing concentrations of GTF showed significant increase in 2-DG transport in a dose dependent manner like insulin (figures 8, 9 and Tables 2, 3). Our experiments have shown that GTF in combination with insulin had a higher increase in 2-DG transport than either insulin alone or GTF alone. A combination of insulin as low as 1 mU/ml and 2.5 mg/ml of GTF had a higher stimulated 2-DG transport than the highest insulin concentration used in our experiments (133 nM) and GTF (20 mg/ml) (Table 2). This

suggests that GTF works in an additive manner with insulin. Other researchers have observed that GTF works in a synergistic manner with insulin (162-167). This additive effect of insulin and GTF observed in this thesis may be due to the ability of GTF to bind to the insulin receptor with insulin then an additive effect in 2-DG transport may occur. Also, the stimulated 2-DG transport is due to GTF binding to the insulin receptor therefore activating the insulin-signalling pathway to stimulate 2-DG transport in 3T3-L1 adipocytes and L6 myotubes. The next sets of experiments were done to determine if GTF binds to the insulin receptor.

Treatments with other compounds that mimic insulin actions work by binding to the insulin receptor. To determine if the effect(s) of GTF are due to binding to the insulin receptor, we studied ^{125}I-insulin binding on 3T3-L1 adipocytes. The data presented in figure 11, showed that the addition of GTF did not change ^{125}I-insulin binding to its receptor on 3T3-L1 adipocytes. Davis et al. showed by kinetic studies that LMWCr has an intrinsic role with insulin without binding to the insulin receptor (160). Our results showed that GTF does not bind to the insulin receptor. GTF might also have an intrinsic role with insulin without binding to the insulin receptor. One possibility can be that GTF enters the cells through other cell receptors such as IGF-1. Future experiments will be done to determine if GTF binds to other cell receptors. Another possibility is that GTF might enter the cells by binding to insulin then entering the cell through its receptor. McCarty (194) stated that GTF binds to insulin then it enters the cell. Other experiments will be done to determine if GTF binds to insulin before entering the cell. GTF binding to insulin and then entering the cell may explain why insulin and GTF in combination have

an additive effect in stimulating 2-DG transport in 3T3-L1 adipocytes and L6 myotubes. Since GTF did not bind to the insulin receptor, the next sets of experiments were done to determine in GTF displaces ^{125}I-insulin from its receptor.

If GTF did not bind to the receptor then would GTF displace ^{125}I-insulin from its receptor? Figure 17 showed that with increasing concentrations of GTF, there was no displacement of ^{125}I-insulin from its receptor. This then corresponds to the previous results that GTF does not bind to the insulin receptor and GTF does not have an effect on stimulating sugar transport through the insulin receptor. If GTF does not work through the insulin receptor, how does it stimulate sugar transport? To answer the question, other experiments were done to determine the effect of GTF on the insulin signaling pathway.

The first step of the insulin cascade is the phosphorylation of the insulin receptor β-chain. The insulin receptor β-chain phosphorylation is an important early signal in the insulin cascade (185, 186). The phosphorylation of insulin receptor β-chain by insulin, GTF and a combination of both GTF and insulin was examined. Our findings showed that in the presence of insulin alone, there was tyrosine phosphorylation of the β-chain (figure 12). The lack of tyrosine phosphorylation of the insulin receptor β-chain by GTF can be due to the GTF not binding to the insulin receptor which stimulates the phosphorylation of the β-chain. When both insulin and GTF were present in combination, the tyrosine phosphorylation of the insulin receptor β-chain was lower than insulin alone. One possible explanation for this observation is that low concentrations of insulin (1 mU/ml) and GTF (5 mg/ml) in combination was used to stimulate tyrosine

phosphorylation compared to the 20mU/ml of insulin used alone, therefore it might not represent the true data. For future experiments new concentrations of insulin (20 mU/ml) and GTF (10 mg/ml) in combination will be used to see if the lower tyrosine phosphorylation is due to lower concentrations used or GTF in the presence of insulin decreases tyrosine phosphorylation of the insulin β-chain. This may be due to the GTF acting like or stimulating a phosphatase such as PTP-1B. Another possibility is that GTF requires binding to insulin to affect tyrosine phosphorylation of the insulin receptor β-chain. Davis et al. (173) showed that LMWCr which is a naturally-occurring oligopeptide found mainly in mammalian liver did not activate tyrosine kinase activity alone, but the presence of insulin was required. If insulin is removed, then the effect of LMWCr on activation of tyrosine kinase is abolished (173). The reason for GTFs apparent inhibition of the tyrosine phosphorylation is presently unknown. The next step of the insulin signaling pathway is the phosphorylation of the IRS-1. Since GTF did not stimulate phosphorylation of the insulin receptor β-chain, our next sets of experiments were to determine if GTF stimulates phosphorylation of the IRS-1.

Figure 22 showed that immunoprecipitation of IRS-1 post treatment for 15 minutes with insulin, GTF and a combination of insulin and GTF stimulates phosphorylation of IRS-1. Once again, the combination of insulin (1 mU/ml) and GTF (5 mg/ml) on phosphorylation of IRS-1 was lower than insulin alone. This can be due to the use of lower concentrations of both insulin and GTF compared to each alone. To test this hypothesis, future experiments will be done to correct these concentrations. Another possibility is that GTF stimulates IRS-1 phosphorylation at an earlier time than insulin.

IRS-1 phosphorylation by GTF might max out before insulin's effect at 15 minutes. Future experiments will be done using different time points to observe if there is any change in IRS-1 phosphorylation by GTF. From our results, GTF does stimulate IRS-1 phosphorylation despite the lack of binding to insulin receptor or tyrosine phosphorylation of the insulin receptor β-chain. The phosphorylation of IRS-1 by GTF can be due to direct phosphorylation of IRS-1 through another pathway or GTF phosphorlates another signaling molecule that interacts with IRS-1. To follow this up, experiments will be done on other receptors such as IGF-1 (mentioned previously) and on other signaling molecules like Grb2 and Ras. Glucose uptake ultimately arises from the transport of glucose into the cell by glucose transporters (47). GTF stimulates sugar transport and phosphorylation of IRS-1, therefore the next step was to determine if the translocation of GLUT 1 and GLUT 4 glucose transporters is stimulated by GTF.

Down stream signals such as IRS-1, PI3-kinase and PKB once activated allow translocation of the glucose transporters from inside the cell to the plasma membrane. It was shown in figure 22 that GTF stimulates phosphorylation of IRS-1 then GTF can affect translocation of GLUT 1 and GLUT 4. We showed through immunoblotting using GLUT 1 and GLUT 4 transporters that GLUT 1 and GLUT 4 are present in both plasma membrane and in the cell with insulin, GTF and a combination of insulin and GTF (figure 18 and 20). GTF stimulates GLUT 1 and GLUT 4 which correlates with our other results in GTF phosphorylation of IRS-1.

Experiments done in this thesis showed that GTF has similar properties as insulin in stimulating sugar uptake. Our results also correlate with in vivo studies done by Mirsky et al. (183). Other experiments were done to observe if GTF has other insulin like properties. One set of experiments were done to show the effects of GTF on isproterenol induced lipolysis.

Figures 14 and 15 showed that like insulin, GTF inhibits lipolysis in the presence of isoproterenol. This inhibition is also dose dependent. GTF in combination with insulin stimulates a higher inhibition of lipolysis than either insulin or GTF alone. These results indicate that GTF has other similar properties as insulin. These results further indicate that GTF and insulin have similar modes of action.

Based on the 2-DG transport and lipolysis data, GTF has similar properties as insulin. GTF mode of action is not through binding to the insulin receptor but may form a complex with insulin by binding to insulin and then entering the cell. GTF might have intrinsic activity once entering the cell. Another possibility is that GTF binds to another receptor on cell surfaces and enters the cell and activates downstream signals of the insulin pathway directly or via other signaling molecules that interact with the insulin pathway. IRS-1 is phosphorylated by GTF which ultimately stimulates sugar uptake by GLUT1 and GLUT4 sugar transporters.

Future studies on characterizing GTFs mode of action will include binding studies with GTF on other cell receptors like IGF-1 and on insulin itself. Other experiments that will be done is characterizing GTFs effect on insulin downstream signals such as PI3-kinase, PKB and other signals associated with the insulin pathway like Grb2, Ras. Also, future experiments will include working with other insulin sensitizing drugs like thiazolidinediones in the presence of GTF. Also, we will test the effects of GTF on other cell lines like the human fibroblast cell lines. Other experiments will be repeated using different concentrations for the insulin receptor β-chain and IRS-1 western blots. Another experiment that will be done is using inhibitors of the insulin pathway such as Wortmannin and tests its effect in the presence of GTF. Much more work must be done to elucidate GTFs mode of action.

References

1. http://arbl.cvmbs.colostate.edu/hbooks/patphys/endocrine/pancreas/insulin.html

2. http://arbl.cvmbs.colostate.edu/hbooks/patphys/endocrine/pancreas/insulin_struct.html

3. M.F. White. The insulin signaling system and the IRS proteins. Diabetologia (1997) 40: 512-517.

4. C. Taha, A. Klip. The insulin signaling pathway. J. Membrane Biol 169 (1999) 1-12.

5. G.D. Holman, M. Kasuga. From receptor to transporter: insulin signaling to glucose transport. Diabetologia (1997) 40 991-1003.

6. I. Goldfine. The insulin receptor: Molecular biology and transmembrane signaling. Endocr.Rev. 8 (1987) 235-255.

7. C.R. Kahn. Membrane receptors for hormones and neurotransmitters. J.Cell.Biol 70 (1976) 261-286.

8. E.O. freed. HIV-1 gag proteins: diverse function in the virus life cycle. Virology 251 (1998) 1-15.

9. H. Geuze, J. Slot, G. Straus, H. Lodish, A. Schwartz. Intracellular site of Asialoglycoprotein receptor-ligand uncoupling: Double-labled immunoelectron microscopy during receptor mediated endocytosis. Cell 32 (1983) 277-287.

10. J. Baenziger, D. Fiete. Separation of two populations of endocytic vesicles involved in receptor ligand sorting in rat hepatocytes. J.Biol.Chem 261 (1986) 7445-7454.

11. S. Marshall. Dual pathways for the intracellular processing of insulin. Relationship between retroendocytosis of intact hormone and the recycling of insulin receptors. J.Biol.Chem.(1985) 13524-13531.

12. J. Levy, J. Olefsky. Retroendocytosis of insulin in rat adipocytes. Endocrinology 119 (1986) 572-579.

13. S. Marshall, A. Green, J. Olefsky. Evidence for recycling of insulin receptors in isolated rat adipocytes. J.Biol.Chem 256 (1981) 11464-11470.

14. S. Marshall. Kinetics of insulin receptor internalization and recycling in adipocytes. Shunting of receptors to a degradative pathway by inhibitors of recycling. J.Biol.Chem. 260 (1985) 4136-4144.

15. S.I. Taylor, D.E. Moller. Mutations of the insulin receptor gene. In: Moller DE (ed) Insulin resistance. John Wiley and Sons, Chichester (1993) 83-121.

16. T. Hunter. Protein kinases and phosphatases: the yin and yang of protein phosphoylation and signaling. Cell 80 (1995) 225-236.

17. D.T. Pang, B. Sharma, J.A. Shafer, M.F. White, C.R. Khan. Predominance of tyrosine phoshorylation of insulin receptors during the insulin response of intact cells to insulin. J Biol Chem 260 (1985) 7131-7136.

18. O.M. Rosen. After insulin binds. Science 237 (1987) 1452-1458.

19. E.P. Feener, J.M. Backer, G.L. King, P.A. Wilden, X. Sun, C.R. Kahn, M.F. White; Insulin stimulates serine and tyrosine phosphorylation in the juxta membrane region of the insulin receptor. J. Biol Chem 268 (1993) 11256-11264.

20. M.F. White, C.R. Kahn. The insulin signaling system. J. Biol Chem 269 (1994) 1-5.

21. D. McClain, H. Maegawa, J. Levy et al.. Properties of a human insulin receptor with a COOH-terminal truncation I. Insulin binding, autophosphorylation and endocytosis. J. Biol Chem 263 (1998) 8904-8912.

22. H. Maegawa, D. McClain, G. Freidneberg et al.. Properties of a human insulin receptor with a COOH-terminal truncation II. Truncated receptors have normal kinase activity but one defective in signaling metabolic effects. J. Biol Chem 263 (1988) 8912-8917.

23. M.F. White, R. Maron, C.R. Kahn. Insulin rapidly stimulates tyrosine phosphorylation of a Mr 185000 protein in intact cells. Nature 318 (1985) 183-186.

24. Z. Songyang, K.L. Carraway III, M.J. Eck et al. Catalytic specificity of protein-tyrosine kinases is critical for selective signaling. Nature 373 (1995) 536-539.

25. X. Sun, P. Rothenberg, C.R. Kahn et al.. The structure of the insulin receptor substrate IRS-1 defines a unique signal transduction protein. Nature 352 (1991) 73-77.

26. X. Sun, D.L. Crimmins, M.G. Jr Myers, M. Miralpeix, M.F. White. Pleiotropic insulin signals are engaged by multisite phosphorylation of IRS-1. Mol Cell Biol 13 (1993) 7418-7428.

27. M.G. Jr Myers, T.C. Grammer, J. Brooks et al. The pleckstrin homology domain in IRS-1 sensitizes insulin signaling. J. Biol Chem 270 (1995) 11715-11718.

28. T.A. Gustafson, W. He, A. Craparo, C.D. Schaub, T.J. O'Neil. Phosphotyrosine-dependent interaction of Shc and IRS-1 with the NPEY motif of the insulin receptor via a novel non-SH2 domain. Mol Cell Biol 15 (1995) 2500-2508.

29. A. Craparo, T.J. O'Neil, T.A. Gustafson. Non-SH2 domains within the insulin receptor substrate-1 and Shc mediate their phosphotyrosine dependent interaction with the NPEY motif of the insulin-like factor 1 receptor. J. Biol Chem 270 (1995) 15639-15643.

30. S.F. Clark, J.C. Molero, D.E. James. Release of Insulin Receptor Substrate Proteins from an Intracellular Complex Coincides with the Development of Insulin Resistance. J.Biol.Chem. 275 (2000) 3819-3826.

31. G. Inoue, B. Cheatham, R. Emkey, C.R. Kahn. Dynamics of insulin signaling in 3T3-L1 adipocytes. J. Biol Chem 273 (1998) 11548-11555.

32. B. Kublaoui, J. Lee, P.F. Pilch. Dynamics of signalling during insulin-stimulated endocytosis of its receptor in adipocytes. J. Biol Chem 270 (1995) 59-65.

33. R. A. Heller-Harrison, M. Morin, M.P. Czech. Insulin regulation of membrane-associated insulin receptor substrate-1. J. Biol Chem 270 (1995) 24442-24450.

34. J.M. Ricort, J.F. Tanti, E. Van Obberghen, Y. LeMarchand Brustel. Different effects of insulin and platelet-derived growth factor on phosphatidylinositol 3-kinase at the subcellular level in 3T3-L1 adipocytes. A possible explaination for their specific effects on glucose transport.Eur. J. Biochem 239 (1996) 17-22.

35. M. Otsu, T. Hiles, I. Gout et al. Characterization of 2 85kD proteins that associate with receptor tyrosine kinases, middle-t pp60C-Src complexes, and PI3-kinase. Cell 65 (1991) 91-104.

36. E.Y. Skolnik, B. Margolis, M. Mohammadi et al. Cloning of Pi3-kinase associated p85 utilizing a novel method for expression cloning of target proteins for receptor tyrosine kinases. Cell 65 (1991) 83-90.

37. J.A. Escobedo, S. Navankasattusas, W.M. Kavanaugh, D.Milfay, V.A. Fried, L.T. Williams.cDNA cloning of a novel 85kD protein that has SH2 domains and regulates binding of PI3-kinase to the PDGF-beta receptor. Cell 65 (1991) 75-82.

38. I.D. Hiles, M.Otsu, S. Volinia et al.. Phosphatidylinositol 3-kinase-structure and expression of the 100kD catalytic subunit. Cell 70 (1992) 419-429.

39. P. Hu, P. Mondino, E.Y. Skolnik, J. Shlessinger. Cloning of a novel ubiquitously expressed human phosphatidylinosital 3-kinase and identification of its binding site on p85. Mol Cell Biol 13 (1993) 7677-7688.

40. M.G. Myers jr., J.M. Backer, X. Sun et al. IRS-1 activates the phosphatidylinositol 3'-kinase by associating with the src homology 2 domains of p85. Proc Natl Acad Sci (USA) 89 (1992) 1411-1415.

41. B. Vanhaesebroeck, D.R. Alessi. The PI3-k-PDK-1 connection: more than just a road to PKB. J. Biochem 346 (2000) 561-576.

42. D.R. Alessi, M. Andjelkovic, B. Caudwell, P. Cron, N. Morrice, P. Cohen, B.A. Hemmings. Mechanism of activation of protein kinase B by insulin and IGF-1. EMBO J. 15 (1996) 6541-6551.

43. A.D. Kohn, f. Takeuchi, R.A. Roth. Akt, a pleckstrin homology domain containing kinase, is activated primarily by phosphorylation. J. Biol Chem 271 (1996b) 21920-21926.

44. M. Andjelkovic, D.R. Alessi, R. Meier, A. Fernandez, N.C.J. Lamb, M. French, P. Cron, P.Cohen, J.M. Lucocq, B.A. Hemmings. Role of translocation in the activation and function of protein kinase B. J. Biol Chem 272 (1997) 31515-31324.

45. K.S. Walker, M. Deak, A. Paterson, K. Hudson, P. Cohen, D.R. Alessi. Activation of protein kinase B beta and gamma isoforms by insulin in vivo and by 3-phosphoinositide-dependent protein kinase-1 in vitro:comparison with protein kinase B alpha. J. Biochem 331 (1998) 299-308.

46. A. Belacossa, J.R. Testa, S.P. Staal, P.N. Tsichlis. A retroviral oncogene, akt, encoding a serine-threonine kinase containing an SH2-like region. Science 254 (1991) 244-247.

47. H. Franklin, M.D. Epstein. Glucose transporters and insulin action. The New England Journal of Medicine 341 (1999) 248-257.

48. T. Kayano, C.F Burant, H. Fukumoto, G.W. Gould et, al. Humal facilitative glucose transporters. Isolation, functional characterization, and gene localization of cDNAs encoding an isoform (GLUTS) expressed in small intestine, kidney, muscle and adipose tissue and an unusual glucose transporter pseudogene-like sequence (GLUT6). J.Biol.Chem. 265 (1990) 13276-13282.

49. I.D. Waddell, A.G. Zomerschoe, M.W Voice, and A. Burchell. Cloning and expression of a hepatic microsomal glucose transport protein. Comparison with liver plasma-membrane glucose-transport protein GLUT 2. Biochem J. 285 (1992) 173-177.

50. A. Burchell. A re-evaluation of GLUT 7. biochem J. 331 (1998) 973.

51. M.O. Carayannopoulos, M. M-y. Chi, Y. Cui, J. M. Pingsterhaus, R. A. Mcknight, M. Mueckler, S. U. Devadkar, and K. H. Moley. GLUT 8 is a glucose transporter responsible for insulin-stimulated glucose uptake in the blastocyst.PNAS. 97 (2000) 7313-7318.

52. K.V. Kandror, P.F. Pilch. Compartmentalizatin of protein traffic in insulin-sensitive cells. Am J. Physiol 271 (1996) E1-E14.

53. G.W. Gould, G.D. Holman. The glucose transporter family: structure, function and tissue-specific expression. Biochem J. 295 (1993) 329-341.

54. K. Ueki, C.M. Yballe, S. M. Brachmann, D. Vincent, J.M Watt, C.R. Kahn, L. C. Cantley. Increased insulin sensitivity in mice lacking p85β subunit of phoshoinositide 3-kinase. PNAS 99 (2002) 419-424.

55. A.D. Kohn, A. Barthel, K.S. Kovacina et al. Construction and characterization of a conditionally active version of the serine/threonine kinase Akt. J. Biol Chem 273 (1998) 11937-11943.

56. M.L. Standaert, L. Galloway, P. Karnam, G. Bandyopadhyay, J. Moscat, R.V. Farese. Protein kinase C-zeta as a downstream-effector of phosphatidylinositol 3-kinase during insulin stimulation in rat adipocytes: potential role in glucose transport. J. Biol Chem 272 (1997) 30075-30082.

57. K. Kotani, W. Ogawa, M. Matsumoto et al. Requirement of atypical protein kinase C lambda for insulin stimulation of glucose uptake but not for Akt activation in 3T3-L1 adipocytes. Mol Cell Biol 18 (1998) 6971-6982.

58. C.C. Cain, W.S. Trimble, G.E. lienhard. Members of the VAMP family of synaptic vesicle proteins are components of glucose transporter-containing vesicles from rat adipocytes. J. Biol. Chem. 267 (1992) 11681-11684.

59. K.I. Timmers, A.E. Clark, M.Omatsu-Kanbe, S.W. Whiteheart, M.K. Bennetts, G.D. Holman, S.W. Cushman. Identification of SNAP receptors in rat adipose cell membrane fractions and in SNARE complexes co-immunoprecipitated with epitope-tagged N-ethylmaleimide-sensitive fusion protein. Biochem J 320 (1996) 429-436.

60. J.M. Olefsky Insulin-stimulated glucose transport minireview series. J. Biol Chem 274 (1999) 1863.

61. J.E. Pessin, D.C. Thurmond, J.S. Elmendorf, K.J. Coker, S. Okada. Molecular basis of insulin-stimulated Glut-4 vesicle trafficking. J. Biol Chem 274 (1999) 2593-2596.

62. A.L. Olson, J.B. Knight, J.E. Pessin. Syntaxin 4, VAMP2, and/or Vamp3/cellubrevin are functional target membrane and vesicle SNAP receptors for insulin-stimulated GLUT 4 translocation in adipocytes. Mol Cell Biol 17 (1997) 2425-2435.

63. S. Rea, L.B. Martin, S. McIntosh, S.L. Macaulay, T. Ramsdale, G. Baldini, D.E. James. Syndet, an adipocyte target SNARE involved in the insulin-induced translocation of GLUT 4 to the cell surface. J Biol Chem 273 (1998) 18784-18792.

64. A. Volchuk, Q. Wang, H.S. Ewart, L.Z. He, M.K. Bennett, A. Klip. Syntaxin 4 in 3T3-L1 adipocytes: regulation by insulin and participation in insulin-dependent glucose transport. Mol Biol Cell 7 (1996) 1075-1082.

65. B. Cheatham, A. Volchuk, C.R. Kahn, L. Wang, C.J. Rhodes, A. Klip. Insulin-stimulated translocation of GLUT 4 glucose transporters requires SNARE-complex proteins. Proc. Natl. Acad. Sci USA 93 (1996) 15169-15173.

66. R.J. Garippa, A. Johnson, J. Park, R.L. Petrush, T.E. McGraw. The carboxyl terminus of GLUT 4 contains contains a serine-leucine-leucine sequence that functions as a potent internalization motif in Chinese hamster ovary cells J. Biol Chem 271 (1996) 20660-20668.

67. H. Nishimura, M.J. Zarnowski, I.A. Simpson. Glucose transporter recycling in rat adipose cells. Effects of potassium depletion. J. Biol Chem 268 (1993) 19246-19253.

68. A.B. Muhlberg, D.E. Warnock, S.L. Schmid. Domain structure and intramolecular regulation of dynamin GTPase. EMBO J. 16 (1997) 6676-6683.

69. R. Urrutia, J.R. Henley, T. Cook, M.A. McNiven. The dynamins:redundant or distinct functions for an expanding family of related GTPases? Proc. Natl. Acad. Sci USA 94 (1997) 377- 384.

70. V. Baron, F. Alengrin, E. Van Obberghen. Dynamin associates with Src-Homology Collagen (Shc) and becomes tyrosine phosphorylated in response to insulin. Endocrinology 139 (1998) 3034-3037.

71 K.Hara, K. Yonezawa, H. Sakaue, A. Ando, et al. 1-Phosphatidylinositol 3-kinase activity is required for insulin stimulated glucose transport but not for RAS activation in CHO cells. Proc. Natl. Acad. Sci. USA 91 (1994) 7415-7419.

72. Y. Yang, P. Santamaria. Dissecting autoimmune diabetes through genetc manipulation of non-obese diabetic mice. Diabetologia 46 (2003) 1447-1464.

73. S.W. Christianson, L.D. Shultz, E.H. Leiter. Adoptive transfer of diabetes into immunodeficient NOD-scid/scid mice. Relative contributions of CD4+ and CD8+ T-cells from diabetic versus prediabetic NOD. Non-Thy-la donors. Diabetes 42 (1993) 44-55.

74. J.W. Yoon, C.S. Yoon, H.W. Lim et al.. Control of autoimmune diabetes in NOD mice by GAD expression or suppression in beta cells. Science 284 (1999) 1183-1187.

75. S. Bonner-Weir. Life and death of the pancreatic beta cells. Trends Endocrinol Metab II (2000) 375-378.

76. Y. Kido, J. Nakac, M.L. Hribal, S. Xuan, A Efstratiadis, D. Accili. Effects of mutations in the insulin-like growth factor signaling system on embryonic pancreas development and beta-cell compensation to insulin resistance. J. Biol Chem 277 (2002) 36740-36747.

77. L.M. 't Hart, A. Fritsche, I. Rietveld et al.. Genetic factors and insulin secretion: gene variants in the IGF genes. Diabetes 53 (2004) 526-530.

78. R.N. Kulkarni, M. Holzenberger, D.Q. Shih, U. Ozcan, M. Stoffel, M.A. Magnuson, C.R. Kahn. Beta-cells specific deletion of the IGF-1 receptor leads to hyperinsulinemia and glucose intolerance but does not alter beta-cell mass. Nat. Genet. 31 (2002) 111-115.

79. S. Xuan, T. Kitamura, J. Nakae, K. Politi, Y. Kido, P.E. Fisher et al. Defective insulin secretion in pancreatic beta cells lacking type 1 IGF receptor. J Clin Invest 110 (2002) 1011-1019.

80. R.A. De Fronzo. The triumvirate: β-cell, muscle, liver: a collusion responsible for NIDDM. Diabetes 37 (1988) 667-687.

81. D. Porter Jr. β-cells in type II diabetes mellitus. Diabetes 40 (1991) 166-180.

82. K.S. Polonsky, J. Sturis, G.I. Bell. Non-insulin-dependent diabetes mellitus: a genetically programmed failure of the β-cell to compensate for insulin resistance. N. Engl. J. Med. 334 (1996) 777-783.

83. T.J. Biden, D. Robinson, D. Cordery, W.E. Hughes, A.K. Beusch. Chronic effects of fatty acids on pancreatic β-cell function. New insights from functional genomics. Diabetes 53 (2004) 5159-5165.

84. G.C. Yaney, B.E. Corkey. Fatty acid metabolism and insulin secretion in pancreatic beta cells. Diabetologia 46 (2003) 1297-1312.

85. N. Abate. Insulin resistance and obesity. The role of fat distribution pattern. Diabetes Care 19 (1996) 292-294.

86. A.H. Kissebah, A.N. Peiris. Biology of regional body fat distribution: relationship to non- insulin-dependent diabetes mellitus. Diabetes Meta. Rev. 5 (1989) 83-109.

87. G. Paolisson, P.A. Tataranni, J.E. Foley, C. Bogardus, B.V. Howard, E. Ravussin. A high concentration of fasting plasma non-esterified fatty acids is a risk factor for the development of NIDDM. Diabetologia 38 (1995) 1213-1217.

88. M.A. Charles, E. Eschwege, N. Thibult, J.R. Claude, J.M. Warnet, G.E. Rosselin, J. Girard, B. Balkau. The role of non-esterified fatty acids in the deterioration of glucose tolerance in Caucasian subjects: results of the Paris prospective study. Diabetologia 40 (1997) 1101-1106.

89. M. Shimarbukuro, Y-T Zhou, M. Levi, R.H. Unger. Fatty acid-induced β-cell apoptosis: a link between obesity and diabetes. Proc. Natl. Acad. Sci. USA 95 (1998) 2498-2502.

90. K. Maedler, G.A. Spinas, D. Dyntar, W. Mortitz, N. Kaiser, M.Y. Donath. Distinct effects of saturated and monounsaturated fatty acids on β-cell turnover and function. Diabetes 50 (2001) 69-76.

91. V. Grill, A. Bjorklund. Dysfunctional insulin secretion in type 2 diabetes: role of metabolic abnormalities. Mol. Life Sci. 57 (2000) 429-440.

92. Y-P Zhou, V.E. Grill. Long term exposure of rat pancreatic islets to fatty acids inhibits glucose-induced insulin secretion and biosynthesis through a glucose fatty acid cycle. J. Clin. Invest. 93 (1994) 870-876.

93. P.C. Schmitz. Signaling aspects of insulin resistance in skeletal muscle: mechanisms induced by lipid over supply. Cell Signal 12 (2000) 583-594.

94. Y. Zick. Insulin resistance: a phosphorylation-based uncoupling of insulin signaling. Trends Cell Biol 11 (2001) 437-441.

95. E. Duplus, M. Glorian, C. Forest; Fatty acid regulation of gene transcription. J. Biol Chem 275 (2000) 30749-30752.

96. A.K. Busch, D. Cordery, G. Denyer, T.J. Biden Expression-profiling of palmitate.

97. J. Xiao, S. Gregersen, M. Kruhoffer, S.B. Pedersen, T.F. Orntoft, K. Hermansen. The effect of chronic exposure of fatty acids on gene expression in clonal insulin-producing cells studies using high density oligonucleotide microarray. Endocrinology 142 (2001) 4777-4784.

98. J. Svedberg, P. Bjorntorp, U. Smith, P. Connroth. Free-fatty acid inhibition of insulin binding, degradation and action in isolated rat hepatocytes. Diabetes 39 (1990) 570-574.

99. P.J. Randle, P.B. Garland, C.N. Hales, E.A. Newsholme. The glucose-fatty acid cycle. Its role in insulin sensitivity and the metabolic disturbances of diabetes mellitus. Lancet i (1963) 785-789.

100. J. Vague. The degree of masculine differentiation of obesities: a factor determining predisposition to diabetes, atherosclerosis, gout, and uric calculous disease. Am. J. Clin. Nutr. 4 (1956) 20.

101. A.J. Hartz, D.C. Rupley, R.D. Kalkhoff, A.A. Rimm. Relationship of obesity to diabetes:influence of obesity level and body fat distribution. Prev Med 12 (1983) 351.

102. A. Garcia-Patterson, L. Erdozain, G. Ginouart, J.M. Adelantado, J.M. Cubero, G. Gallo, A. deLeiva, R. Corcoy. Inhuman gestational diabetes mellitus congenital malformations are related to pre-pregnancy body mass index and to security of diabetes. Diabetologia 47 (2004) 509-514.

103. L. Wei-Dong, D. Chuanhui, L. Ding, Z. Hongyu, A.R. Price. An obesity-related locus in chromosome region 12q23-24. Diabetes 53 (2004) 812-820.

104. S. Kaisa et al.A large set of Finnish affected sibling pair families with type 2 diabetes suggests susceptibility loci on chromosomes 6, 11 and 14. Diabetes 53 (2004) 821-829.

105. R.A. De Fronzo. The triumvirate: β-cell, muscle, liver: a collusion responsible for NIDDM. Diabetes 37 (1988) 667-687.

106. D. Porte jr.. β-cells in type 2 diabetes mellitus. Diabetes 40 (1991) 166-180.

107. K.S. Polonsky, J. Sturis, G.I. Bell. Non-insulin-dependent diabetes mellitus:a genetically programmed failure of the β-cell to compensate for insulin resistance. N Engl J Med 334 (1996) 777-783.

108. L. Simonsen, O. Henriksen, L.H. Enevoldsen, J. Bülow. The effect of exercise on regional adipose tissue and splanchmic lipid metabolism in overweight type 2 diabetes subjects. Diabetologia 47 (2004) 652-659.

109. D. David, J.R. Daugaard, M.E. Young, A. Saha, D. Vavvas et al. Exercise diminishes the activity of acetyl-COA carboxylase in human muscle. Diabetes 49 (2000) 1295-1300.

110. D. Accili, A. Cama, F. Barbetti, H. Kadowaki, T. Kadowaki, S.I. Taylor. Insulin resistance due to mutations of the insulin receptor gene: an overview. JEndocrinol Invest. 15 (1992) 676-679.

111. G.W. Cline, K.F. Petersen, M. Krssak, J. Shen, R.S. Hundal, Z. Trajanoski, S. Inzucch A. Dresner, D.L. Rothman, G. Shulman. Impared glucose transport as a cause of decreased insulin-stimulated muscle glycogen synthesis in type 2 diabetes. N. Engl. J. Med. 341 (1999) 240-246.

112. M. Roden, T.B. Price, G. Perseghin, K.F. Petersen, D.L. Rothman, G.W. Cline, G.I. Shulman. Mechanism of free fatty-acid-induced insulin resistance in humans. J. Clin. Invest. 97 (1996) 2859-2868.

113. M.F. White. Perspective: the insulin signaling system – a common link in the pathogenesis of type 2 diabetes. Endocrinology 141 (2000) 1917-1921.

114. D.J. Withers, D.J. Burks, H.H. Towery, S.L. Altamuro, C.L. Flint, M.F. White. IRS-2 coordinates IGF-1 receptor-mediated β-cell development and peripheral insulin signaling. Nat. Genet. 23 (1999) 32-40.

115. E. Araki, M.A. Lipes, M.E. Patti, J.C. Bruning, B.L. Haag III, R.S. Johnson, C.R. Kahn. Alternative pathway of insulin signaling mice with targeted disruption of the IRS-1 gene. Nature 372 (1994)186-190.

116. H. Tamemoto, T. Kadowaki, K.Tobe et al. Insulin resistance and growth retardation in mice lacking insulin receptor substrate-1. Nature 372 (1994) 182-186.

117. D.J. Withers, J.S. Gutierrez, H. Towery, D.J. Burks et al. Disruption of IRS-2 causes type 2 diabetes in mice. Nature 391 (1998) 900-904.

118. J. Huang, T. Imamura, J.M. Olefsky. Insulin can regulate Glut 4 internalization by signaling to Rab5 and the motor protein dynein. PNAS 98 (2001) 13084-13089.

119. Q. Wang, R. Somwar, P.J. Bilan et al. Protein kinase Bl Akt participates in Glut-4 translocation by insulin in L6 myoblasts. Mol. Cell Biol 19 (1999) 4008-4018.

120. U. Smith, E. Carvalho, E. Mosialou, F. Beguinot, P. Formisano, C. Rondinone; PKB inhibition prevents the stimulatory effect of insulin on glucose transport and protein translocation but not the antilipolytic effect in rat adipocytes. Biochem Biophy Res Commun 268 (2000) 315-320.

121. E. Carvalho, B. Eliasson, C. Wesslau, U. Smith. Impaired phosphorylation and insulin-stimulated translocation to the plasma membrane of protein kinase Bl Akt in adipocytes from type II diabetic subjects. Diabetologia 43 (2000) 1107-1115.

100

122. G. Seidner, M.G. Alvarez, J.I. Yeh, et al. Glut-1 deficiency syndrome caused by haploinsufficiency of the blood brain barrier hexose carrier. Nat. Genet. 18 (1998) 188-191.

123. R. Santer, R. Schneppenhein, A. Dombrowski, H. Gotze, B. Steinmann, J. Schaub. Mutations in Glut-2, the gene for the liver type glucose transporter, in patients with Fanconi-Bickel syndrome. Nat. Genet. 17 (1997) 324-326.

124. M.L. Standaert, K. Yoshinori, M.P. Sajan, G. Bandyopadhyay, R.V. Farese. Cbl, IRS-1 and IRS-2 mediate effects of rosiglitazone on PI3 k, Pkc-λ and glucose transport in 3T3-L1 adipocytes. Endocrinology 143 (2002) 1705-1716.

125. D. Kramer, R. Shapiro, A. Adler, E. Bush, C.M. Rondinone. Insulin-sensitizing effect of rosiglitazone (BRL-49653) by regulation of glucose transporters in muscle and fat of Zucker rats. Metabolism 50 (2001) 1294-1300.

126. P.W. Young, D.R. Buckle, B.C.C. Cantello, H. Chapman, J.C. Clapham et al. Identification of high affinity binding sites for the insulin sensitizer rosiglitazone (BRL-49653) in rodent and human adipocytes using a radio iodinated ligand for peroxisomal proliferator-activated receptor γ. J. P. Et 284 (1998) 751-759.

127. R. Hernandez, T. Teruel, M. Lorenzo. Rosiglitazone produces insulin sensitization by increasing expression of the insulin receptor and its tyrosine kinase activity in brown adipocytes. Diabetologia 46 (2003) 1618-1628.

128. A. Miura, M.P. Sajan, M.L. Standaert, G. Bandyopadhyay, D.M. Franklin, R.L. Currie, R.V. Farese. Cbl PYXXM motifs activate the p85 subunit of phosphatidylinositol 3-kinase, Crk, atypical protein kinase C and glucose transport during thiazolidinedione action in 3T3-L1 and human adipocytes. Biochem 42 (2003) 14335-14341.

129. J. Rieusset, C. Chambrier, K. Bouzakri, E. Dusserre, J. Auwerx, J.P. Riou, M. Laville, H. Vidal The expression of the p85 subunit of phosphatidylinositol 3 kinase is induced by activation of the peroxisome proliferator-activated receptor λ in human adipocytes. Diabetologia 44 (2001) 544-554.

130. T. Albrektsen, K.S. Frederiksen, W.E. Holmes, E. Boel, K. Taylor, J. Fleckner. Novel genes regulated by the insulin sensitizer rosiglitazone during adipocyte differentiation. Diabetes 51 (2002) 1042-1051.

131. J.M. Lehmann, L.B. Moore, T.A. Oliver Smith, W.O. Wilkinson, T.M. Willson, S.A. Khewer. An antidiabetic thiazolidinedione is a high affinity ligand for peroxisome proliferator activated receptor gamma (PPARgamma). J. Biol Chem 270 (1995) 12963-12966.

132. P. Tontonez, E. Hu, R.A. Graves, A.I. Budavari, B.M. Spiegelman. mPPARgamma2: tissue-specific regulator of an adipocyte enhancer. Genes Dev 8 (1994) 1224-1234.

133. A. Vidal-Puig, R.V. Considine, M. Jimenez-Linan, A. Werman, W.J. Pories, J.F. Caro, J.S. Flier. Peroxisome proliferator-activated receptor gene expression in human tissues. Effects of obesity, weight loss, and regulation by insulin and glucocorticoids. J. Clin. Invest. 99 (1997) 2416-2422.

134. D. Auboeuf, J. Rieusset, L. Fajas, P. Vallier, V. Freering, J. Riou, J.P. Riou, B. Staels, J. Auwerx, M. Laville, H. Vidal. Tissue distribution and quantification of the expression of mRNAs of peroxisome proliferator-activated receptors and live X receptor-alpha in humans:no alteration in adipose tissue of obese and NIDDM patients. Diabetes 48 (1997) 1319-1327.

135. I. Barroso, M. Gurnell, V.E. Crowley, M. Agostini, J.W. Schwabe, M.A. Soos et al. Dominant negative mutations in human PPAR gamma associated with severe insulin resistance, diabetes mellitus and hypertension. Nature 402 (1999) 880-883.

136. J. Moitra, M.M. Mason, M. Olive, D. Krylov, O. Gavrilova, B. Marcus-Samuels, L. Feigenbaum, E. Lee, T. Aoyama, M. Eckhaus, M.L. Reitman, C. Vinson. Life without white fat: a transgenic mouse. Genes Dev. 12 (1998) 3168-3181.

137. I. Shimomura, R.E. Hammer, J.A. Richardson, S.I. Kemoto, Y. Bashmaka, J.L. Goldstein, M.S. Brown. Insulin resistance and diabetes mellitus in transgenic mice expressing nuclear SREBP-1c in adipose tissue: model for congenital generalized lipodystrophy. Genes Dev. 12 (1998) 3182-3194.

138. M.C. Cam, R.W. Brownsey, J.H. McNeil. Mechanism of vanadium action: insulin-mimetic or insulin-enhancing agent. Can. J. Physiol. Pharmacol 78 (2000) 829-847.

139. J.H. McNeil. Lack of in vivo effect of vanadium on Glut-4 translocation in white adipose tissue of streptozotocin-diabetic rats. Metabolism 23 (2001) 296-302

140. C.E. Heyliger, A.G. Tahiliani, J.H. McNeil; Effect of vanadate on elevated blood glucose and depressed cardiac performance of diabetic rats. Science (Washington, D.C.) 227 (1985) 1474-1477.

141. S.M. Brichard, W. Okitolonda, J.C. Henquin. Long term improvement of glucose homeostasis by vanadate in obese hyper insulinemic fa/fa rats. Endocrinology 125 (1988) 2048-2053.

142. G. Boden. Role of fatty acids in the pathogenesis of insulin resistance and NIDDM. Diabetes 46 (1997) 3-10.

143. M.R. Paquet, R.J. Romanek, R.J. Sargeant. Vanadate induces the recruitment of Glut-4 glucose transporter to the plasma membrane of rat adipocytes. Mol. Cell. Biochem 109 (1992) 149-155.

144. J.C. Molero, C. Martinez, A. Andres, J. Satrustegui, J.M. Carrascosa. Vanadate fully stimulates insulin receptor substrate-1 associated phosphatidylinositol 3-kinase activity in adipocytes from young and old rats. FEBS Lett 425 (1998) 298-304.

145. E. Tsiani, E. Bogdanovic, A. Sorisky, L. Nagy, I.G. Fantus. Tyrosine phosphatase inhibitors, vanadate and pervanadate, stimulate glucose transport and Glut translocation in muscle cells by a mechanism independent of phosphatidylinositol 3-kinase and protein kinase C. Diabetes 47 (1998) 1676-1686.

146. J. Wang, V.G. Yuen, J.H. McNeil. Effect of vanadium on insulin sensitivity and appetite. Metabolism 50 (2001) 667-673.

147. W. Mertz; Proc. 7th Inter. Cong. Nutr. 5 (1967) 689-692.

148. W. Mertz. Chromium occurrence and function in biological systems. Physiol. Rev. 49 (1969) 163-239.

149. W. Mertz. Human requirements basic and optimal. Ann. N.Y. Acad. Sci. 199 (1972) 191-201.

150. K. Schwarz, W. Mertz. Chromium (III) and the glucose tolerance factor. Arch. Biochem. Biophys. 85 (1959) 292.

151. R.A. Anderson. Chromium metabolism and its role in disease processes in man. Clin. Physiol. Biochem. 4 (1986) 31-41.

152. R.A. Anderson; In trace elements in health and disease. Norstedt Stockholm (1985) 110-119.

153. W. Mertz. Chromium in human nutrition: a review. J. Nutr. 123 (1993) 626-633.

154. R.A. Anderson, M.M. Polansky, N.A. Bryden, S.J. Bhathena, J.J. Canary. Effects patients with symptoms chromium on of supplemental of reactive hypoglycaemia. Metabolism 36 (1987) 351-355.

155. R. Riales, M.J. Albrink. Effect of chromium chloride supplementation on glucose tolerance and serum lipids including high-density lipoprotein of adult men. Am. J. Clin. Nutr. 34 (1981) 2670-2678.

156. M. Beran, R. Stahl, M. Beran jr.. Glycaemic activity of chromium (III)-β-nicotinamide adenine dinucleotide phosphate complex and its presence in yeast extracts. Analyst 120 (1995) 979-981.

157. K.L. Olin, D. Stearns, W. Armstrong, C.L. Keen, J. Trace. Elem Electrolytes Health Dis.11 (1994) 182.

158. R.A. Anderson, N.A. Bryden, M.M. Polansky, K. Gautschi; J. Trace Elem. Exper. Med. 9 (1996) 11 and references therein.

159. A. Yamamoto, O. Wada, T. Ono. Isolation of a biologically active low-molecular-mass chromium compound from rabbit liver. Eur. J. Biochem 165 (1987) 627-631.

160. A. Yamamoto, O. Wada, H. Suzuki. Purification and properties of biologically active chromium complex from bovine colostrums. J. Nutr. 118 (1988) 39-45.

161. C.M. Davis, H.K. Sumrall, J.B. Vincent. A biologically active form of chromium may activate a membrane phosphotyrosine phosphatase (PTP). Biochemistry 35 (1996) 12963-12969.

162. K. Schwarz and W. Mertz. A glucose tolerance factor and its differentiation from factor 3. Arch. Biochem. Biophys. 72 (1957) 515.

163. N. Mirsky, A. Weiss, Z. Dori. Chromium in biological systems I. Some observations on glucose on glucose tolerance factor in yeast. J. Inorg. Biochem. 13 (1980) 11-21.

164. H.E. Morgan, C.F. Whitfield. Curr. Top. Membr. Transp. 4 (1973) 255.

165. G.A. Bray. Anal. Biochem 1 (1960) 279.

166. J.M. Olefsky. Mechanisms of the ability of insulin to activate the glucose-transport system in rat adipocytes. Biochem. J. 172 (1977) 137.

167. E.S. Holdsworth, G. Appleby; Assays of glucose tolerance factor and its mode of action, studied with brewer's yeats. J. Inorg. Biochem. 21 (1984) 31-44.

168. S.J. Haylock, P.D. Buckley, L.F. Blackwell; Separation of biologically active chromium containing complexes from yeast extracts and other sources of glucose tolerance factor (GTF) activity. J. Inog. Biochem 18 (1983) 195-211.

169. N. Mirsky, A. Weiss, Z. Dori. The effect of glucose tolerance factor in glucose uptake by yeast cells. J. Inorg. Biochem 15 (1981) 275.

170. K.F. Kingry, A.C. Royer, J.B. Vincent; Nuclear magnetic resonance studies of chromium (III) pyridinecarboxylate complexes. J. Inorg. Biochem 72 (1998) 79-88.

171. E.M. O'Donoghue, J.A. Cooper, T.G. Jackson, P.R. Shepherd, P.D. Buckley, L.F. Blackwell; Identification of peptides from autolysates of saccharomyces cerevisiae that exhibit glucose tolerance factor activity in a yeast assay. Int. J. Biochem 22 (1990) 841-846.

172. C.M. Davis, J.B. Vincent. Chromium oligopeptide activates insulin receptor by tyrosine kinase activity. Biochem 36 (1997) 4382-4385.

173. C.M. Davis, J.B. Vincent. Isolation and characterization of a biologically active chromium oligopeptide from bovine liver. Arch. Biochem. Biophys. 339 (1997) 335-343.

174. V.G. Zetic, V.S. Tomas, S. Grba, L. Lutilsky, D. Kozlek. Chromium uptake by saccharomyces cerevisiae and isolation of glucose tolerance factor from yeast biomass. J. Biosci 26 (2001) 217-223.

175. A. Knochel, M. Mayer. Dipeptide complexes of chromium (III) models of the glucose tolerance factor (GTF). Abstracts 440.

176. S.J. Haylock, P.D. Buckley, L.F. Blackwell. The relationship of chromium to the glucose tolerance factor II. J. Inorg. Biochem. 19 (1983) 105-117.

177. N. Mirsky, A. Weiss, Z. Dori. Chromium in biological systems I. Some observations on glucose on glucose tolerance factor in yeast. J. Inorg. Biochem. 13 (1980) 11-21.

178. D.L. Hwang, A. Lev-Ran, T. Papoain, W.K. Beech. Insulin-like activity of chromium-binding fractions from brewers yeast. J. Inorg. Biochem. 30 (1987) 219-225.

179. D.M. Davies, E.S. Holdsworth, J.L. Sherriff; The isolation of glucose tolerance factors from brewers yeast and their relationship to chromium. Biochem. Med. 33 (1985) 29. 311.

180. D.D. Held, E. Gonzalez-Vergara, H.M. Goff. Isolation of a non-chromium insulin enhancing factor from brewers yeast. Fedn. Proc. 43 (1984) 472.

181. K. Barseghian. Glucose tolerance factor and method of making same. International Patent Application A61k 31/47 BOID 15/04, 15/08 (Publication No. WO 87/03200).

182. E.W. Toepfer, W. Mertz, M.M Polansky, E.E. Roginski, W.R. Wolff. Preparations of chromium-containing material of glucose tolerance factor activity from brewer's yeast extracts and by synthesis. J. Agric. Food Chem. 25 (1977) 162.

183. N. Mirsky. Glucose tolerance factor reduces blood glucose and free fatty acids levels in diabetic rats. J. Inorg. Biochem. 49 (1993) 123-128.

184. J.B. Vincent. Relationship between glucose tolerance factor and low-molecular-wieght chromium-binding substance. J. Nutr. 124 (1994) 117-118.

185. T. Okada, Y. Kawano, T. Sakakibara, O. Hazeki, M. Ui. Essential role of phosphatidylinositol 3-kinase in insulin-induced glucose transport and antilipolysis in rat adipocytes. J. Biol. Chem. 269 (1994) 3568-3573.

186. C.R. Kahn. Banting lecture, insulin action, diabetogenes and the cause of type 2 diabetes, Diabetes. 43 (1994) 1066-1084.

187. B.C. Reed, S.H. Kaufman, J.C. Mackall, and M.D. Lane. Alterations of insulin binding accompanying differentiation of 3T3-L1 adipocytes. Proc. Natl. Acad. Sci. USA. 74 (1977) 4876-4880.

188. O.H. Lowry, N.J. Rosebrough, A.L. Farr, R.J. Randall. Protein measurement with the folin phenol reagent. J. Biol. Chem. 193 (1951) 265-275.

189. C. Cammalleri, R.J. Germinario. The effects of protease inhibitors on basal and insulin-stimulated lipid metabolism insulin binding and signaling. J. Lipid Res. 44 (2003) 103-108.

190. B.B. Hoffman, H. Chang, Z.T. Farahbakhsh, G.M. Reaven. Inhibition of lipolysis by adenosine is potentiated with age. J. Clin. Invest. 74 (1984) 1750-1755.

191. A. Noma, H. Okabe, M. Kita. A new colorimetric micro-determination of free fatty acids in serum. Clin. Chem. Acta. 43 (1973) 317-320.

192. Cory, A.H. et al.Use of an aqueous soluble tetrazolium/formazan assay for cell growth assays in culture.Cancer Commun. 1991 Jul;3 (7):207-12

193 I. Berdicevsky, N. Mirsky, Effects of insulin and glucose tolerance factor (GTF) on growth of Saccharomyces cerevisiae. Mycoses.37 (1994) 405-410

194. MF. McCarty, the Therapeutic Potential of Glucose Tolerance Factor. Medical Hypotheses: 6 (1980) 1177-1189.