Cellular and Molecular Mechanisms Underlying the Effect of *Tinospora crispa* (Miers ex Hook. f. & Thom) on Glucose Uptake

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Thesis Title	Cellular and Molecular Mechanisms Underlying the Effect of Tinospora crispa (Miers ex Hook. f. & Thom) on Glucose Uptake
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ชื่อวิทยานิพนธ์ กลไกการออกฤทธิ์ระดับเซลล์และโมเลกุลในการพากลูโคสเข้าสู่เซลล์ของสารสกัดจากบอระเพ็ด

ผู้เขียน นางสาวกุสุมาลย์ น้อยผา

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บทคัดย่อ

บอระเพ็ด (*Tinospora crispa*) มีสรรพคุณตามแพทย์แผนไทยในการรักษาโรคเบาหวาน และมีรายงานการศึกษาบ้างในสัตว์ทดลอง แต่รายงานการศึกษาด้านกลไกยังมีน้อยมาก ดังนั้น ในการศึกษาวิจัยครั้งนี้มีวัตถุประสงค์เพื่อทดสอบการออกฤทธิ์ลดน้ำตาลของสมุนไพรบอระเพ็ดโดย ใช้เซลล์กล้ามเนื้อลายของหนู (L6 myotubes) ที่เพาะเลี้ยงในห้องปฏิบัติการเป็นแบบจำลอง ผลการศึกษาพบว่าสารสกัดน้ำจากบอระเพ็ด (TCc) ที่ความเข้มข้น 4 mg/ml สามารถเพิ่มการพาน้ำตาลกลูโคสเข้าสู่เซลล์กล้ามเนื้อลายของหนูได้ ต่อมาได้ใช้ตัวทำละลายที่เหมาะสมในการแยกสารสกัดกึ่งบริสุทธิ์จากสารสกัดส่วนน้ำของบอระเพ็ด ร่วมกับการทดสอบสรรพคุณในการออกฤทธิ์ พบว่าหนึ่งในสารสกัดกึ่งบริสุทธิ์ (TCf) นี้สามารถออกฤทธิ์พากลูโคสเข้าสู่เซลล์กล้ามเนื้อได้อย่างมีนัยสำคัญทางสถิติด้วยปริมาณเข้มข้นน้อยกว่าเดิม 10 เท่า (0.4 mg/ml) การศึกษากลไกการออกฤทธิ์ในระดับเซลล์และโมเลกุล พบว่าสารสกัดบอระเพ็ดกระตุ้นการเพิ่มปริมาณของตัวพากลูโคสชนิด GLUT1 และ GLUT4 และกระตุ้นการทำงานของเอ็นไซม์ Extracellular Signal-Regulated Kinases1/2 (ERK1/2) ได้ นอกจากนี้ในการศึกษาครั้งนี้ ยังพบว่า ตัวยับยั้งการสร้างโปรตีนของเซลล์ (protein synthesis inhibitor, cycloheximide) และตัวยับยั้งการทำงานของเอ็นไซม์ MEK1/2 (PD98059) สามารถยับยั้งการออกฤทธิ์ของสารสกัดในการพากลูโคสเข้าสู่เซลล์กล้ามเนื้อได้ โดยสรุป สมุนไพรบอระเพ็ดมีสรรพคุณในการลดระดับน้ำตาลในเลือดได้ โดยการออกฤทธิ์พาน้ำตาลเข้าสู่เซลล์กล้ามเนื้อโดยอาศัยการเพิ่มปริมาณโปรตีน และ mRNA ของตัวพากลูโคสชนิด GLUT1

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ABSTRACT

Tinospora crispa (TC) has been used for years as diabetic remedy in Thai-traditional medicine. However its biological activity study has received little attention in literatures up to date. Using an in vitro model of cultured L6 myotubes, a significant enhancement of glucose uptake and glucose transporter protein (GLUT1 and GLUT4) expression by the crude aqueous extract of TC (TCc at 4 mg/ml) have been demonstrated. On a further investigation using bioassay-guided fractionation approach, a similar biological activity was reproduced by one potent fraction of TC (TCf) at 10-fold reduction in dosage (0.4 mg/ml). The up-regulation of GLUT1 protein and glucose transport was accompanied by a significant increased in GLUT1 mRNA expression and phosphorylation of the extracellular signal regulated kinase (ERK1/2). In addition, the enhancement of glucose transport was blocked by specific kinase inhibitors, MEK1/2 (PD98059) and protein synthesis inhibitor (cycloheximide). In conclusion, the enhancement of glucose transport by *T. crispa* in L6 myotubes is mediated by the up-regulation of GLUT1 protein and mRNA expression via both insulin and non-insulin signaling pathways.

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LIST OF ABBREVIATION AND SYMBOLS

Ab	=	antibody
APS	=	ammonium persulfate
ATP	=	adenosine triphosphate
bp	=	base pair (s)
BSA	=	bovine serum albumin
°C	=	degree Celsius
cm	=	entimeter
dATP	=	deoxyadenosine-5'-triphosphate
dCTP	=	deoxycytidine-5'-triphosphate
dGTP	=	deoxyguanosine-5'-triphosphate
dTTP	=	deoxythymidine-5'-triphosphate
dNTPs	=	dATP+dCTP+dGTP+dTTP
DMSO	=	dimethylsulfoxide
DNA	=	deoxyribonucleic acid
DTT	=	dithiothreital
EDTA	=	ethylenediaminetetraacetic acid
et al.	=	et ali (Latin) and others
EtBr	=	ethedium bromide
FBS	=	fetal bovine serum
g	=	gram
h	=	hour
i.e	=	id. Est, for example
IgG	=	Immunoglobulin
KDa	=	kilo Dalton (s)
M	=	Molar
mA	=	miliampare
mg	=	milligram
min	=	minute
ml	=	milliliter
mM	=	milimolar
mm	=	millimeter

LIST OF ABBREVIATION AND SYMBOLS (CONTINUTES)

MMLV	=	Moloney Murine Leukemia Virus Reverse Transcriptase
mol	=	mole
mRNA	=	messenger RNA
MW	=	molecular weight
nM	=	nanomolar
nm	=	nanometer
nmole	=	nanomole
nt	=	nucleotide (s)
OD	=	optical density
p	=	p value
PBS	=	phosphate buffer saline
PCR	=	polymerase chain reaction
PAGE	=	polyacrylamide gel electrophoresis
rpm	=	revolution per minute
RT-PCR	=	reverse trancripstase-polymerase chain reaction
sec	=	second
SDS	=	sodium dodecyl sulfate
TAE	=	Tris-acetate, EDTA
Taq	=	thermos aquaticus
TBE	=	Tris-borate, EDTA
TBS	=	Tris-buffed saline
TBS-T	=	Tris-buffed saline Tween20
TEMED	=	N,N,N',N'-tetramethylethylenediamine
Tris-HCl	=	Tris-(hydroxymethyl)-aminoethan hydrochloride acid
µg	=	microgram
µl	=	microliter
µM	=	micromolar
U	=	unit (s)
UV	=	ultraviolet

LIST OF ABBREVIATION AND SYMBOLS (CONTINUTES)

V	=	volt
v/v	=	volume per volume
w/v	=	weight per volume
α	=	alpha
β	=	beta
γ	=	gramma
λ	=	lamda
δ	=	delta

CHAPTER 1

INTRODUCTION

1.1 Background and significance of study

Diabetes mellitus is a heterogeneous group of disorders, characterized by the disturbance of glucose metabolism and raised plasma glucose concentration. The World Health Organization (The expert committee on the diagnosis and classification of diabetes mellitus, WHO 2002) has prepared a number of classification schemes of diabetes mellitus; nowadays the following is mostly accepted: insulin-dependent diabetes mellitus (IDDM, type 1 diabetes), non-insulin-dependent diabetes mellitus (NIDDM, type 2 diabetes), gestational diabetes mellitus (GDM) and other types.

The *type 2 diabetes* is more common than *type 1 diabetes* and comprises approximately 90% of all individuals with diabetes. It is also becoming increasingly common in younger people and outnumbers *type 1 diabetes*, even in the very young. It also seems clear that the rising prevalence rates mirror changes in lifestyle, notably nutrition, physical inactivity and urbanization. According to WHO, in 2006 it was estimated that, globally, there were already 246 million people affected with diabetes. By the year 2025, 380 million people will suffer from this disease (International Diabetes Federation, 2006).

Oral hypoglycemic drugs and dietary manipulation represent the biggest roles in therapy; insulin is sometimes required to correct hyperglycemia. However, do we have an alternative way to treat diabetes such as natural remedies using herbal and medicinal plants. According to the world ethno-botanical information, there are about 1,000 plants used in the control of diabetes mellitus (Alarcon-Aguilara et al., 1998). In the developing world where conventional medicines are not readily available, traditional treatments for diabetes remain the major form of therapy. However, only a few of these plants used in traditional remedies have undergone comprehensive scientific investigation, and the WHO has recommended that research focus should now be directed in this area. Examples of traditional plant treatments for diabetes (Grover et al, 2002) are from i) fruits such as white mulberry (*Morus alba*), black berry (*Eugenia jambolana*), pomegranate (*Punica granatum*) and banana (*Musa sapientum*); ii) vegetables like onion (*Allium cepa*), garlic (*Allium sativum*), holy basil (*Ocimum sanctum*) and ivy guard (*Coccinia indica)*; iii)

herbs like shoe flower (*Hibiscus rosa sinensis*), Fenugreek (*Trigonella foenum-graecum*) and madagasca periwinkle (*Vinca rosea*), ect.

The decoction of *Tinospora crispa* (*T. crispa*, TC) stem has been used traditionally to relieve diabetes sufferers in Thailand for a long time. Nevertheless, like many other herbals, there have been few scientific reports on the antiglycemic effects of *T. crispa*. One early study on *T. crispa* reported that a water extract of *T. crispa* significantly lowered the blood glucose levels and increased plasma insulin levels in alloxan-induced moderately diabetic rats (Noor and Ashcroft, 1989). In later studies, Noor and co-workers were able to show a reduction in blood glucose level in moderately diabetic rats using a water extract of *T. crispa*, and later demonstrated that the hypoglycemic effect was probably due to the insulinotropic activity of the extract (Noor et al, 1989 and Noor and Ashcroft, 1998). Interestingly, a reduction in blood glucose levels due to the peripheral utilization of blood glucose, especially in the muscle tissues, has never been demonstrated.

In our current study, we decided to investigate for further scientific research on its anti-diabetic activity. Using a strategic approach based on the knowledge of our ancient wisdom, the aqueous extract from the dried stem of *T. crispa* was prepared. The crude extract of *T. crispa* was initially evaluated for its effect on glucose transport using a rat muscle cell-line, the L6 myotubes and further investigated its downstream regulatory mechanisms via the insulin signaling glucose transport pathways. The biological activity studies include: i) the measurement of H^3-2D-Glucose uptake in L6 myotubes at time and dose dependent effects of *T. crispa* in the absence and presence of specific inhibitors of insulin signaling glucose transport pathways; ii) the western blot analysis of glucose transporter 1 and 4 (GLUT1 and GLUT4) proteins; and iii) the activation of GLUT1 and GLUT4 genes expression using mRNA analysis. All experiments are carried out in triplicates with a minimum of 3 independent assays. The in vitro studies involve the various batches of crude extract and separated fraction concentrates. The bioactivity of certain fractions and sub-fractions are performed throughout the studies in order to eventually isolate the active compounds and determine its chemical structures.

The first beneficial outcomes of this study secured the scientific evidence of support on the antidiabetic activity of *T. crispa* with understanding on the mode of its action on the peripheral muscle tissues. Second, we obtained the preclinical information for the further study of *T. crispa* as a potential drug therapy in patients with *type 2 diabetes*.

Finally, this study established the in-vitro bio-assay model for future studies of antiglycemic activity of medicinal plants and natural products.

1.2 Literature review

1.2.1 Glucose Metabolism and Homeostasis

1.2.1.1 Dietary sugar, digestion and absorption process

Dietary sugar: simple sugars versus complex carbohydrates

The term carbohydrates includes monosaccharides and disaccharides, which are sometimes called simple sugars and polysaccharides which are sometimes called complex carbohydrates or starch and fiber

The term monosaccharide means one sugar molecule. Foods contain three common monosaccharides; glucose, fructose and galactose.

The term disaccharide means two sugar molecules. Two monosaccharides combine to form a disaccharide. Three important disaccharides are maltose (glucose+glucose), sucrose (glucose+fructose) and lactose (glucose+galactose).

The term polysaccharide means many sugar molecules. Dietary polysaccharides contain hundreds of sugar molecules and include complex carbohydrates or starch and fiber.

Simple sugars and complex carbohydrates or starches occur naturally in many foods that also supply other nutrients including milk, fruits, vegetables, breads, cereals and grains. Sugars also are added to foods during processing and preparation. Most sugars found naturally in foods or added to foods are disaccharides or two sugar molecules. The body can not tell the difference between natural sugars and added sugars because they have the same chemical structure.

Our bodies can only absorb monosaccharides or single sugar molecules. During digestion enzymes break down disaccharides into two monosaccharides which can be absorbed by the body. Digestive enzymes also break down complex carbohydrates or starches which contain hundreds of sugar molecules into monosaccharides or single sugar

molecules for absorption. Certainly the body can not tell the difference between monosaccharides that come from the break down of simple sugars or from a complex carbohydrate.

Digestion process

Polysaccharides and disaccharides must be digested to monosaccharides prior to absorption and the key players in these processes are the brush border hydrolases, which include maltase, lactase and sucrase. Dietary lactose and sucrose are ready for digestion by their respective brush border enzymes. Starch, as discussed previously, is first digested to maltose by amylase in pancreatic secretions and saliva in some species.

Dietary lactose, sucrose and maltose derived from digestion of starch diffuse in the small intestinal lumen and come in contact with the surface of absorptive epithelial cells covering the villi where they engage with brush border hydrolases; maltase cleaves maltose into two molecules of glucose, lactase cleaves lactose into a glucose and a galactose, and sucrase cleaves sucrose into a glucose and a fructose.

At long last, we are ready to actually absorb these monosaccharides. Glucose and galactose are taken into the enterocyte by co-transport with sodium using the same transporter. Fructose enters the cell from the intestinal lumen via facilitated diffusion through another transporter.

Absorption of glucose: Transport across the intestinal epithelium

Absorption of glucose, or any molecules for that matter, entails transport from the intestinal lumen, across the epithelium and into the blood stream (Fig 1.). The transporter that carries glucose and galactose into the enterocyte is the sodium-dependent glucose transporter, known formally as SGLUT1 (Wright, 1993). As the name indicates, this molecule transports both glucose and sodium into the cell and in fact, will not transport either alone. Fructose may cross the luminal membrane via the facilitated transporter GLUT5 (Kayano, 1990). This transporter is responsible for the entry of fructose into the intestinal enterocytes (Burant, 1992), but it does not transport glucose or galactose.

Once inside the enterocyte, glucose or galactose and fructose must be exported from the cell into the blood stream. Glucose is transported out of the enterocyte

through a different transporter (called GLUT2) in the basolateral membrane as shown in Figure 1. The GLUT2 transporter serves as the major route of glucose exit from the cell as well as entry of glucose from the blood stream into enterocytes (Thorens, 1993). Glucose then diffuses down its concentration gradient into capillary blood within the villus. Fructose also crosses the basolateral membrane via the activity of GLUT2 (Cheeseman, 1993).

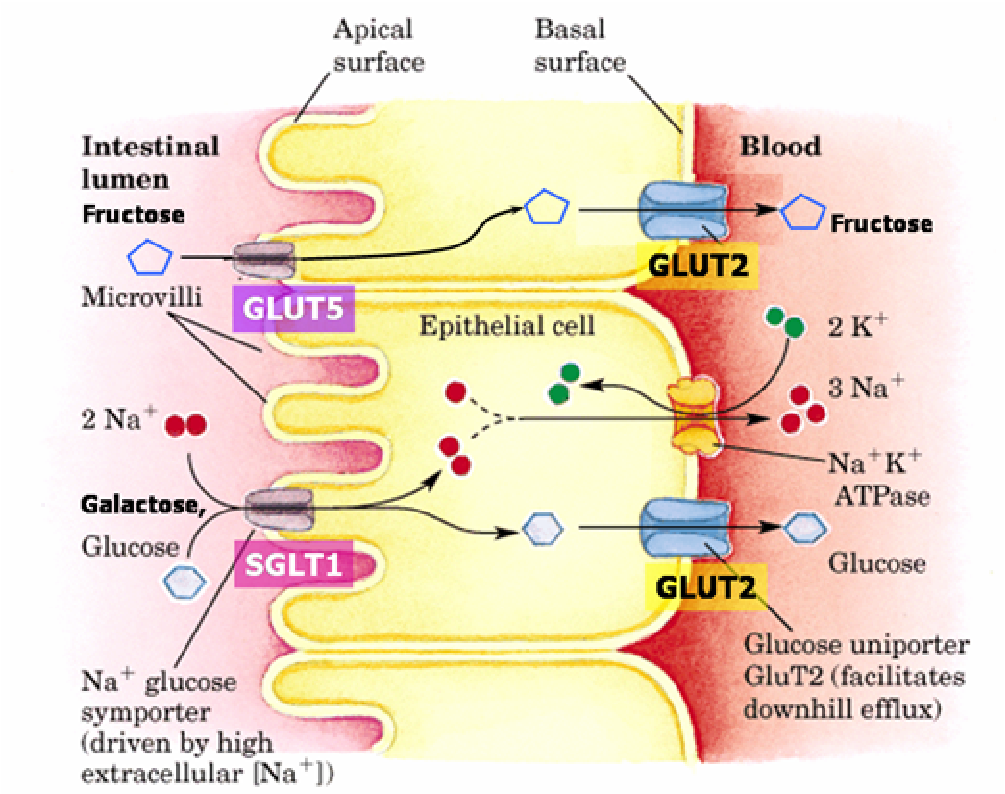

Figure 1. Glucose transport in enterocytes. The Na^+-dependent glucose transporter (SGLT1) is located in the apical membrane and two sodium molecules transports ($2Na^+$) coupled with glucose or galactose and water. This transport is driven by the inwardly directed Na^+ gradient, which is maintained by the activity of Na^+/K^+ ATPase in the basolateral membrane. The facilitative GLUT2 transporter transports sugars across the basolateral membrane, whereas the facilitative GLUT5 transporter transports fructose across the apical membrane (adapted from Wright, 1993)

1.2.1.2 Glucose metabolism

Normally an individual oscillates between the high insulin/ low-glucagon state and the low-insulin/high glucagon state. The high-insulin/low-glucagon state occurs during the ingestion of food and for several hours after a meal (absorptive, postprandial or fed state). The low insulin/ high-glucagon state occurs during fasting. The early period of fasting, lasting between 6 and 12 h after meal, is called the postabsorptive state. Beyond that, it is refer to either as "prolonged fasting"or "starvation".

The postprandial (absorptive) phase

The macronutrients in a standard meal of 90 g of carbohydrate, 30 g of protein and 20 g of fat will be utilized by the body in a typical manner. The absorption of dietary carbohydrate causes a rise in circulating glucose level (up to 15 mM) which together with other signals trigger release of insulin (Stumpel and jungerman, 1997). The liver takes up glucose and perhaps as much as 20 g of the glucose in a meal is then deposited as glycogen (Figure 2). The remaining glucose (70 g) passes through the liver, causing peripheral plasma glucose levels to 6 to 7 mM. Over the next 2 h, the brain will take up approximately 15-20 g of glucose to be oxidized directly as fuel. Most of the glucose, however, will be taken up in an insulin-dependent manner (facilitative transporter GLUT4) by skeletal muscle (45 g) and adipose tissue (2 g). In skeletal muscle, approximately the other half will be oxidized and half stored as glycogen. In adipose tissue, most will be used for synthesis of glycerol 3-phosphate for triacylglycerol synthesis and a small quantity may be metabolized as to lactate. During the absorptive phase, there is evidence that the net production of lactate by the liver and other tissues provides an important fuel for the kidney, heart, and possibly the colon.

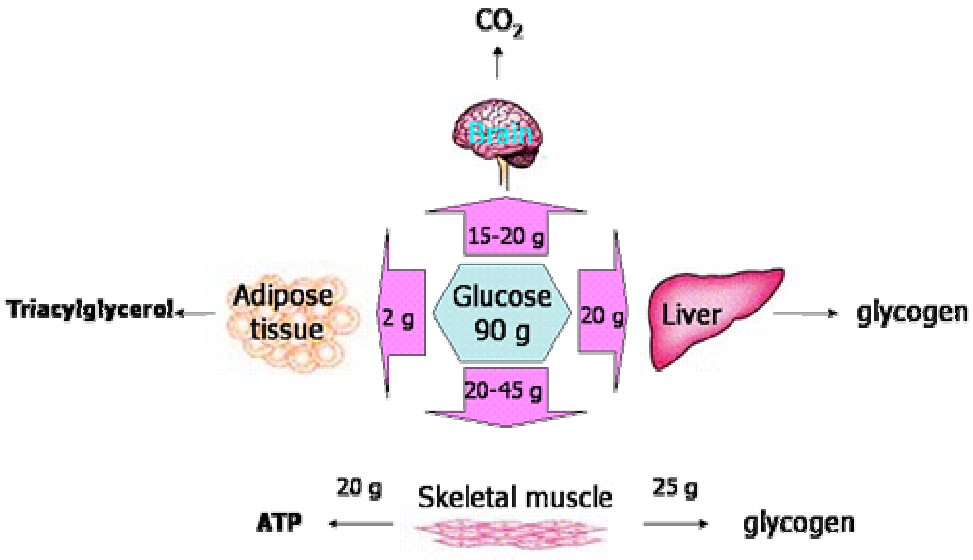

Figure 2 . Fate of dietary carbohydrate (glucose) from one meal during the absorptive phase (~2h). From Watford M, Goodridge AG. 2000

Postabsorptive phase

By convention, the basal or post-absorptive state is defined as the metabolic condition that prevails in the morning after an overnight (10-14 hours) fast. Maintenance of the fasting plasma glucose concentration is primarily the responsibility of the liver (DeFronzo RA and Ferrannini E, 1987). The liver provides glucose for all tissues of the body either by breaking down its own stores of glycogen or by synthesizing glucose from gluconeogenic precursors, of which more important ones are lactate, pyruvate, and glycerol, alanine, and other gluconeogenic amino acids. The central role of the liver in providing a constant supply of glucose to the body is related to the present of glucose-6-phosphatase, which catalyses the conversion of glucose-6-phosphate to glucose within the hepatocytes. Although a number of tissues, including muscle and adipocytes, possess the enzymatic machinery necessary to degrade glycogen and to synthesize glucose-6-phosphate from lactate, and amino acid, they either completely lack or possess too little of a key enzyme, glucose-6-phosphatase, to release significant amounts of free glucose into the circulatory system

In the fasting or post-absorptive state, the fasting plasma glucose concentration in a healthy adult is maintained within a very narrow range, 65 to 105

mg/dL (3.6-5.8 mM). Under basal conditions, insulin-independent tissue, the brain (50-60%) and splanchnic organs (20- 25%)- account for the majority of total body glucose utilisation. Muscle, an insulin-dependent tissue, is responsible for most of the remaining 20-25% of glucose disposal within the fasting state (DeFronzo RA et al, 1988 and 1992). The basal rate of tissue glucose uptake is precisely equaled by an equivalent rate of glucose output by the liver. After the ingestion or infusion of glucose, this delicate balance between hepatic glucose production and tissue glucose utilization is disrupted and the maintenance of normal glucose homeostasis in the fed state depends upon three processes that occur simultaneously in a coordinated, and tightly integrated fashion: (1) in response to hyperglycemia, insulin secretion is stimulated; (2) the combination of hyperinsulinemia and hyperglycemia augments glucose uptake by splanchnic (liver and gut) and peripheral (primarily muscle) tissues; (3) both insulin and glucose suppress hepatic glucose production

1.2.1.3 Glucose Homeostasis in *Type 2 Diabetes*

Classification of diabetes

Diabetes is a metabolic disorder characterized by resistance to the action of insulin, insufficient insulin secretion, or both (The expert committee on the diagnosis and classification of diabetes mellitus, WHO 2002). The major clinical manifestation of the diabetic state is hyperglycemia. However, insulin deficiency and/or insulin resistance are also associated with disturbances in lipid and protein metabolism. The vast majority of diabetic patients are classified into one of the two broad categories: type 1diabetes, which is caused by an absolute deficiency of insulin, and type 2 diabetes, which is characterized by the presence of insulin resistance with an inadequate compensatory increase in insulin secretion. In addition, women who develop diabetes during their pregnancy are classified as having gestational diabetes. Finally, there are a variety of uncommon and diverse types of diabetes which are caused by infections, drugs, endocrinopathies, pancreatic destruction, and genetic defects. These unrelated forms of diabetes are classified separately.

Type 2 Diabetes

Type 2 Diabetes (T2DM) is characterized by abnormal glucose homeostasis leading to hyperglycemia, and its pathogenesis appears to involve complex interactions between genetic and environmental factors. It is believed by many that the primary defect in T2DM is represented by insulin resistance, which is already present at the very early stage of the prediabetic state. While initially the beta cell is able to compensate for this resistance, overt diabetes occurs when the beta cells become exhausted. It should be noted, however, that an alternative hypothesis proposes that the primary defect in T2DM is due to mild dysregulation in insulin secretory mechanisms that leads to overt diabetes following the secondary superimposition of insulin resistance (Kahn, 2003).

Irrespective of whether the initial primary defect is due to insulin resistance of beta cell dysfunction, it is universally appreciated that T2DM is a complex and heterogeneous polygenic disease whose exact genetic causes continue to remain elusive. This complexity is underscored by the realization that in addition to peripheral tissue resistance to insulin action and a failure of the beta cell to overcome this resistance, T2DM is characterized by a variable inability of the liver to properly suppress hepatic glucose release and by adipose tissue-derived hormones and cytokines that antagonize insulin action (Figure 3).

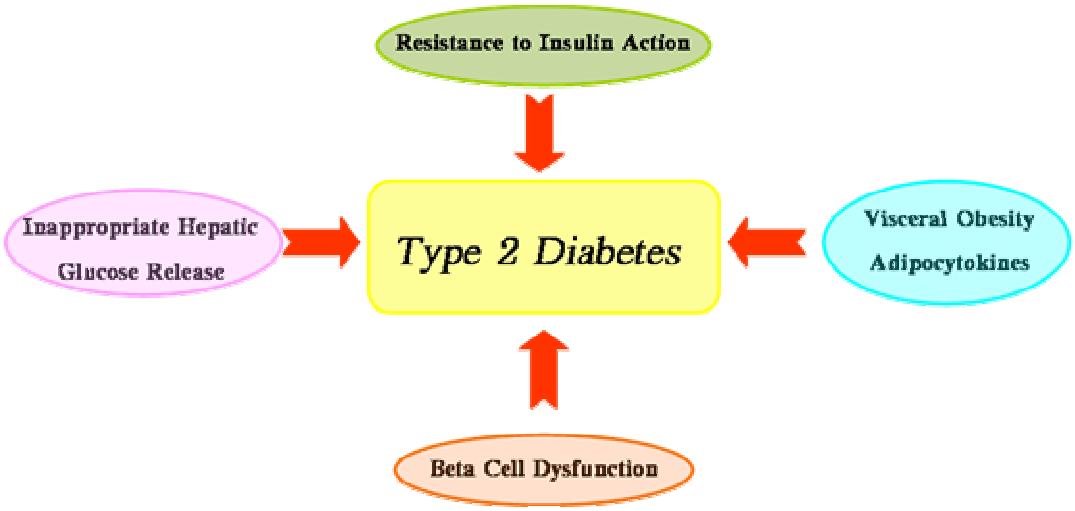

Figure 3. Pathophysiology of *Type 2 Diabetes*. *Type 2 Diabetes* is a multi-gene disorder with four major areas of dysfunction. The hallmark of this disease that has long been recognized is peripheral resistance to insulin action. This resistance occurs in a setting of

variable levels of beta cell dysfunction, which leads to an inadequate capacity to release insulin in response to the degree of resistance, in conjunction with inappropriate hepatic glucose release in the setting of hyperglycemia. A major contributor to this resistance is visceral obesity and the production of adipocytokines that antagonize insulin action.

Glucose Homeostasis in *Type 2 Diabetes*

Whole body glucose homeostasis depends upon a delicate balance between hepatic glucose output and glucose utilization by insulin-dependent tissues (adipocytes and skeletal muscle) and insulin-independent tissues (brain and splanchnic organs) (Wallberg-Henriksson, 1987, DeFronzo et al, 1992). Skeletal muscle accounts for at least 80% of glucose disposal during glucose and insulin infusion, (DeFronzo et al, 1992) whereas adipose tissue accounts for much less (Bjorntorp et al 1971, Bjorntorp and Sjostrom, 1978). Thus, skeletal muscle is quantitatively the most important tissue involved in maintaining glucose homeostasis under insulin-stimulated conditions (DeFronzo et al, 1992).

The pathophysiology of *Type 2 diabetes* involves impairments in both insulin action and insulin secretion (DeFronzo 1988, Kahn 1994, Porte 1991). Insulin sensitivity is determined by the ability of insulin to promote glucose uptake and utilization. Thus, in insulin-resistant conditions, there is decreased glucose clearance in response to insulin. Insulin regulates glucose homeostasis primarily through suppression of hepatic glucose production and stimulation of peripheral (and to a lesser degree, splanchnic) glucose uptake (Weyer et al, 1999). Clinical studies in man have demonstrated the impaired ability of insulin to promote glucose clearance in type 2 diabetic subjects assessed after oral glucose administration (Ludvik et al, 1997), during euglycemic hyperinsulinemic clamp studies (DeFronzo et al, 1985), or by nuclear magnetic resonance spectrometry (Shulman 2000). The most conclusive evidence for defective insulin sensitivity in T2DM comes from euglycemic hyperinsulinemic clamp studies, in which total body glucose clearance is shown to be reduced in type 2 diabetic subjects compared with age and weight-matched controls (DeFronzo et al, 1985). Furthermore, in vivo human studies suggest that the primary site of reduced insulin-mediated glucose uptake is located in the peripheral (muscle) tissue (DeFronzo et al, 1985, Baron et al, 1991). Decreased insulin-mediated glucose clearance seen in T2DM has also been demonstrated in humans at

risk for development of diabetes, including persons with obesity, hypertension, hyperlipidemia, or a strong family history of disease (Reaven et al, 1991, Martin et al, 1992). Thus, extensive research on the development of T2DM has been focused on cellular and molecular processes of insulin signaling (Saltiel and Kahn, 2001, Virkamaki et al, 1999).

1.2.2 Mammalian Glucose Transporters (GLUTs)

1.2.2.1 Classification of sugar transporters

Glucose derived from the diet is transferred from the lumen of the small intestine, and both dietary glucose and glucose synthesized within the body have to be transported from circulation into target cells. These processes involve the transfer of glucose across plasma membranes and these occur via integral transport proteins. These transporters comprise two structurally and functionally distinct groups, whose members have been identified over the past two decades, namely: (i) the Na^+ -dependent glucose co-transporters (SGLT, members of a larger family of Na-dependent transporters, gene name solute carrier family 5A (SLC5A)) (Wright, 2001); (ii) the facilitative Na^+-independent sugar transporters (GLUT family, gene name SLC2A) (Mueckler, 1994; Joost and Thorens, 2001).

Sodium-dependent glucose transporters (SGLT)

The SGLTs transport glucose (and galactose) with different affinities, via a secondary active transport mechanism. The Na^+-electrochemical gradient provided by the Na^+/K^+ ATPase pump is utilized to transport glucose into cells against its concentration gradient. This form of glucose transport takes place across the lumenal membrane of cells lining the small intestine and the proximal tubules of the kidneys. The first of this type of glucose transport protein to be cloned was the high-affinity transporter from rabbit intestine, SGLT1 (Hediger et al. 1987). The human analogue soon followed by homology cloning (Hediger et al. 1989). Amino acid comparisons of the human SGLT range from 57.71% sequence identity. SGLT1 has a limited tissue expression and is found essentially on the

apical membranes of small-intestinal absorptive cells (enterocytes) and renal proximal straight tubules (S3 cells).

A second Na^+-glucose transporter, SGLT2, is of low affinity and is predominantly expressed on the apical membrane of renal convoluted proximal tubules (S1 and S2 cells) (Wells et al. 1992; Kanai et al. 1994). It is currently accepted that in the kidney, SGLT2 (low affinity, high capacity) transports the bulk of plasma glucose from the glomerular filtrate. Any remaining glucose is recovered by SGLT1 (high affinity, low capacity) thus preventing glucose loss in the urine. However, controversy exists as to whether SGLT2 is the major renal glucose transporter (Hediger et al. 1995; Wright, 2001).

Over recent years, data collected from homology cloning and the Human Genome Project has indicated the presence of additional members of the SGLT-like transporters, which are currently under investigation (Wright, 2001). Additional members, SGLT3-6, have been assigned but await complete functional and structural characterization.

Facilitative glucose transporters

The facilitative transporters (GLUT) utilise the diffusion gradient of glucose (and other sugars) across plasma membranes and exhibit different substrate specificities, kinetic properties and tissue expression profiles. The first transporter to be isolated, GLUT1, was cloned from a HepG2 cell line (Mueckler et al. 1985). Identification of other members of the GLUT family followed over a five-year period to yield four glucose transporters (GLUT1-4) and a fructose transporter (GLUT5). Two further members were briefly included but have since been discarded. One gene, for GLUT6, was deemed to be a pseudogene (Kayano et al. 1990) and GLUT7 was found to be a sequencing artifact (Burchell, 1998). The GLUT6 and GLUT7 names have since been assigned to other gene products (Table 1).

Recently, additional family members have been identified on the basis of sequence similarity. However, the pace at which these new genes were identified and classified by independent groups initially caused confusion in their terminology. A consensus has since been reached (Joost et al. 2002) in which the thirteen members have been named GLUT1 to GLUT12, and HMIT (H^+-coupled myo-inositol transporter). It is considered that all members of the family have now been identified (Joost and Thorens,

2001). In 2002, Wu and Freeze identified a new one member of GLUT, GLUT14, a duplicon of GLUT3, specifically expressed in testis as alternative splice forms (Wu and Freeze, 2002).

Table 1. Summary of the extended GLUT family

Protein	Previous name, No. of amino acid	Gene Name	Chromosome Localization	Accession Nos.		Primary References
				cDNA	Gene	
GLUT1	GTR1, 492	SLC2A1	1p35-31.3	AC023331	K03195	Mueckler et al, 1985
GLUT2	GTR2, 524	SLC2A2	3q26.2-27	AC068853	J03810	Fukumoto et al, 1989
GLUT3	GTR3, 496	SLC2A3	12p13.3	AC007536	J04069	Kayano et al, 1988
GLUT4	GTR4, 509	SLC2A4	17p13	AC003688	M20747	Fukumoto et al, 1989
GLUT5	GTR5, 501	SLC2A5	1p36.2	AC041046	J05461	Kayano et al, 1990
GLUT6	GTR6, GLUT9,507	SLC2A6	9q34	AC002355	Y17803	Doege et al, 2000
GLUT7	GTR7, 528	SLC2A7	1p36.2	AL356306	-	Waddell et al, 1992
GLUT8	GTR8, GLUTX1, 477	SLC2A8	9	AL445222	Y17081	Carayannopoulos et al, 2000
GLUT9	GTR9, GLUTX, 511/540	SLC2A9	4p15.3-16	AC005674	AF210317	Phay et al, 2000
GLUT10	GTR10, 541	SLC2A10	20q12-13.1	AC031055	AF321240	McVie-Wylie et al, 2001
GLUT11	GTR11, GLUT10, 496	SLC2A11	22q11.2	AP000350	AJ271290	Doege et al, 2001
GLUT12	GTR12, GLUT8, 617	SLC2A12	6q23.2	AL449363	-	Rogers et al, 2002
HMIT	-, 618/629	SLC2A13	Ambiguous	AJ315644	AJ315644	Uldry et al, 2001
GLUT14	a retroposon of SLC2A3, 497/520	SLC2A14	12p13.3	-	AF481878/9	Wu and Freeze, 2002

The facilitative sugar transporters are predicted to have twelve membrane-spanning regions with intracellular located amino- and carboxyl-termini. Alignments of the amino acid sequences show that there is 39-65% identity between the well-characterized human GLUT1 to GLUT5, and at least 28% identity between GLUT1 and each of the new GLUTs (Bell et al, 1990, Joost and Thorens, 2001). Sequence comparisons of all members reveal the presence of "sugar transporter signatures" (Joost and Thorens, 2001) and these consist of numerous conserved glycine and tryptophan residues, which are regarded as being essential for general facilitative transporter function. Structural and functional characteristics of individual GLUT members are at varying degrees of completion. Based on a dendrogram (Fig 4), from a multiple sequence alignment of the extended GLUT family, three subclasses (I-III) are apparent, which also share common sequence motifs (Joost and Thorens, 2001).

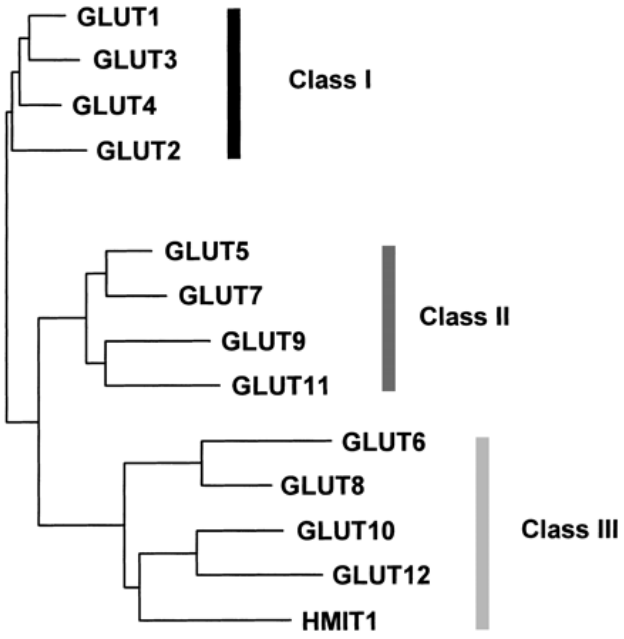

Figure 4. Dendrogram of a multiple alignment of all members of the extended GLUT family (from Joost et al, 2002).

1.2.2.2 GLUT Structure

The common features revealed by sequence alignment and analysis of all the above-mentioned transporters include 12 predicted amphipathic helices arranged so that both the N- and C-termini are at the cytoplasmic surface (Figure 5)(Mueckler et al, 1985). As shown in Figure 5, the signature sequences of the GLUT family are highlighted. Transmembrane domains (TM) are labeled 1 to 12. The sites of glycosylation (N) are shown. Conserved domains and amino acids are also included. The tryptophan residues 388 and 412 (numbering of GLUT1) are part of the cytochalasin B binding site. Major differences between classes I and II and class III are the position of the large extracellular loop containing the N-glycosylation site(s), the proline- containing motif between transmembrane domains (TM) TM6 and TM7, and the presence of a dileucine motif in the amino-terminal tail of class-III transporters (except for GLUT10). For class I transporters, the TMs proposed to form the glucose transport channel are highlighted by gradient shading, and regions which interact with hexose substrates and the GLUT inhibitor cytochalasin B are indicated (Holman and Rees, 1987, Cairns et al, 1987).

These may also constitute part of hydrogen bonding channel allowing hexoses which are accepted at the exofacial site access to the inner binding site of the transporter. Release of sugars at the inner site may be controlled by conformational changes occurring in helices 10 and 11, where highly conserved tryptophan and proline residues are present. Molecular modeling and molecular dynamics studies suggest that prolines 383 and 385 are particularly important in facilitating an alternate opening and closing of the external site of these transporters. Ligand binding and labelling studies suggest some structural separation of external and internal binding sites. The bis-mannose labelling site has been mapped to helix 8 and helix 9 (Holman and Rees, 1987). However, the inside-specific ligand cytochalasin B labels a region between helices 10 and 11 (Holman and Rees, 1987, Cairns et al, 1987), while the diterpine compound IAPS-forskolin labels helix 10 (Wadzinski et al, 1988) and/or helix 9 (Schurmann et al, 1992).

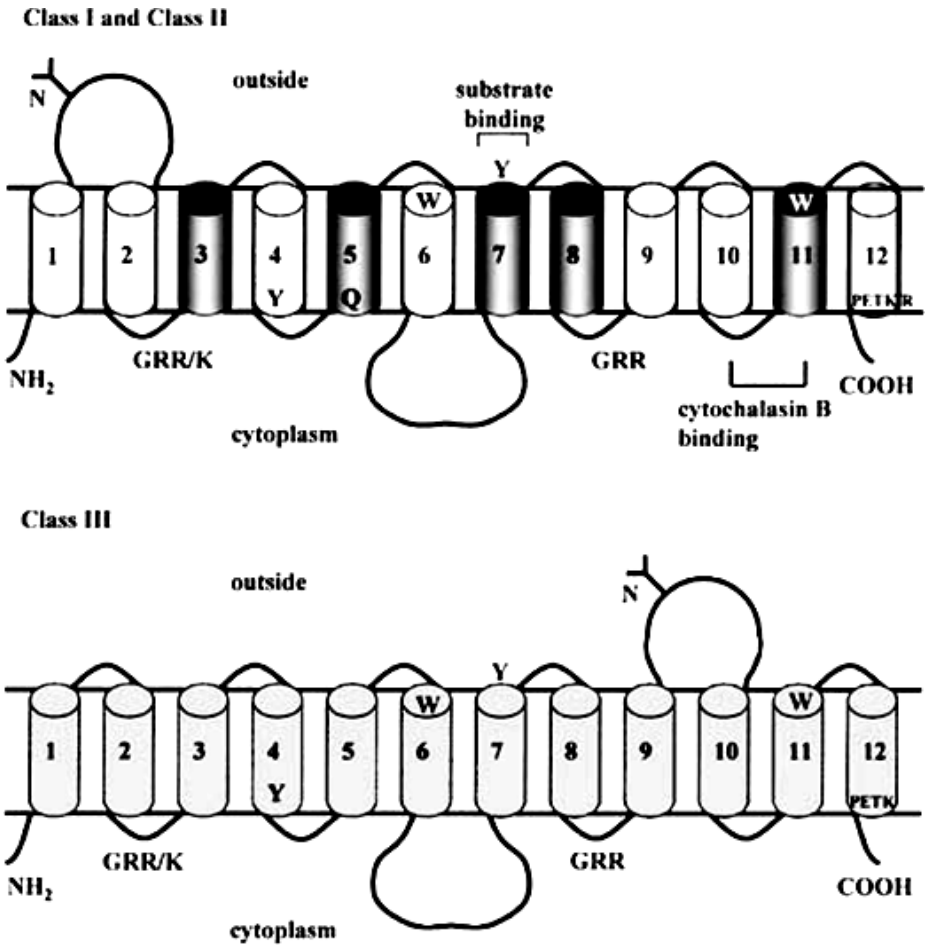

Figure 5. Schematic model of the structure of classes I and II (upper panel) and class III (lower panel) members of the GLUT family (from Mueckler et al, 1985). E: glutamic acid, G: glycine, K: lysine, L: leucine, P: praline, Q: glutamine, R: arginine, T: threonine, W: tryptophan, Y: tyrosine.

1.2.2.3 Function and tissue-specific expression of glucose transporter family

Glucose transporters are expressed in every cell of the body, as might be anticipated from the key role of glucose in providing metabolic energy and building blocks for the synthesis of biomolecules. The specific physiological role of the isoforms expressed in tissues involved in the control of glucose homeostasis, i.e. muscle, adipose tissue, liver, pancreatic β-cells and brain, has been studied in greatest detail. Indeed, in these tissues

glucose transporters play important roles in the control of glucose utilization, glucose production and glucose sensing and their dysregulated expression may underlie pathogenetic mechanisms leading to development of diabetes mellitus, but also other specific monogenic diseases. The function and tissue expression of glucose transporter family was summarized in Table 2.

Table 2 Summarized the function and tissue expression of glucose transporter family.

Isoform	Class	Main tissue localization	Insulin sensitive?	Functional characteristics (transport)	Present in skeletal muscle?	Present in adipose tissue?
GLUT1	I	Erythrocytes, brain, and ubiquitous	No	Glucose	Yes	Yes
GLUT2	I	Liver, islets, intestine, kidney	No	Glucose (low affinity); fructose	No	No
GLUT3	I	Brain (neuronal)	No	Glucose (high affinity)	No	Yes (m)
GLUT4	I	Muscle, fat, heart, brain	Yes	Glucose (high affinity)	Yes	Yes
GLUT5	II	Intestine, testis, kidney	No	Fructose; glucose (very low affinity)	Yes	Yes
GLUT6	III	Spleen, leukocytes, brain	No	glucose	No	n.d.
GLUT7	II	Not determine	n.d.	n.d	n.d.	n.d.
GLUT8	III	Testis, blastocyst, brain other tissues	No (Yes in blastocytes)	glucose	Yes (m)	Yes (m)
GLUT9	II	Liver, kidney	n.d.	n.d	No	n.d.
GLUT10	III	Liver, pancreas	No	glucose	Yes (m)	n.d.
GLUT11	II	Heart, muscle	No	Glucose (low affinity); fructose (long form)	Yes (m)	No
GLUT12	III	Heart, prostate, muscle, Intestine, and fat	Yes	n.d	Yes	Yes
HMIT	III	Brain	n.d.	H+-myo-inositol	No (m)	Yes (m)
GLUT14	I	Testis	n.d.	glucose	n.d.	n.d.

n.d.; Not determine, m; mRNA only

1.2.3 The Glucose Transporters of Skeletal Muscle

Skeletal muscle is the major consumer of glucose in the body following a meal as well as during exercise. Glucose is taken up into the tissue through facilitated diffusion by two glucose transporters, GLUT1 and GLUT4. The molar ratio of GLUT1:GLUT4 polypeptides in skeletal muscle plasma membranes is 0.6 to 1.0 (Klip and Marette 1992). The presence of both transporters at the muscle surface has also been documented by immunofluorescence (Marette et al, 1992b). These results suggest that both transporters could play an important role in basal glucose uptake i.e. that in the non-fed state at rest. These results suggest that both transporters could play an important role in basal glucose uptake, i.e. that in the non-fed state at rest. However, in the whole muscle there is much more GLUT4 transporter than GLUT1. The majority of the GLUT4 protein is found in an intracellular organelle in the basal state, from where it can be rapidly translocated to both the plasma membrane and its continuous invaginations known as transverse tubules (Marette et al, 1992a, Dudek et al, 1994). The resulting increase in cell surface glucose transporters rapidly increases the rate of glucose uptake into the muscle. Interestingly, human muscle also expresses another member of the GLUT family, GLUT5, which is primarily a fructose transporter located on the cell surface and copurifies with isolated plasma membranes (Hundal et al, 1992).

1.2.3.1 GLUT1 transporter

GLUT1 was the first transporter to be characterized by molecular cloning, and its cDNA was isolated from an expression library using antibodies against the human erythrocyte glucose transporter (Mueckler et al, 1985). GLUT1 is found in almost every tissue with different levels of expression in different cell types. The expression level usually correlates with the rate of cellular glucose metabolism. It is also expressed highly in blood-tissue barriers, in particular, in the endothelial cells forming the blood-brain barrier (Maher et al, 1994).

The transport of glucose may be described as an alternating conformer model in which the transporter has mutually exclusive binding sites located on the extracellular (import site) and on the intracellular face (export site) of the transporter. Binding of glucose to one site induces the transporter to switch to the opposite

conformation, a process that is accompanied by a movement of the substrate across the plasma membrane. GLUT1 transports glucose with a Km of ~3 mM. Other transported substrates are galactose, mannose and glucosamine (Uldry et al, 2002). Glucose transport by GLUT1 is sensitive to several inhibitors. Many of them are competitive inhibitors of sugar binding, either to the extracellular or the cytosolic sugar binding sites. Cytochalasin B binds to the inner surface of GLUT1 (Baldwin and Lienhard GE. 1989) and inhibits its glucose transport activity with an IC50 of 0.44 μM. Also acting on the same intracellular site is the diterpene toxin forskolin (Morris et al, 1991).

Glucose transport activity of GLUT1 is inhibited by $HgCl_2$ (IC50 3.5 μM), phloretin (IC50 49 μM) phlorizin (IC50 355 μM) (Kasahara and Kasahara, 1996) and 4,6-O-ethylidene-d-glucose (IC50 12 mM), which binds to the external glucose binding site (Mueckler 1994).

Once cDNA sequences for the transporter isoforms became available, studies directed toward understanding the molecular mechanisms controlling their expression could be performed. Alterations in glucose transporter abundance, tissue distribution and subcellular localization were examined in response to numerous physiological stimuli. The ability to alter glucose transporter gene expression may produce agents utilizable against diabetes, cancer, viral infection and other diseases that alter glucose homeostasis. Thus, an understanding of what controls the expression of these genes and how they can be manipulated representes a challenge for both the biochemical and pharmaceutical communities. It is clear that an increased knowledge of the molecular processes controlling the expression of this family of proteins has far reaching implications.

Regulation of GLUT1 transcription by Serum-Related Growth Factors

Hiraki et al. (1988) and Kitagawa et al. (1989, 1991) analyzed the effect of serum and serum components on GLUT1 gene expression was listed in Table 3 Several growth factors and the reported effects they have on GLUT1 expression. These studies demonstrated that serum stimulation of quiescent fibroblasts produced a dramatic increase in glucose transporter activity, protein and mRNA levels. Both groups reported an approximate 5- to 10-fold increase in GLUT1 mRNA following serum stimulation, with maximum response occurring within 4 hr. Serum induction of GLUT1 expression occurred on a rapid time scale. Maximum effects can be seen by 4 hr, with measurable increases in

GLUT1 activity, protein and mRNA accumulation as early as 2 hr (Hiraki et al., 1988; Kitagawa et al., 1989).

Table 3. The effect of serum and growth factors on GLUT1 expression

Factor	Fold of stimulation		
	Hiraki et al., 1988	Kitagawa et al., 1989	Kitagawa et al., 1991
Serum	5-10	7.6	4.5
PDGF	5	7.5	-
FGF	7.5-15.5	8.0	4.0
EGF	7.5-15.5	1.68	1.0
Insulin	-	2.17	0.9
IGF- 1	No effect	-	-
TGF-β	-	-	-
PMA	5	6.13	1.8

PDGF, platelet-derived growth factor; FGF, fibroblast growth factor; EGF, epidermal growth factor; IGF-1, insulin-like growth factor-l; TGF-β, transforming growth factor; PMA, phorbol 12-myristate 13-acetate.

GLUT1 mRNA Stability

The turnover of mRNA is a highly regulated process able to control gene expression by determination of the content of a particular mRNA transcript. The abundance of a particular transcript is dependent both on its rate of synthesis and the decay. The decay rates (or half-lives) have been demonstrated to vary greatly within the same cell. Thus, mRNA stability should not be considered an alternative to transcriptional regulation, but as a partner with it. Growth factor and oncogene transcripts are characterized by rapid turnover rates, while structural protein transcripts, such as actin or tubulin, have considerably longer half-lives (Peltz et al., 1991). Even within the same cell line, RNA turnover rates for a particular transcript can change as the result of hormonal treatment, nutrient depletion or differentiation. Our understanding of the control of mRNA turnover is expanding rapidly, and several recent reviews focusing on mechanisms of RNA stability in greater detail have been published (Peltz et al., 1991, Peltz and Jacobson, 1992).

Most, if not all, mitogens appear to regulate GLUT1 expression through predominately transcriptional mechanisms. However, a number of investigators have shown that an alteration in GLUT1 mRNA stability occurs in response to various stimuli. These studies are summarized in Table 4. It is notable that a majority of the treatments displayed in Table 4 have profound effects on cellular energy stores. For example, glucose deprivation and inhibition of oxidative phosphorylation alter cellular ATP levels, and in this situation, stabilization of GLUT1 mRNA would permit synthesis of more transporters and a subsequent increase in the glucose uptake potential. Similarly, exposure of cells to cytokines and growth factors may tax cellular energy stores, requiring increased uptake of glucose. As detailed above, stabilization of mRNA, resulting in the synthesis of more protein, is an effective way to accomplish this task. Treatment of the 3T3-Ll adipocytes with arachidonic acid, which depleted GLUT4 mRNA and protein, resulted in stabilization of GLUT1 mRNA and increased GLUT1 protein levels (Tebbey et al., 1994). Following arachidonate treatment, basal glucose uptake is elevated to levels typical of an insulin-stimulated state. Following exposure of the cells to arachidonic acid treatment, the stabilization of GLUT1 mRNA may reflect an adaptive mechanism that responds to the GLUT4 depletion (Tebbey et al., 1994).

Table 4. Factors that affect GLUT1 mRNA turnover

Cell type	Treatment	Control half-life	Treated half-life	Fold of stimulation	Reference
Quiescent 3T3-Ll fibroblasts	Tumor necrosis factor (TNF)	43 min	200 min	5	Cornelius et al., 1990; Stephens et al., 1992
Quiescent 3T3-Ll fibroblasts	Phorbol esters	50 min	300 min	6	Stephens et al., 1992
Quiescent 3T3-Ll fibroblasts	Okadaic acid	50 min	270 min	5	Stephens et al., 1992
BALB/c 3T3 fibroblasts	PDGF*	47 min	160 min	4	Rollins et al., 1988
Quiescent NIH 3T3-Ll fibroblasts	Vanadate	30-60 min	90-120 min	2-3	Mountjoy and Flier, 1990
L6 muscle cells	Insulin	2.0-2.5 hr	4.0-4.5 hr	2	Maher and Harrison, 1990
L6 muscle cells	Glucose deprivation	2.0-2.5 hr	4.0-4.5 hr	2	Maher and Harrison, 1990
L6 muscle cells	Insulin &glucose deprivation	2.0-2.5 hr	8.0-8.5 hr	4	Maher and Harrison, 1990
L6 muscle cells	Tunicamycin	2.0-2.5 hr	4.0-4.5 hr	2	Maher and Harrison, 1991
Clone 9 kidney cells	Azide	2.3 hr	8.0 hr	4	Shetty et al., 1993
3T3 F442A preadipocyte	Insulin	2.2 hr	5.7 hr	2.5	Sandouk et al., 1993
3T3 F442A preadipocyte	Pioglitazone	2.2 hr	3.6 hr	3.6	Sandouk et al., 1993
3T3 F442A preadipocyte	Insulin and pioglitazone	2.2 hr	>24 hr	>10	Sandouk et al., 1993
Bovine brain endothelial cells	Glucose deprivation	3.6 hr	6.4 hr	2	Baodo and Pardridge, 1993
3T3-Ll adipocytes	Arachidonic acid	13.1 hr	>24 hr	>2	Tebbey et al, 1994

* PDGF ;Platelet-derived growth factor

1.2.3.2 GLUT4 transporter

GLUT4 was cloned from human (Fukumoto et al, 1989), rat (Birnbaum, 1989, Charron et al, 1989) and mouse tissues (Kaestner et al, 1989). GLUT4 has a K_m for glucose transport of ~5 mM. In addition, GLUT4 can also transport dehydroascorbic acid and glucosamine (K_m ~3.9 mM). The transporting activity is inhibited by cytochalasin B (IC50 0.1-0.2 μM), phloretin (IC50 10 μM) and phlorizin (IC50 140 μM) (Kasahara and Kasahara, 1997). Recently, the protease inhibitor indinavir has been shown to inhibit GLUT4 activity (IC50 50 μM) non-competitively in adipocytes. GLUT4 is the major glucose transporter of brown and white adipose tissues and both skeletal and cardiac muscles.

GLUT4 has two internalization sequences, a dileucine repeat present at the C-terminal tail and a FxxY motif (phenylalanine, unknown or unspecificed aminoacid and tyrosine) in the amino-terminal end. These motifs are responsible for GLUT4 association with an intracellular tubulo-vesicular compartment in basal low plasma insulin conditions (Al-Hasani et al, 2002). Binding of insulin to its cell surface receptor leads to a rapid translocation of GLUT4 to the cell surface, resulting in an increased in cellular glucose transport activity. GLUT4 translocation to the plasma membrane can also be stimulated by exercise. This involves a signalling pathway different from that activated by insulin. This pathway may be regulated by the AMP-activated protein kinase (AMPK). The kinetics of GLUT4 cell surface translocation and endocytosis has been extensively studied using the photoaffinity label 4-(1-azi-2,2,2-trifluoroethyl)-benzoyl-1, 3-bis(d-mannos-4-yloxy)-2-propylamine, ^{3}HATB- BMPA (Holman et al, 1990). This has been applied to the study of GLUT4 recycling in adipocytes and muscles of animals and humans following insulin signalling and exercise training. The ability of insulin to stimulate glucose uptake relies on a complex signalling cascade (Saltiel and Pessin, 2002) that is still not completely understood. A defect in the ability of insulin to regulate this metabolic event is one of the key physiological dysfunctions of *Type 2 Diabetes*. A decreased expression of GLUT4 may also cause insulin resistance. However, in *Type 2 Diabetes* decreased expression of GLUT4 is observed only in adipose tissue and not in muscle, although the latter is responsible for ~90% of glucose utilization in the post-prandial state and is thus a major site of insulin resistance.

Tissue-specific disruption of the GLUT4 in muscle results in profound reductions in basal glucose transport and the near absence of stimulation by insulin or contraction. (Zisman et al, 2000). Disruption of GLUT4 in adipose tissue shows results in markedly impaired insulin-stimulated glucose uptake in adipocytes and, surprisingly, to insulin resistance in muscle and liver. This may be due to altered secretion by adipocytes of factors that could regulate insulin sensitivity in these other tissues (Abel et al, 2001). Selective deletion of GLUT4 from the heart induces modest cardiac hypertrophy associated with increased myocyte size. Basal and isoprotenerol-stimulated isovolumic contractile performance is unaffected (Abel et al, 1999).

Regulation of GLUT4 transcription by Insulin

Chronic insulin stimulates GLUT4 biosynthesis. Animal studies have shown that hyperinsulinemia due to insulin infusion results in elevated GLUT4 protein levels in adipocytes (Kahn et al., 1987, Cusin et al., 1990), which parallels with previous reports of the hyper-responsiveness of this tissue (Wardzala et al., 1985). Kahn et al. (1987) demonstrated that in the chronically insulin-stimulated state, the concentration of GLUT4 in the plasma membrane was 38% higher in adipocytes from insulin-treated rats, paralleling the 55% increase in insulin-stimulated glucose transport activity in the intact cell. In a similar study, hyperinsulinemia was shown to increase GLUT4 protein and mRNA in rat adipose tissue (Cusin et al., 1990). In muscle, the data are less clear. The effect of hyperinsulinemia on glucose transport in rat skeletal muscle correlates closely with the GLUT4 content of the fiber type and depends on circulating glucose levels. In human muscle from individuals with insulin-dependent diabetes mellitus, no direct regulatory role of plasma insulin levels on GLUT4 expression is evident (Kahn et al., 1992). In a study of the effects of diabetes on the myocardial glucose transport system in rats (Garvey et al., 1993), it was concluded that transport rates were decreased due to pretranslational suppression of the GLUT4 transporter, and synthesis could be corrected by insulin therapy. In an in vitro model system, Flores-Riveros et al. (1993) demonstrated that insulin rapidly induced down-regulation of GLUT4 gene expression in 3T3-Ll murine adipocytes. In this system, chronic insulin exposure (24 hr) down-regulated GLUT4 mRNA and protein content by repressing transcription of the gene to 25% of its initial rate and by decreasing the half-life of the mRNA from 9 hr to 4.6 hr. Consistent with these

results, in vitro studies with L6 myotubes in culture, Klip and colleagues demonstrated that high insulin levels, and not insulin deficiency, resulted in a reduction in GLUT4 mRNA (Koivisto et al., 1991). These findings suggested that at least in animal and cell culture models, insulin diminished both GLUT4 gene transcription and mRNA stability, which contribute, at least in part, to the mechanism of insulin resistance observed in hyperinsulinemia. However, this appears not to be true in humans, as Friedman et al. (1992) found that insulin resistance in the skeletal muscle of morbidly obese patients with and without non-insulin-dependent T2DM cannot be causally related to the cellular content of GLUT4.

Regulation of GLUT4 transcription by exercise

Numerous studies of exercise training have demonstrated an increase in whole-body insulin sensitivity. Maximal glucose transport is increased in skeletal muscle with endurance exercise training by means of an increase in GLUT4 protein content (Neufer et al., 1992; Friedman et al., 1990). Ploug et al. (1990) and Goodyear et al. (1992) reported an increase in Vmax for glucose transport after exercise training, which resulted from an increase in activation and in the content of GLUT4 translocated to the plasma membrane. The training-induced increase in GLUT4 protein has been reported to be associated with a 1.5-fold increase in GLUT4 mRNA content, suggesting that exercise training may regulate GLUT4 gene expression at the level of gene transcription and/or message stability (Ploug et al., 1990). The increment of gene transcription was confirmed by Neufer and Dohm (1993) using nuclear transcription run-on assays. However, the significant increased in GLUT4 protein with exercise training has not been established.

In the year 2006, through analysis of myocyte enhancer factor 2 (MEF2) regulation, McGee and Hargreaves proposed a model of transcriptional regulation of GLUT4 during exercise in human skeletal muscle (Fig 6) that involves the derepression by Histone deacetylase (HDAC5) following its phosphorylation by adenosine monophosphate-activated protein kinase (AMPK) , the coactivation by peroxisome proliferator-activated receptor gamma coactivator 1α (PGC- 1α) and finally the phosphorylation by p38 mitogen-activated protein kinase, p38 MAPK (McGee and Hargreaves, 2006). As consensus MEF2 binding domains are found on the promoter regions of many exercise responsive genes, it is possible that these smilar regulatory

mechanisms mediate many of the transcriptional responses to exercise. As such, they could provide therapeutic targets for the treatment and management of metabolic diseases in type 2 diabetes.

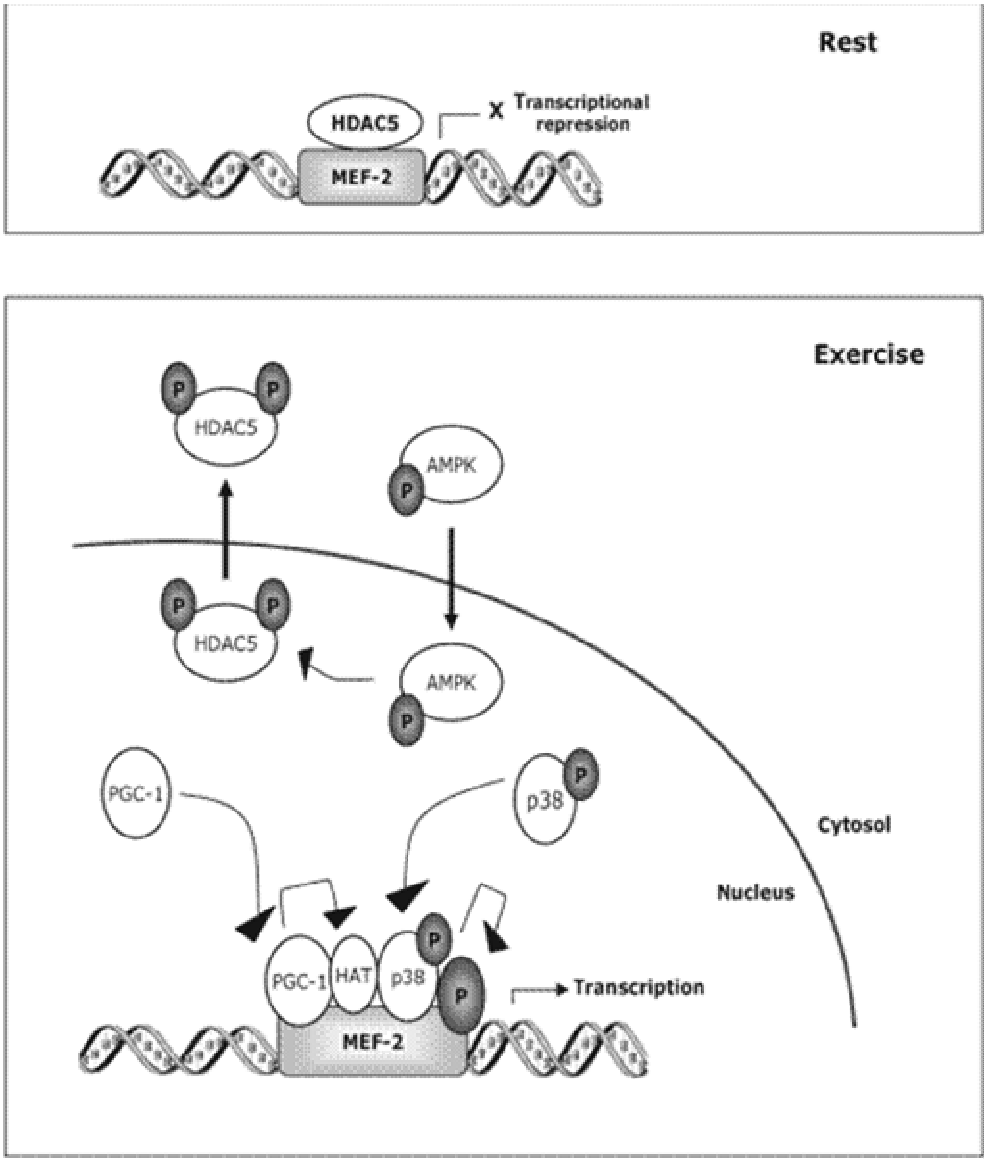

Figure 6. Schematic diagram of the proposed regulation of myocyte enhancer factor 2 (MEF2) in contracting human skeletal muscle. at rest, MEF2 is associated with HDAC5, which renders MEF2 transcriptionally inactive. During exercise, phosphorylation of HDAC5 by AMPK results in the dissociation from MEF2 and nuclear export. PGC-1α associates with MEF2 presumably to recruit cofactors with Histone acetyl transferase (HAT) activity. Exercise increases nuclear p38 phosphorylation and association with MEF2.

Myocyte enhancer factor 2 is phosphorylated on various threonine residues by a mitogen activated protein kinase (MAPK) in a sequence specific manner. These events result in enhanced MEF2 transcriptional activity (McGee and Hargreaves, 2006).

GLUT4 mRNA stability

Previous data have demonstrated that exposure of the 3T3-Ll adipocytes to TNF treatment resulted in a significant destabilization of the GLUT4 mRNA (Stephens and Pekala, 1992). After 48 hr of TNF treatment, the half-life of GLUT4 mRNA decreased from 9.5 hr to 4.5 hr. This destabilization effect could be detected only after 12 hr of TNF treatment. Regulation of GLUT4 gene expression by decreased the stability of the mRNA was supported by recent studies in the same cell line with insulin (Flores-Riveros et al., 1993) and arachidonic acid (Tebbey et al., 1994), both of which decreased GLUT4 mRNA half-life in the same manner as TNF.

Regulated membrane trafficking of the insulin- responsive glucose transporter 4

In unstimulated muscle or adipose cells, more than 90% of GLUT4 is intracellular and a mere 4-10% resides at the plasma membrane (PM), the result of slow exocytosis and fast endocytosis (Satoh et al, 1993, Li et al, 2001). Electron microscopy or immunofluorescence studies in primary or cultured adipocytes detect the majority of GLUT4 in tubulo-vesicular structures in the perinuclear region and cytosol (Piper et al 1991, Slot et al 1991, Marette et al 1992a). Upon subcellular fractionation, GLUT4 colocalizes in part with markers of the *trans*-Golgi network (TGN), Golgi complex, and rough endoplasmic recticulum (RE). In the basal state, chemical ablation of the transferrin receptor (TfR)-rich compartments of 3T3-L1 adipocytes spares about half of the GLUT4 complement, leading to the concept that a specialized compartment harbors GLUT4 that may or may not be able to recycle to the PM in the basal state (Livingstone et al 1996, Zeigerer et al 2002). In L6 myoblasts stably expressing myc-tagged GLUT4 and in 3T3-L1 adipocytes transiently expressing hemagglutinin (HA)-tagged GLUT4-eGFP [enhanced green fluorescent protein (GFP)], all the transporters recycle in the basal state, and insulin markedly accelerates such recycling (Li et al, 2001, Zeigerer et al 2002).

However, in 3T3-L1 adipocytes stably overexpressing HA-tagged GLUT4 (achieved by retrovirus gene transfer), a proportion of transporters fail to recycle (Coster et al, 2004). Hence, the existence of a static pool of GLUT4 in adipose cells is a matter of current debate, and solving this may require careful analysis of the behavior of the transfected GLUT4 in each study.

Whether static or dynamic, half of the GLUT4 molecules in 3T3-L1 adipocytes and L6-GLUT4myc myoblasts escape colocalization with TfR or GLUT1 and instead colocalize with the soluble N-ethylmaleimide-sensitive factor attachment protein receptors (v-SNARE) vesicle-associated membrane protein2 (VAMP2) (Sevilla et al, 1997, Malide et al, 1997, Volchuk et al, 1994). This latter protein segregates away from the TfR (Martin et al, 1996) and is required for fusion of the GLUT4 vesicles mobilized by insulin with the PM. Interestingly, VAMP2 is not involved in the fusion of the continuously recycling GLUT4 vesicles with the PM. Moreover, GLUT4 recruitment to the cell surface by platelet-derived growth factor or hyperosmolarity is also independent of VAMP2 and instead appears to involve the vesicular (v)- SNARE TI-VAMP/VAMP7 (Randhawa et al 2004, Torok et al 2004). These observations suggest the existence of a GLUT4 specialized compartment that can be regulated by insulin and is characterized by the presence and functional requirement of VAMP2 for final fusion with the PM. Names assigned to such a compartment include specialized compartment (SC) and GLUT4-storage vesicles (GSV). Furthermore, these findings suggest that the GLUT4 vesicles reaching the PM in response to insulin may differ from those arriving in the basal state. Such a conclusion is also supported by studies comparing the segregation and recycling of GLUT4 and the TfR in 3T3-L1 adipocytes, suggesting that insulin stimulates the transfer of GLUT4 from the RE to SC/GSV as well as translocation of GLUT4 from SC/GSV to the PM (Zeigerer et al 2002, Karylowski et al, 2004). However, a contrasting model proposes that GLUT4 molecules stored in elements of the TGN recycle back to the RE from where they are stimulated to exit to the PM in response to insulin (Shewan et al, 2003, Govers et al, 2004). Further studies are required to reconcile these models. In both models, GLUT4 is dynamically regulated by insulin, not only its mobilization toward PM but also its traffic between endosomal compartments (Figure 7.).

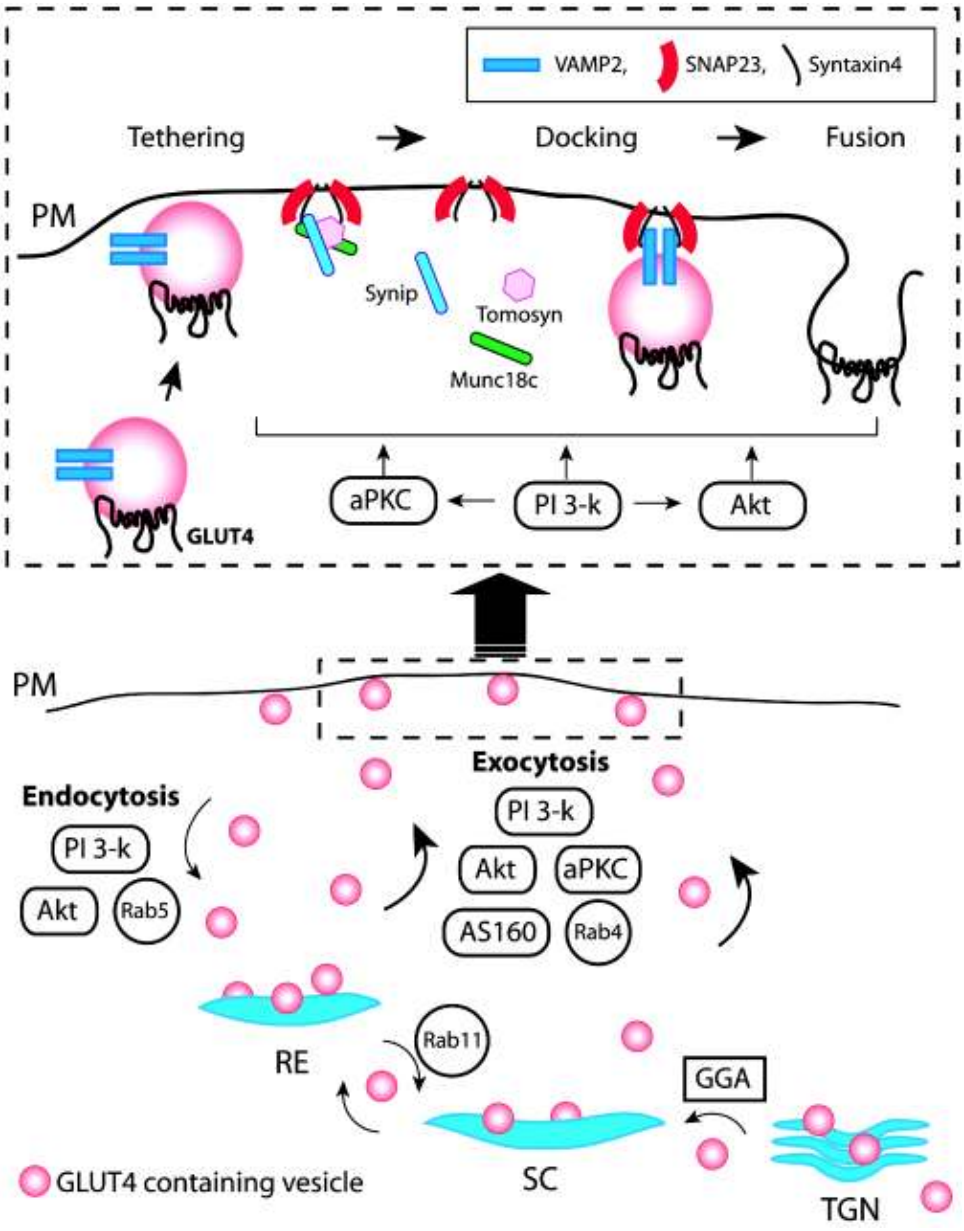

Figure 7. Schematic representation of GLUT4 traffic to and from the plasma membrane. Newly synthesized GLUT4 enters the insulin-responsive SC (also called GSV) from the trans-Golgi network (TGN), regulated by Golgilocalized, γ-ear-containing, Arf-binding protein (GGA). Insulin accelerates GLUT4 exocytosis from SC/GSV and possibly also from the recycling endosomes (RE) resulting in accumulation of GLUT4 at the plasma membrane (PM). Involvement of exocyst components is predicted to allow GLUT4-containing vesicle movement to appropriate sites for docking and fusion. soluble N-ethylmaleimide-sensitive factor attachment protein receptors (SNARE) mechanisms accomplish these processes along with accessory proteins. The involvement of small

GTPases at distinct steps is indicated. At the PM, class IA PI 3-kinase (PI3-k) input regulates vesicle fusion with the PM. Proteins involved in GLUT4 vesicle tethering and fusion with the PM is illustrated within the dotted-line square. (Ishiki and Klip 2005)

The relative GLUT4 distribution throughout organelles of cells from non-stimulated and insulin-stimulated brown adipose tissue was shown in Figure 8. Cryosections of brown adipose tissue were immunolabelled with anti-GLUT4 antibody and gold-conjugated Protein A. Gold particles were counted and assigned to the following organelles (Slot et al, 1991). This was the first immunocytochemical analysis of GLUT4 in insulin-sensitive cells and showed that insulin caused a striking redistribution of GLUT4 from intracellular tubulo-vesicular elements to the plasma membrane.

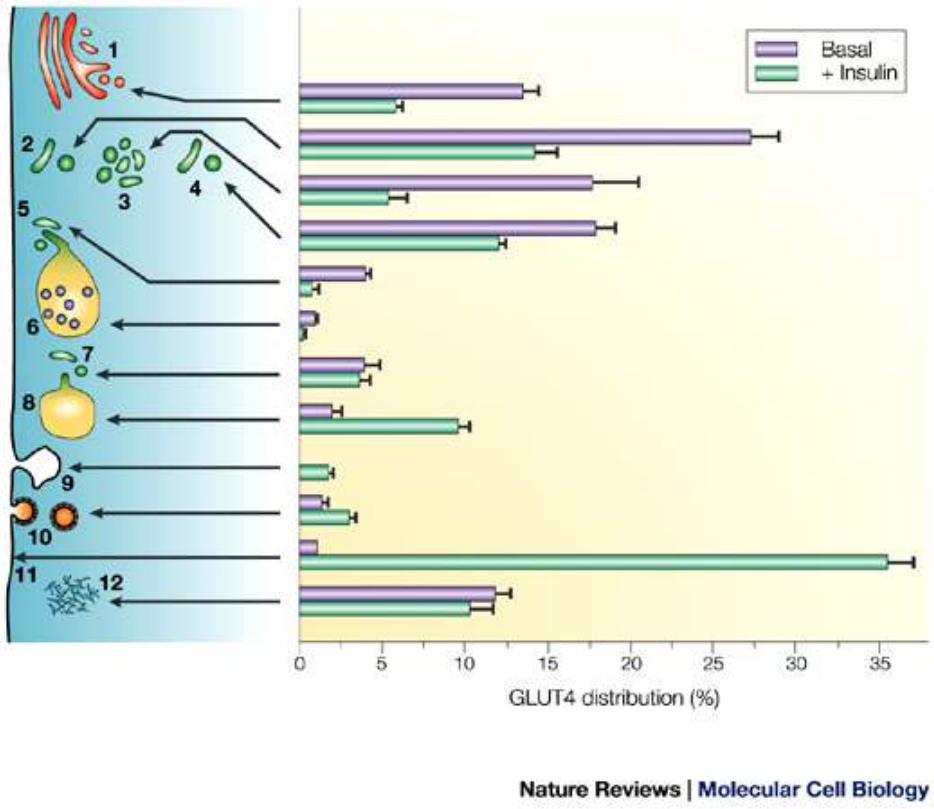

Figure 8. Relative GLUT4 distribution throughout organelles of cells from non-stimulated and insulin-stimulated brown adipose tissue. Cryosections of brown adipose tissue were immunolabelled with anti-GLUT4 antibody and gold-conjugated Protein A. Gold particles were counted and assigned to the following organelles: (1) trans-Golgi network (TGN); (2) tubulo-vesicular (T-V) elements located underneath the plasma membrane; (3) clusters of T-V elements; (4) T-V elements distributed throughout the cytoplasm; (5) T-

V elements connected or close to late endosomal vacuoles (6); (7) T-V elements connected or close to early endosomal vacuoles (8); (9) non-coated invaginations of the plasma membrane; (10) coated pits and vesicles; (11) plasma membrane; (12) cytoplasm. The bar graph shows the relative distribution of GLUT4 throughout these organelles. (Bryant et al, 2002)

GLUT4 translocation

At least two discrete signaling pathways have been implicated in insulin-regulated GLUT4 translocation (Figure 9). The first involves the lipid kinase phosphatidylinositol 3-kinase (PI3K), (Yang et al, 1996) and the second involves the proto-oncoprotein c-Cbl (Ribon and Saltiel, 1997, Ribon et al, 1998). Insulin binds to its receptor on the surface of target cells. This binding induces a conformational change in the receptor, and leads to activation of its tyrosine-kinase domain, which is located within the intracellular portion of its β-subunits. On activation, the receptor phosphorylates several proximal substrates, including members of the insulin-receptorsubstrate family (IRS-1 and IRS-2 being the most important in muscle and fat cells) and c-Cbl. Tyrosine-phosphorylated IRS proteins, which are thought to be held in close proximity to the plasma membrane through association with the underlying cytoskeleton, recruit more effector molecules, such as PI3K, to this location. Substantial evidence indicates that the Class 1A PI3K might have an important role in insulin-stimulated GLUT4 translocation, although the role for other PI3K isoforms cannot be excluded. In muscle and fat cells, two important targets of PI3K that have been shown to play a role in insulin-stimulated GLUT4 translocation are; 1) the serine/threonine kinase, Akt/protein kinase B (PKB) and 2) the atypical protein kinase C (PKC) isoform, PKCζ. PI3K activates Akt by generating polyphosphoinositides in the inner leaflet of the plasma membrane. This acts as a docking site for Akt through its pleckstrin homology domain, thereby bringing it in close proximity to its upstream regulatory kinase, phosphatidylinositol-dependent kinase-1 (PDK-1). The second, putative signalling pathway that has been shown to have a role in insulin-stimulated GLUT4 translocation operates independently of PI3K and involves a dimeric complex that comprises c-Cbl and the c-Cbl-associated protein CAP. Intriguingly, whereas many growth factors trigger the activation of PI3K, Akt and PKCζ in various cell types, the c-Cbl-CAP pathway, including the tyrosine phosphorylation and the expression

of CAP, are unique to muscle and fat cells. Insulin triggers the movement of this dimeric c-Cbl-CAP complex into cell-surface lipid rafts through association with the raft protein flotillin. Inhibition of this process inhibits insulin-stimulated GLUT4 translocation in adipocytes (Watson et al, 2001). Tyrosine-phosphorylated c-Cbl then recruits a complex of CrkII, an adaptor protein, and C3G into lipid rafts. C3G is a guanine-nucleotide exchange factor for the Rho-like GTPase, TC10. Because TC10 is constitutively localized to lipid rafts, this catalyses GTP loading and, consequently, activation of TC10.

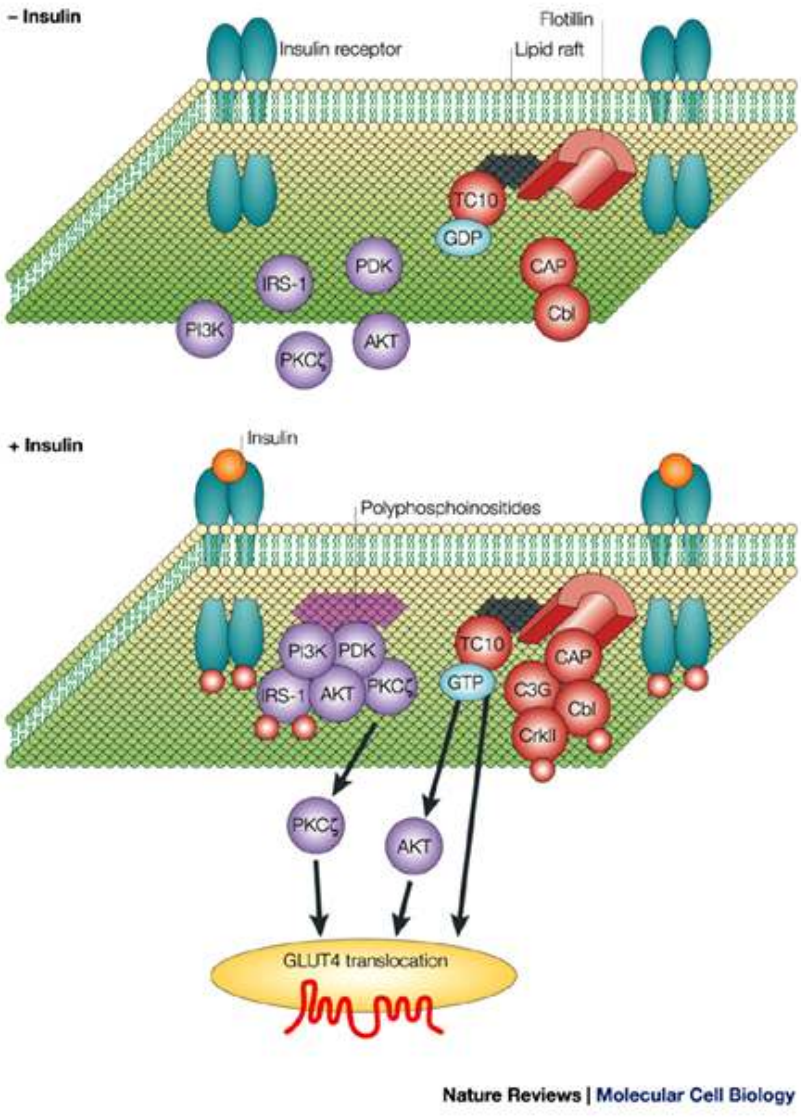

Figure 9. Insulin signalling pathways that control glucose transport in muscle and fat cells. Two discrete signaling pathways have been implicated in insulin-regulated GLUT4

translocation. The first involves the lipid kinase phosphatidylinositol 3-kinase (PI3K), and the second involves the proto-oncoprotein c-Cbl (Bryant et al, 2002).

Intrinsic activation of GLUT4

Can translocation of GLUT4 to the plasma membrane account for the stimulatory effects of insulin on glucose transport in muscle and fat cells, or is its ability to transport glucose also subject to regulation? Several recent studies have shown that insulin- stimulated transport and GLUT4 translocation can be dissociated from each other under certain conditions, which indicates that there might be further means of regulating the transport properties of GLUT4 -a phenomenon previously referred to as 'intrinsic activation'. First, kinetic studies in L6 myotubes have indicated that the insulin-dependent arrival of GLUT4 at the cell surface precedes the increase in glucose uptake by several minutes (Somwar et al 2001a). Intriguingly, a similar effect is not observed in adipocytes (Karnieli et al, 1981, Molero et al, 2001), which raises the possibility that transporters that translocate more slowly might account for the increase in glucose uptake in L6 cells. Second, a discrepancy in the dose-response effects of wortmannin on insulin-stimulated glucose transport compared with GLUT4 translocation have been observed in both 3T3-L1 adipocytes (Hausdorff et al, 1999) and L6 cells (Somwar et al, 2001b). In both of these studies, glucose uptake was inhibited at a much lower dose of wortmannin than GLUT4 translocation, which indicates that these two processes are clearly dissociated. Finally, an inhibitor of the mitogen-activated protein kinase (MAPK) isoform p38 inhibits insulin-stimulated glucose uptake without any apparent effect on GLUT4 translocation (Sweeney et al, 1999). In addition to these studies, several agents such as leptin (Sweeney et al, 2001), isoproterenol (Joost et al, 1986) and dibutyryl cyclic AMP (Lawrence et al, 1992) decrease glucose uptake, whereas cycloheximide (Clancy et al, 1991) and adenosine (Joost et al, 1986) increase glucose uptake without affecting the amount of GLUT4 at the plasma membrane. An important limitation of the intrinsic-activation hypothesis is that a plausible biochemical mechanism for intrinsic activation of GLUT4 is yet to be described. It is most likely that intrinsic activation involves some type of covalent or structural change in GLUT4. Several possible mechanisms, such as phosphorylation (James et al, 1989), nucleotide binding (Piper et al, 1993) and (at least in the case of GLUT1) the formation of homo-oligomers (Zottola et al, 1995) has been proposed.

Moreover, it has been reported that GLUT4 can be detected in both clathrin coated pits (Robinson et al, 1992) and caveolae (Ros-Baro et al, 2001) at the cell surface in adipocytes, and it is possible that within these subdomains, the structure of GLUT4, and consequently its activity, is constrained in some way.

1.2.4 Signaling mechanisms that regulate glucose transport in muscle and fat cells

In muscle and fat cells, glucose transport can be activated by at least two separate pathways. One stimulated by insulin, insulin mimicking agents, and insulin-like growth factors; and the other activated by muscle contraction/exercise and hypoxia (Figure 10). PI3-kinase is involved in the insulin-glucose transport and glucose transporter 4 (GLUT4) translocation activities. While the activation of 5'-AMP-activated kinase (AMPK) has been identified as one of the regulator of insulin-independent glucose transport in response to metabolic stress.

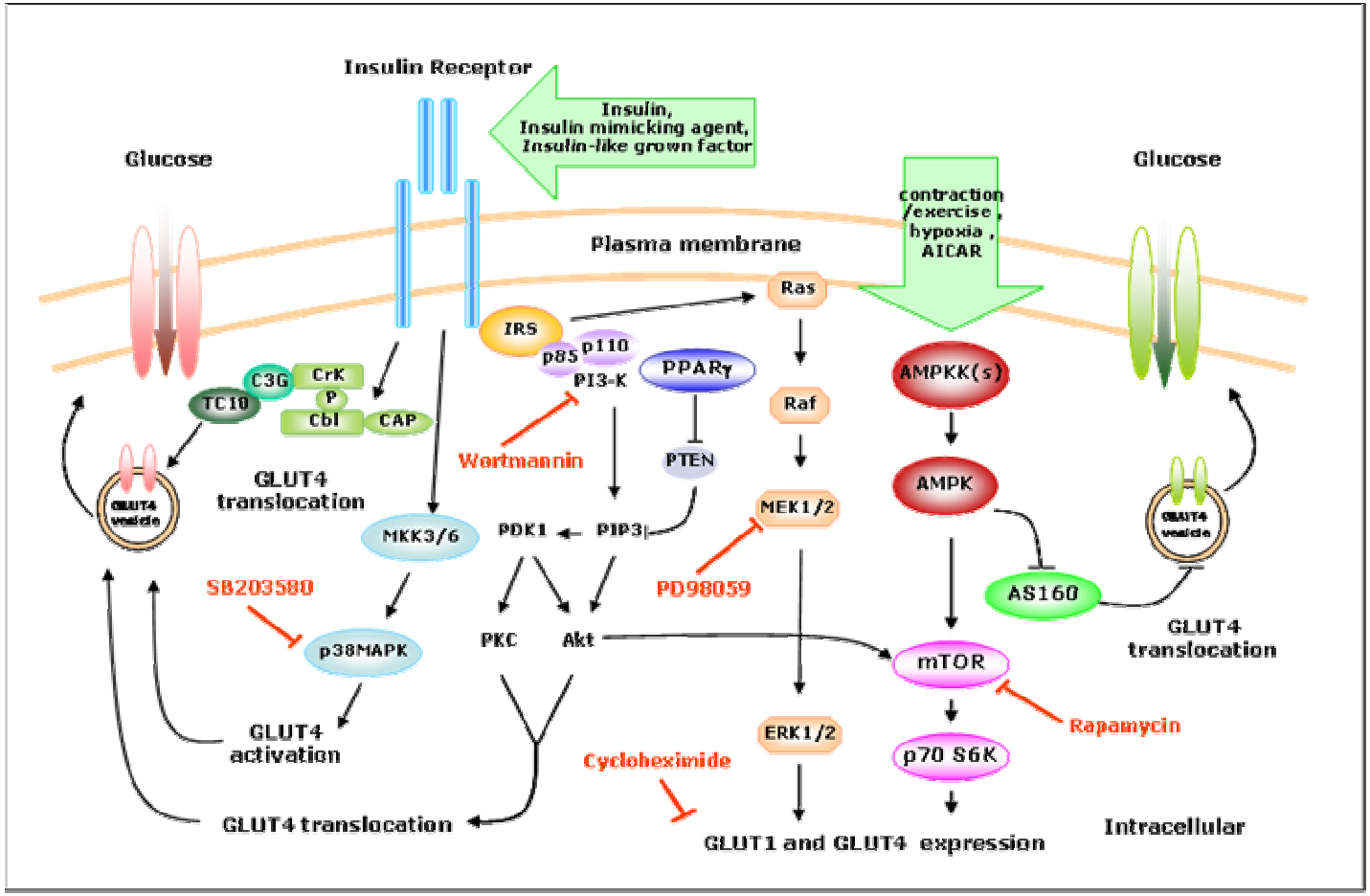

Figure 10. Schematic of pathways regulating glucose transport in adipose tissue and skeletal muscle. Modified from Bryant et al, 2002, Sakamoto and Goodyear, 2002, Furtado LM et al, 2003, Rudich and Klip, 2003, Fujii et al, 2004, Pereira and Lancha, 2004, Dugani and Klip, 2005, and Ishiki and Klip, 2005, Kim KY et al, 2007.

1.2.4.1 Insulin-Dependent Signaling Pathway that control glucose transport via GLUT4 translocation

Insulin signalling commences with binding to the insulin receptor (IR), a tyrosine kinase receptor, composed of two extracellular α-subunits and two transmembrane β-subunits linked by disulphide bonds (Figure 11). The cytoplasmic domain of the β-subunit harbours an ATP-binding site and has low tyrosine kinase activity that is stimulated upon insulin binding to the α-subunits. As a result, the β-subunits undergo intramolecular trans-phosphorylation (Tyr-960) (for review see Van Obberghen et al, 2001). The phosphorylated receptor binds and subsequently phosphorylates tyrosine residues on a number of important proteins such as the insulin receptor substrates (IRS-1/2/3/4), SHC, Grb-1, Gab-1, and APS. In addition, the insulin receptor is subjected to phosphorylation at serine and threonine sites by other kinases and, under most circumstances, such phosphorylation diminishes the tyrosine kinase activity of the receptor as part of feedback or other regulatory mechanisms (Strack et al, 2000).

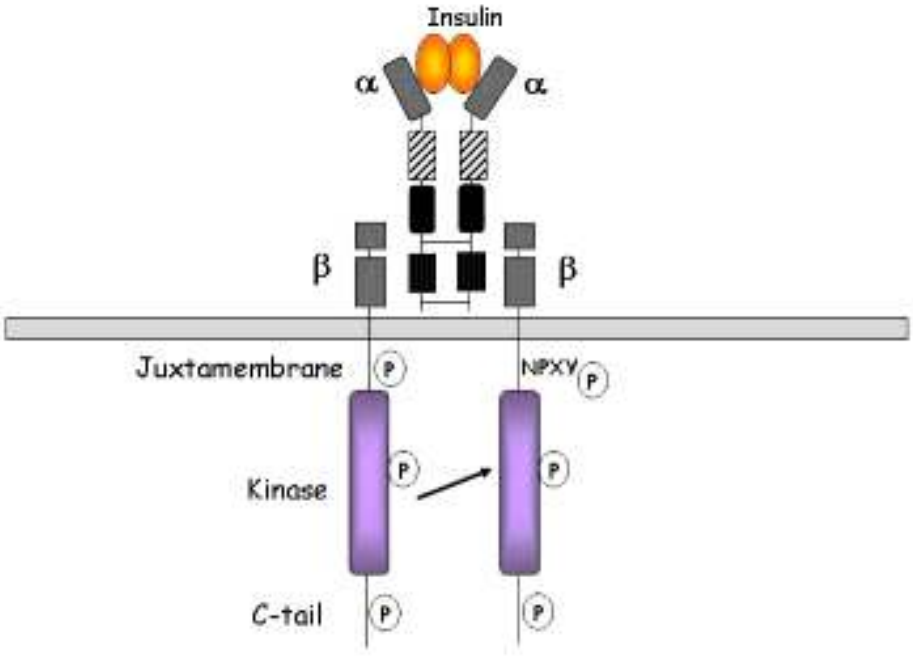

Figure 11. Activation of insulin receptor. The insulin receptor consists of 2 extracellular α subunits that bind insulin and 2 transmembrane β subunits with tyrosine kinase activity.

Insulin binding to the α subunit induces the transphosphorylation of one β subunit by another on specific tyrosine residues in an activation loop, resulting in the increased catalytic activity of the kinase. The receptor also undergoes autophosphorylation at other tyrosine residues in the juxtamembrane regions and intracellular tail. The activated IR then phosphorylates tyrosine residues on intracellular substrates (From Chang et al, 2004).

The IRS proteins are adaptors without intrinsic catalytic activity. Studies ranging from gene knockout in animals to gene silencing in cells culture support the involvement of IRS-1 in insulin-induced peripheral glucose uptake and GLUT4 translocation (Araki et al, 1994, Huang et al, 2005, Yamauchi et al, 1996). Other proteins, particularly IRS-2, may also participate in adipose cell glucose uptake (Fasshauer et al, 2000). Tyrosine phosphorylation of unique motifs on IRS creates docking sites for SH2 domains such as those in SHP-2 protein tyrosine phosphatase, and the small adaptor proteins Grb-2, Crk, and Nck. Critical for metabolic outcomes, IRS phosphorylation on the YMXM motif promotes interaction with the SH2 domain of the p85 subunit of class 1A PI3- kinase, thereby activating the catalytic activity of its p110 subunit. While IRS-1 has a major impact in muscle and fat, and IRS-2 is more relevant in liver and pancreatic beta cells (for review see White, 2002), IRS-3 and IRS-4 appear to contribute little to the metabolic effects of insulin action.

Phosphatidylinositol 3-Kinase-Dependent Signaling pathway

The stimulation of glucose uptake by insulin is mediated by phosphatidylinositol (PI) 3-kinase-dependent and -independent pathways (Watson et al, 2004, Saltiel and Pessin, 2003). Upon tyrosine phosphorylation, IRS proteins interact with the p85 regulatory subunit of PI 3-kinase, leading to the activation of the enzyme and its targeting to the plasma membrane. The enzyme generates the lipid product phosphatidylinositol 3,4,5-trisphosphate (PIP3), which regulates the localization and activity of numerous proteins (Shepherd, 2005). PI 3-kinase activation is attenuated by PIP3 dephosphorylation via 3′ phosphatases such as PTEN (Maehama and Dixon, 1999) or 5′ phosphatases such as SHIP2 (Pesesse et al, 1997, Habib et al, 1998).

In mammals, PI3 kinases comprise of three isoforms of p110 (α, β, and δ) and at least eight regulatory subunits generated by alternative splicing of three genes:

p85α, p85β, and p55γ. Although PI3-kinase is a dual specificity enzyme displaying lipid and serine kinase activity, its major action is towards phosphoinositides. In vitro, the enzyme phosphorylates the D-3 position on the inositol ring of phosphoinositides to generate phosphatidylinositol-3-phosphate (PI-3-P), phosphatidylinositol- 3,4-bisphosphate (PI-3,4-P2), and phosphatidylinositol- 3,4,5-trisphosphate (PI-3,4,5-P3). However, upon in situ insulin-stimulation, the major product is PI- 3,4,5-P3 (for review see Fruman et al,1998). Through diverse pharmacological and molecular strategies, it was demonstrated that class IA PI3-kinase is essential for insulin-stimulated recruitment of GLUT4 to the plasma membrane and its consequent increased in glucose uptake.

PI3-kinase plays an essential role in glucose uptake and GLUT4 translocation. Inhibition of the enzyme with pharmacological inhibitors such as wortmannin completely blocks the stimulation of glucose uptake by insulin (Okada et al, 1994). In addition, overexpression of dominant-interfering forms of PI 3-kinase can block glucose uptake and GLUT4 translocation, and overexpression of constitutively active forms can partially mimic insulin action (Martin et al,1996, Sharma et al,1998). Targeted deletion of the p85 PI 3-kinase regulatory subunit in mice results in increased insulin sensitivity (Ueki et al 2003, Mauvais-Jarvis et al. 2002, Terauchi et al, 1999). Conversely, gene knockout of the catalytic subunit results in insulin resistance and glucose intolerance (Brachmann et al, 2005). Collectively, these studies demonstrate that PI 3-kinase is required for insulin-stimulated glucose transport.

Insulin-stimulated increases in PIP3 result in the recruitment and/or activation of pleckstrin homology (PH) domain-containing proteins, including various enzymes, their substrates, adapter molecules, and cytoskeletal proteins. Among these is the Ser/Thr kinase PDK1, which phosphorylates and activates several downstream kinases, including Akt1 through 3, protein kinase C (PKC)ζ/Λ, and serum and glucocorticoid-inducible kinase (SGK) (Mora et al, 2004). PIP3 appears to mediate the translocation of Akt to the plasma membrane, via its PH domain (Corvera and Czech, 1998). Additionally, the protein kinase mTOR (mammalian target of rapamycin), complexed to the regulatory protein rictor, has been recently identified as PDK2 (phosphoinositide- dependent kinase 2), which phosphorylates Ser473 on Akt (Sarbassov et al, 2005). Overexpression of a membrane-bound form of Akt in 3T3L1 adipocytes resulted in increased glucose transport and localization of GLUT4 to the plasma membrane (Kohn et al, 1998, Kohn et al,

1996). Insulin-stimulated GLUT4 translocation was also inhibited by expression of a dominant-interfering Akt mutant (Wang et al. 1999, Cong et al. 1997).

PI3-K-Independent Signaling pathway: The CAP/Cbl pathway and lipid rafts

Several studies have shown that insulin-stimulated GLUT4 translocation operates independently of PI3K and involves a dimeric complex that comprises c-Cbl and the c-Cbl-associated protein CAP (Figure 12) (Ribon and Saltiel, 1997, Bryant et al, 2002 and Liu et al, 2003). Cbl and the adaptor protein CAP are recruited to the insulin receptor by APS (Liu et al, 2002). Once tyrosine phosphorylated by the receptor, Cbl can recruit the adaptor protein CrkII to lipid rafts, along with the guanyl nucleotide exchange factor, C3G (Chiang et al, 2001). C3G can then activate the GTP-binding protein TC10, which resides in lipid rafts (Watson et al, 2001). The correct spatial compartmentalization of these signaling molecules in the lipid raft microdomain appears to be essential for insulin-stimulated GLUT4 translocation and glucose transport, as these insulin-mediated events are abolished by dominant-interfering mutants of CAP that prevent the localization of Cbl to lipid rafts (Baumann et al, 2000). However, the involvement of this pathway has been debated by recent RNA interference-based analysis of Cbl, CAP, and CrkII function in regulation of GLUT4 by insulin (Mitra et al, 2004). Gene silencing of Cbl or CAP or CrkII in 3T3-L1 adipocytes fails to attenuate insulin-stimulated glucose transport or myc-tagged GLUT4-GFP translocation at either sub-maximal or maximal concentrations of insulin. The dose response relationship for insulin stimulation of glucose transport in primary adipocytes derived from Cbl knockout mice is also identical to insulin action on adipocytes from wild type mice (Mitra et al, 2004). Furthermore, the likelihood that the Cbl cascade plays an important role in GLUT4 regulation is also dampened by studies in skeletal muscle examining this pathway (Thirone et al, 2004, JeBailey et al, 2004 and Wadley et al 2004).

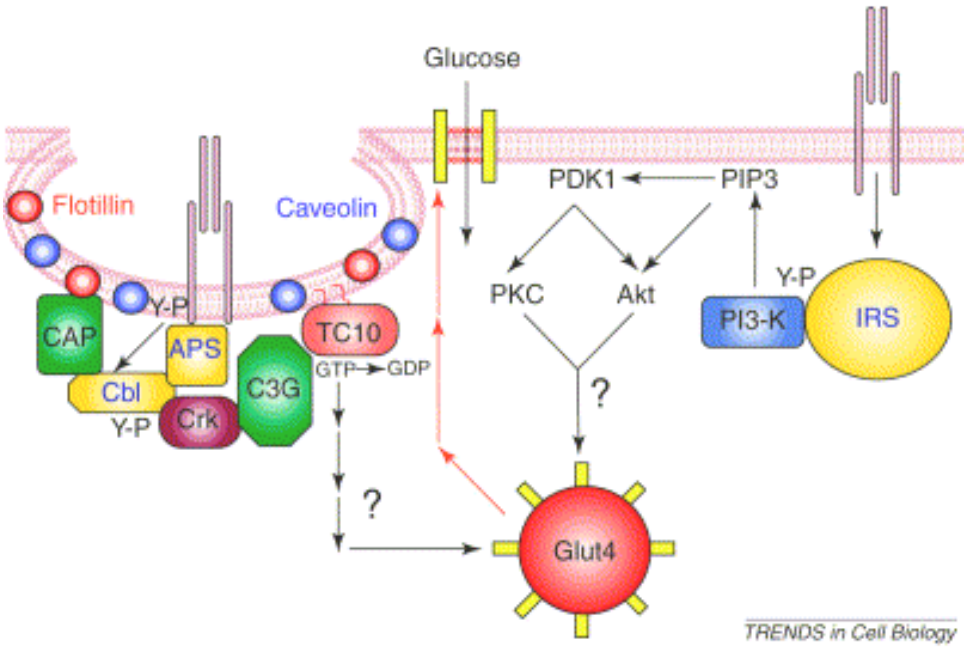

Figure 12. TC10 pathway regulated GLUT4 translocation. A separate pool of the insulin receptor can also phosphorylate the substrates Cbl and APS. Cbl interacts with CAP, which can bind to the lipid raft protein flotillin. This interaction recruits phosphorylated Cbl into the lipid raft, resulting in the recruitment of CrkII. CrkII binds constitutively to the exchange factor C3G, which can catalyze the exchange of GDP for GTP on the lipid-raft-associated protein TC10. Upon its activation, TC10 interacts with a number of potential effector molecules, including CIP4, Exo70, and Par6/Par3/PKCλ, in a GTPdependent manner. (Saltiel and Pessin, 2002)

1.2.4.2 Insulin-Dependent Signaling Pathway that control glucose transport via GLUT4 intrinsic activation: p38 Mitogen-Activated Protein Kinase (MAPK) pathway

p38 (also known as CSBP, mHOG1, RK, and SAPK2) is the archetypal member of the second MAPK-related pathway in mammalian cells (Han et al, 2002, Lee et al, 1994). The p38 module consists of several MAPKKKs, including MEKKs 1 to 4, (MEKK1-4), MLK2 and -3, DLK, ASK1, Tpl2 (also termed Cot), and Tak1, the MAPKKs MEK3 and MEK6 (also termed MKK3 and MKK6, respectively), and the four known p38 isoforms (α,β,γ and δ) (Figure 13) (reviewed in reference Kyriakis and

Avruch, 2001). In mammalian cells, the p38 isoforms are strongly activated by environmental stresses and inflammatory cytokines but not appreciably by mitogenic stimuli. Most stimuli that activate p38 also activate JNK, but only p38 is inhibited by the anti-inflammatory drug SB203580, which has been extremely useful in delineating the function of p38 (Lee et al, 1994).

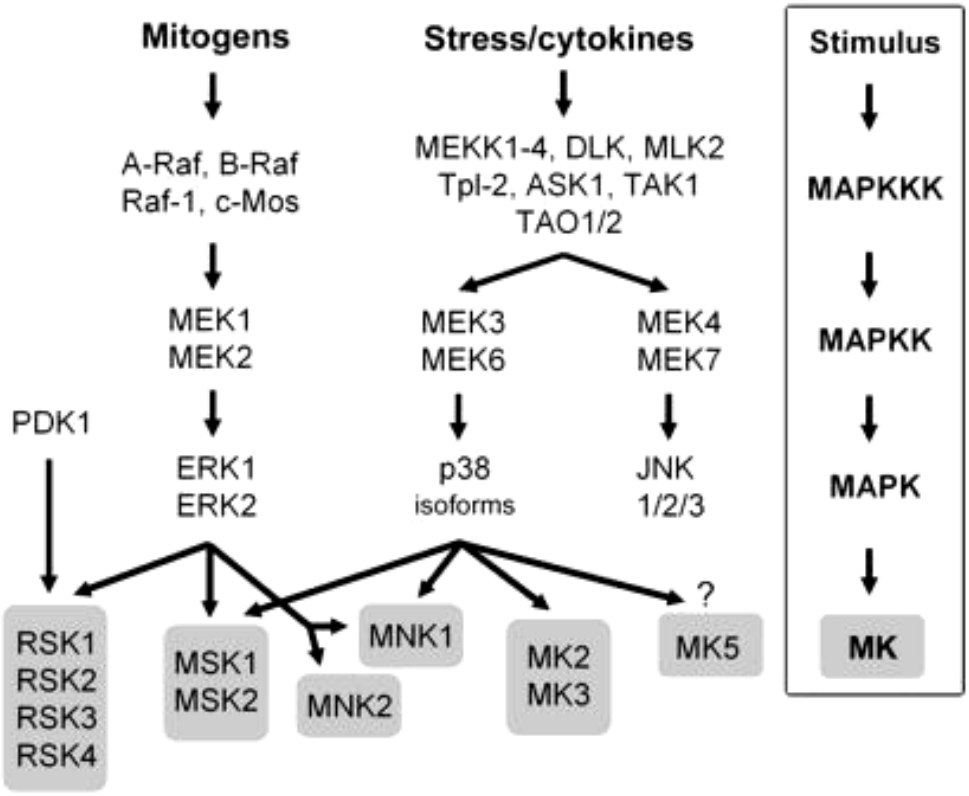

Figure 13. Signaling cascades leading to activation of the MKs. Mitogens and cellular stresses lead to activation of the ERK1/2 and p38 cascades, which in turn phosphorylate and activate the five subgroups of MKs. (reviewed in Kyriakis and Avruch, 2001)

The pyridinyl imidazoles SB202190 and SB203580 are the most widely used inhibitors of p38a MAPK and b (Lee et al. 1994). These compounds inhibit p38α and p38β MAPK but not the γ and δ isoforms (Kumar et al. 1999). In contrast, a structural analogue, SB202474, is a poor inhibitor of p38 MAPKs (Lee et al. 1994). Although the Ki for inhibition of p38 MAPK by SB203580 and SB202190 is in the nanomole range, concentrations in the range 1-10 μM are required to inhibit the enzyme

in intact cells (Eyers et al. 1999). At 10 μM, SB202190 or SB203580 fully inhibit p38α and β MAPK in diverse cell types.

In a series of studies, it made the unexpected discovery that pre-incubation of L6 myotubes or 3T3-L1 adipocytes with SB202190 or SB203580 reduced insulin-induced 2-deoxyglucose uptake by a maximum of 60% yet did not alter basal uptake (Sweeney et al. 1999, Somwar et al. 2001). Pre-incubation of L6 myotubes with SB202474 -inactive towards p38 MAPK -did not result in any significant change in 2-deoxyglucose uptake under basal or insulin-stimulated conditions. Pre-incubation of cells with the active SB203580 also reduced the stimulation of 3-O-methylglucose uptake to the same extent as 2-deoxyglucose uptake (Somwar et al. 2001), suggesting that these pyridinyl imidazoles reduce the stimulation of glucose transport and not of post-transport glucose phosphorylation. The dose-response of inhibition of glucose uptake paralleled the inhibition of phosphorylation of endogenous targets of p38 MAPK (Somwar et al. 2001). SB202190 and SB203580 also reduced the insulin response of glucose uptake in isolated skeletal muscle (Somwar et al. 2000).

Two azaazulene inhibitors of p38 MAPK, A291077 and A304000, produced similar results to those achieved with pyridinyl imidazole inhibitors in that pre-incubation of L6 myotubes with these inhibitors reduced insulin-stimulated hexose uptake (Somwar et al., 2002). These azaazulene derivatives are chemically distinct from the pyridinyl imidazole inhibitors, strengthening the concept that inhibition of p38 MAPK by diverse agents reduces insulin's ability to stimulate glucose uptake.

The most striking observation arose from measurements of cell surface GLUT4 levels, detected via the exposed myc epitope. Pre-incubation of cells with SB202190, SB203580, A291077 or A304000 did not alter the 2.5-fold increase in plasma membrane GLUT4myc in response to insulin. This held true for inhibitors used in concentrations of up to 50 μM. These results indicate that the reduction in insulin-stimulated glucose uptake in cells pre-incubated with p38 MAPK inhibitors was not because of a decrease in the number of GLUT4 translocated to the plasma membrane. The reduction in insulin response was also not because of a direct inhibition of GLUT4 or GLUT4myc by the pyridinyl imidazoles or azaazulene derivatives. This is because: (1) neither compound affected basal glucose uptake, yet this function is mediated by GLUT4myc in L6 myotubes ; (2) the agents inhibited glucose uptake only when given to

cells prior to and along with insulin, but not when added directly to the glucose transport assay for equivalent times (Somwar et al. 2002).

The above evidence further prompts the hypothesis that insulin increases glucose uptake not only through the recruitment of transporters to the plasma membrane but also by increasing the activity of these transporters. This activation step is prevented by inhibitors of p38 MAPK.

1.2.4.3 Insulin-Dependent Signaling Pathway that control glucose transport via GLUT1 transcription

The mammalian ERK1/2 module, also known as the classical mitogen kinase cascade, consists of the MAPKKKs A-Raf, B-Raf, and Raf-1, the MAPKKs MEK1 and MEK2, and the MAPKs ERK1 and ERK2 (Figure 13). ERK1 and ERK2 have 83% amino acid identity and are expressed to various extents in all tissues (reviewed in reference Chen et al, 2001). They are strongly activated by growth factors, serum, and phorbol esters and to a lesser extent by ligands of the heterotrimeric G protein-coupled receptors, cytokines, osmotic stress, and microtubule disorganization (Lewis et al, 1998). MEKK1/2/3 and c-Mos kinases are also known to act as MAPKKKs in this pathway. While the proto-oncogene c-mos appears to play an important role during meiosis, gene disruption studies suggest that MEKK1/2/3 may have limited impact on or redundant contributions to activation of the ERK1/2 pathway (Xia et al, 2000, Yujiri et al, 1998).

ERK1/2 are distributed throughout quiescent cells, but upon stimulation, a significant population of ERK1/2 accumulates in the nucleus (Chen et al, 1992, Gross et al, 1999, Lenormand et al, 1993). While the mechanisms involved in nuclear accumulation of ERK1/2 remain elusive, nuclear retention, dimerization, phosphorylation, and release from cytoplasmic anchors have been shown to play a role (reviewed in reference Pouyssegur, 2002). ERK1/2 signaling has been implicated as a key regulator of cell proliferation, and for this reason, inhibitors of the ERK pathway are entering clinical trials as potential anticancer agents (Kohno and Pouyssegur. 2003). Two structurally unrelated compounds are commonly used to specifically inhibit the ERK1/2 pathway in cultured cells. Both U0126 and PD98059 are noncompetitive inhibitors of MEK1/2/5 and prevent stimulation-mediated activation of ERK1/2/5 (reviewed in reference Ballif and Blenis, 2001).

As is the case for other growth factors, insulin stimulates the mitogen-activated protein (MAP) kinase extracellular signal regulated kinase (ERK). This pathway involves the tyrosine phosphorylation of IRS proteins and/or Shc, which in turn interact with the adapter protein Grb2, recruiting the Son-of-sevenless (SOS) exchange protein to the plasma membrane for activation of Ras. The activation of Ras also requires stimulation of the tyrosine phosphatase SHP2, through its interaction with receptor substrates such as Gab-1 or IRS1/2. Once activated, Ras operates as a molecular switch, stimulating a serine kinase cascade through the stepwise activation of Raf, MEK and ERK. Activated ERK can translocate into the nucleus, where it catalyses the phosphorylation of transcription factors such as p62TCF, initiating a transcriptional programme that leads to cellular proliferation or differentiation (Boulton et al, 1991). Blockade of the pathway with dominant negative mutants or pharmacological inhibitors prevents the stimulation of cell growth by insulin, but has no effect on the metabolic actions of the hormone (Lazar et al, 1995).

1.2.4.4 Insulin-Independent Signaling Pathway : 5′-AMP-Activated Kinase(AMPK) Signaling

AMPK is the mammalian homologue of the sucrose non fermenting 1 protein kinase in yeast, which plays a key role in the adaptation of yeast to nutrient stress (Hardie et al. 1998). AMPK is a heterotrimer formed by an α subunit, which contains the catalytic domain, and by the β and γ subunits, which are important in maintaining the stability of the heterotrimer and for substrate specificity (Hardie et al. 1998, Kemp et al. 1999). AMPK is activated through Thr172 phosphorylation of the a subunit by one or more upstream kinases (AMPK kinase) and allosterically by increases in AMP:ATP and creatine:phosphocreatine (Hardie et al. 1998, Kemp et al. 1999). The α subunit has two known isoforms (α1 and α2) and contains the catalytic domain that transfers a high-energy phosphate from ATP to serine and threonine residues on a number of different target proteins. The α subunit also contains the specific threonine residue (Thr172) that functions as an activating phosphorylation site for one or more upstream AMPK kinase. Multiple isoforms of β (β1, β2) and γ (γ1, γ2, γ3) regulatory subunits have also been identified, which are essential for full enzymic activity and may have many other functions, including localization of the AMPK molecule within the cell and binding to glycogen.

While AMPK is regarded as a ubiquitous enzyme, the expression pattern of individual α, β and γ subunits occurs in a tissuespecific manner. Thus, the composition of the AMPK heterotrimer can vary greatly from tissue to tissue.

Both the α1 and α2 catalytic isoforms are expressed in skeletal muscle. These isoforms share 90% amino acid sequence identity in the catalytic domain and 60% amino acid sequence identity outside this domain. Both isoforms have a molecular mass of approximately 63 kDa (Stapleton et al. 1996, Thornton et al. 1998). The α2 isoform represents approximately 66% of the total expression level of α subunit mRNA in human skeletal muscle (Fujii et al. 2000, Musi et al. 2001a). Although both α1 and α2 isoforms are distributed throughout the cytosol of skeletal muscle, there is some evidence that a substantial percentage of α2 AMPK is associated with nuclei (Salt et al. 1998, Ai et al. 2002), suggesting that this isoform may also play a role in regulating gene transcription. The α1 and α2 isoforms are present in muscles composed of both slow-twitch (type I) and fast-twitch (type IIa and IIb) fibres.

The β regulatory subunits of AMPK have an approximate molecular mass of 30-40 kDa and at least the β1 subunit is capable of being modified post-translationally by phosphorylation and myristoylation (Warden et al. 2001). These modifications may enable the β subunits not only to regulate AMPK catalytic activity, but also to target the AMPK heterotrimer to the nuclei, cytoplasm and membranes within cells. Fast-twitch muscle fibres (types IIa and IIb) appear to contain both β1 and β2 AMPK, whereas red slow-twitch fibres may contain only the β1 isoform (Chen et al. 1999a).

AMPK γ subunits may function in the regulation of AMPK catalytic activity and sensitivity to AMP (Cheung et al. 2000). Several γ subunit mutations have been identified and can have profound effects on skeletal muscle fuel reserves. Both mRNA (Cheung et al. 2000) and protein (Durante et al. 2002) of γ1, γ 2 and γ3 isoforms have been detected in skeletal muscle, although the fibre-type expression pattern of individual isoforms is not entirely clear. The γ1 isoform has been shown to be similarly expressed in red and white muscle, γ2 is weakly expressed in red muscle and abundantly expressed in white muscle and γ3 has been detected only in red muscle (Durante et al. 2002).

AMPK activation stimulates GLUT 4 translocation. There is a considerable amount of data supporting the hypothesis that AMPK plays a role in the stimulation of

glucose uptake. AMPK is indispensable for the effects of AICAR and hypoxia to stimulate muscle glucose uptake. This enzyme also seems to play an important role in contraction-stimulated glucose uptake into muscle; however, other pathways may be involved in regulating this process as well (Figure 14). Currently, the search for downstream molecules regulating AMPK mediated glucose uptake is an area of intense research. The effects of AMPK on glucose metabolism in the muscle and other organs such as the liver make this molecule an attractive target for pharmacological treatment of type 2 diabetes.

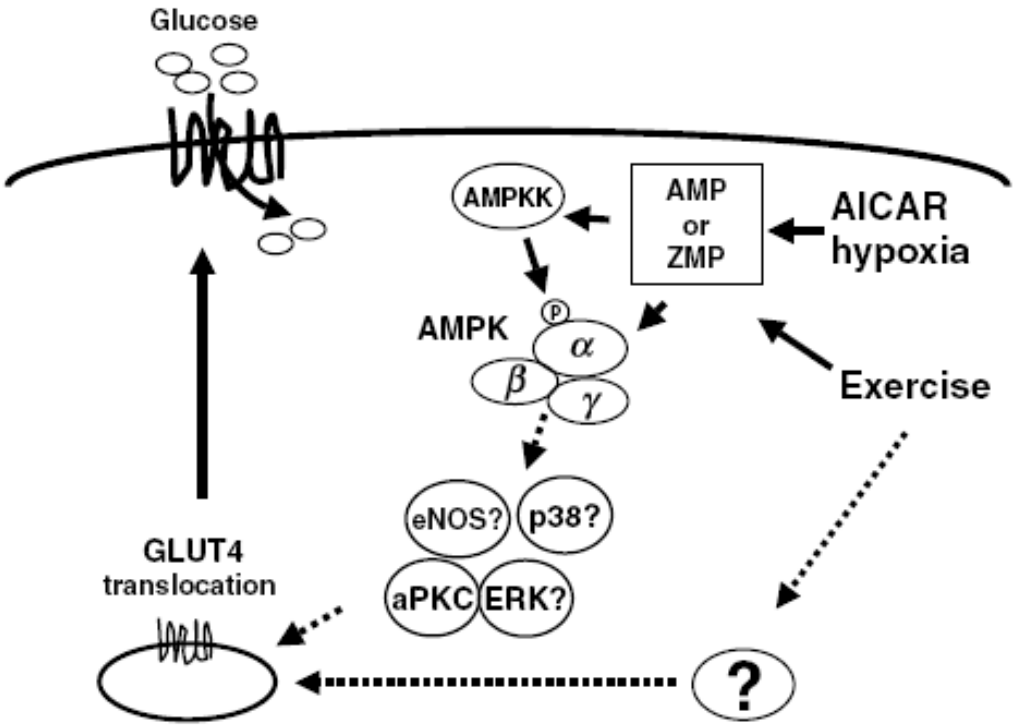

Figure 14. Stimulation of glucose uptake by AMPK. Hypoxia and AICAR lead to increased glucose uptake by activating AMPK through mechanisms that involve phosphorylation by an upstream kinase (AMPKK) and allosteric modification. AMPK, by acting on unidentified targets, then causes glucose transporter translocation. Exercise stimulates cellular glucose uptake partially through AMPK, however, other unknown mechanisms may also be involved. AMPK, AMP-activated protein kinase; AMPKK, AMPK-kinase; AICAR, 5-aminoimidazole-4-carboxamide ribonucleoside; aPKC, atypical protein kinase C isoforms; eNOS, endothelial nitric oxide synthase; ERK, extracellular signal-regulated kinase; p38 MAPK, p38 mitogen-

activated protein kinase; ZMP, 5-aminoimidazole-4- carboxamide ribonucleotide. (Musi and Goodyear, 2003)

In clone 9 cells, 3T3 L1 pre-adipocytes, and C2C12 myoblasts, cells which only express GLUT1 glucose transporters, 5-aminoimidazole-4-carboxamide ribonucleoside (AICAR) stimulates glucose uptake but does not increase GLUT1 content in the plasma membrane (Abbud et al. 2000). Thus, the authors propose that in these cells AICAR stimulates glucose uptake by activating pre-existing plasma membrane GLUT1 transporters. However, in cultured papillary muscle cardiomyocytes (Russell et al. 1999) and perfused rat hindlimb muscles (Kurth-Kraczek et al. 1999) AICAR leads to increased GLUT4 translocation. Furthermore, overexpression of consituvely active AMPK in H-2K^{b} skeletal muscle cells stimulates GLUT1 and GLUT4 translocation accompanied with increased glucose uptake (Fryer et al. 2002). It is therefore quite likely that AMPK stimulates glucose uptake by increasing glucose transporter translocation (Fryer et al. 2002).

While increasing evidence indicates that activation of AMPK increases cellular glucose uptake, downstream molecules involved in this process have not been clearly identified. As shown in Figure 14, nitric oxide (NO) has been implicated in the regulation of muscle glucose uptake during exercise (Balon, 1999). The p38 mitogen-activated protein kinase (MAPK) has also been proposed to be involved in contraction stimulated glucose uptake (Somwar et al. 2000). It has recently been proposed that the effects of AMPK on glucose uptake are mediated through the extracellular signal-regulated kinase (ERK), proline-rich tyrosine kinase-2 (PYK-2), phospholipase D (PLD) and by atypical protein kinase C (aPKC) isoforms (Chen et al. 2002). Therefore, further research is needed to clarify the potential involvement of these molecules in AMPK-mediated metabolic responses.

AMPK activation regulates gene expression. In Sprague-Dawley rats, a swimming exercise stimulated peroxisome proliferator-activated receptor γ co-activator-1 (PGC-1) gene expression in muscle, and this effect was reproduced by incubating isolated muscles with AICAR for 18 h (Terada et al, 2002). This suggests that AMPK may induce exercise-stimulated mitochondrial biogenesis through PGC-1, and gives further support to the hypothesis that AMPK is involved in the cellular adaptations to exercise training by regulating gene transcription. Similar to the effects of exercise training, both in vivo and in vitro chronic AICAR treatment in rats increases GLUT4 protein content and

hexokinase activity (Holmes et al, 1999 and Ojuka et al, 2000). In line with this, AICAR treatment rapidly induced GLUT4 gene transcription in muscle within a few hours of administration (Zheng et al, 2001).

1.2.5 Diabetes treatment option

Diabetes physicians have traditionally focused on treatments to reduce hyperglycemia and the potential treatments for lowering glucose shall be considered here in a rather theoretical way because this may illuminate the mechanisms by which some of the evaluated plant treatments can have an effect.

The general mechanisms for potential diabetes treatments were shown in Table 5.

Table 5 The general mechanisms for potential diabetes treatments

Mechanism	example
Delayed gastric emptying time	Glucagon-like peptide 1 (GLP-1), Cholecytokinin(CCK)
Reduce absorption of dietary carbohydrate	Alpha-glucosidase inhibitor (acarbose), Guar gum
Increase pancreatic insulin secretion	Sulphonylureas, glitinides, GLP-1, liraglutide, exendin-4
Insulin replacement	Insulin injection, pancreas transplant
Insulinomimetic agents, Reduce hepatic glucose output	Metformin, insulin, glucagon antagonists
Increase insulin sensitivity	Metformin, thiazolidinediones , exercise

Source: Baynes, 2006

The rising incidence and prevalence of diabetes, especially in developing countries, means that governments and individuals will be faced with serious challenges. People without access to modern medicines, as well as those in developed countries who avoid drug use, will look for naturally occurring, botanically based therapies. Phytomedicines may be more culturally acceptable as diabetic treatments and easier to

incorporate into a lifestyle than taking a tablet or giving injections. The use of medicinal plant on a daily basis, however, may not be possible because of the seasonal unavailability of the fresh produce and their unpalatability.

The continuing surge in prevalence of diabetes mellitus worldwide will demand new solutions to be found for prevention or treatment. Since man dose not appear inclined to improve its exercise habits to avoid corpulence, the emphasis over the next few decades is likely to be more on treating diabetes and perhaps screenings for asymptomatic individuals. Despite major advances in scientific knowledge about whole-body physiology and the molecular mechanisms involved in glucose homeostasis; this has not been translated into an armamentarium of new drug treatments for diabetes. Discovery of compounds with antidiabetic activity in traditional plant remedies remains a medically legitimate and potentially a commercially rewarding activity.

1.2.5.1 Plants for Glycemic Control in Diabetes

According to the world ethnobotanical information on medicinal plants, there are about 1,000 plants used in the control of diabetes mellitus (Alarcon-Aguilara et al., 1998). In areas of the developing world where conventional medicines are not readily available, traditional treatments for diabetes remain the major form of therapy. However, few of these plants used in traditional remedies have undergone comprehensive scientific investigation, and the World Health Organization has recommended that research focus should now be directed in this area.

In Thailand, there are many plants traditionally used by locals to treat diabetes. In 1971, investigators from the Ministry of Public Health, Bangkok and the Federal Republic of Germany, under the program to evaluate Thai medicinal herbs, first reported the hypoglycemic activity of 12 of Thai medicinal plants said to cure diabetes. The results were shown that in no case could any remarkable action be established. Therefore, they summarized that if some of these drugs really can be benefitial to the diabetic patient, the action can not be of an insulin-like nature (Mueller-Oerlinghausen B et al, 1971). In more currently studies, it has been shown that the 4-hydroxybenzoic acid, a constituent of the root of *Pandanus odorus* Ridl. Pandanaceae (Thai name: Toei-hom) decreased the plasma glucose levels in diabetic rats (Peungvicha et al, 1998a), while

water extract of the whole plant of *Piper sarmentosum* Roxb., locally known as Chaplu, decreased blood sugar levels in streptozotocin-diabetic rats (Peungvicha et al,1998b).

1.2.5.2 *Tinospora crispa* Miers ex Hook. f. & Thom.

Botanical description

Tinospora crispa (Linn.), a plant of Menispermaceae family, is a greenish-grey woody climber with warted stem of 1-1.5 cm in diameter and very long filiform aerial roots (shown in Figure 15). The simple leave of cordate-shape with acuminate apex has 5-7 nerves arising from the base.The small pale-yellow flowers, either solitary raceme or fasicled of 5-20 cm long is usually arising from the older leafless stem. Pubescence consists of six sepals, petals, and stamens. Fruits are ellipsoidal drupe, containing seeds with aril pulp (Hook, 1975).

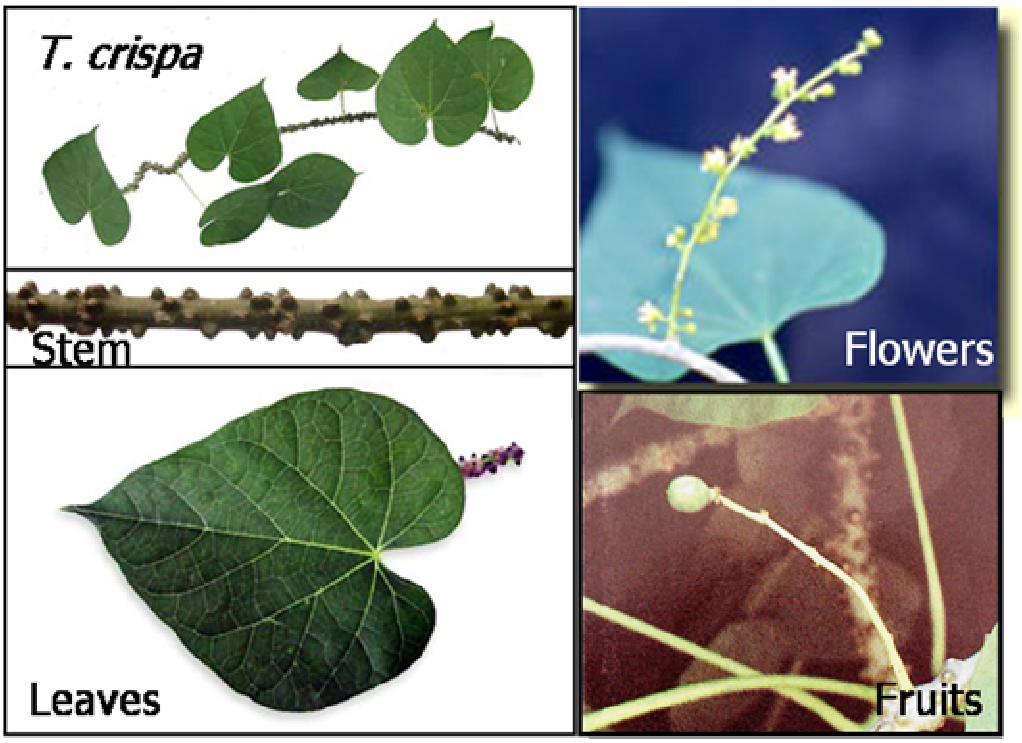

Figure 15. *Tinospora crispa* Miers ex Hook. f. & Thom. (Menispermaceae)

Ecology and distribution

Boraphet, the common name in Thai of this plant, can be found in evergreen forests all over the country. It distributes in moist, mixed deciduous forests and grown in houses. The plant is also found in tropical and subtropical India, in tropical Asia at altitudes up to 1000 meters, in Philippines, Indonesia, Malaysia and Vietnam. (Forman, 1991)

The efficacies of *T. crispa* on ethnophamacological used reviewed from of the claimed efficacies in Thai traditional medicine reported by Farnsworth and Bunyapraphatsara, 1992 are shown in Table 6.

Table 6 The ethnomedicinal uses of *T. crispa* reported by Farnsworth and Bunyapraphatsara, 1992

Parts of uses	Ethnomedicinal uses
Roots	Alleviation of toxicity from fever, Enhancing appetite, Treatment of cerebral malarial, Fever due to abnormalities of a combination of the following origins (Semha, Pitta, Wata, Kamdao, Lohita), Septicemia
Stem	Longevity, Health promotion, Enhancing the appetite, Treatment of septicemia, Exanthematous fever, Cerebral malarial, Complications of fever during smallpox, Severe disorders of Lohita, Thirsty, Hiccough, Small pox, Antipyretic, Element tonic
Leaves	Longevity, Sweet voice, Decrease of obesity, Treatment of septicemia, Exanthematous fever, Abscess pain, Skin diseases, Pruritic rash and Cerebral concussions, Antipyretic, Antimalarial, Antihelmintic, Element tonic, Skin tonic
Flowers	Treatment of diseases of the oral and ear cavities, Antihelmintic,
Fruits	Treatment of mucous bloody stools, Septicemia, Hiccough
Not specified part used	Longevity, Enhancing appetite, Decrease of obesity, Dental care, Treatment of bodily discomfort, fever, headache, hemorrhoids, Uterine infections, Cervicitis, Oropharyngeal systems from gastroenteric diseases, Biliary diseases, Antidiabetic , Antimalarail.

Chemical composition

The chemical constituents of *T. crispa* previously reported in literatures is summarized in Table 7

Table 7 Chemical composition of *T.crispa*

Parts	Groups of chemical	Chemicals	References
Stems	Alkaloid	Palmatine	Bisset and Nwaiwu,1983
		Berberine	Bisset and Nwaiwu,1983
		Jatrorrhizine	Bisset and Nwaiwu,1983
		Tembetarine	Bisset and Nwaiwu,1983
		Choline	Bisset and Nwaiwu,1983
		N-trans-feruloytryramine	Fukuda et al.,1983
		N-cis-feruloyltyramine	Fukuda et al.,1983
		N-formylannonaine	Pachaly et al.,1992
		N-formylnornuciferine	Pachaly et al.,1992
		N-acetylnornuciferine	Pachaly et al.,1992
	Diterpenoid glycoside	Borapetoside A	Fukuda et al.,1986
	Furanoditerpene	Borapetoside B	Murakoshi et al.,1993
	Glycoside	Tinocrisposide	Fukuda et al.,1983
		Borapetoside C-G	Fukuda et al.,1986
		Borapetoside H	Fukuda et al.,1986
	Phenolic glycoside	Tinotuberide	Fukuda et al.,1983
	Diterpenoid	Borapetol A	Fukuda et al.,1986
		Borapetol B	Murakoshi et al.,1993
	Monolignan	Siringin	Murakoshi et al.,1993
	Phenolic compound	Secoisolariciresinol	Cavin et al.,1997
Leaves	Alkaloid	Palmatine	Bisset and Nwaiwu,1983
	Furanoid diterpenes	Tinotufolin A-B	Fukuda et al.,1993
		Tinotufolin C-F	Fukuda et al.,1993
	Other groups	Galactans	Sinha, 1960
		Mathylpentosan	Sinha, 1960
Root	Alkaloid	Berberine	Bisset and Nwaiwu,1983
Branch	Alkaloid	Palmatine	Bisset and Nwaiwu,1983

Pharmacological activities

Antiglycemic Activity

The efficacy of aqueous extarct *T. crispa* to improve diabetic conditions by virtue of its action on endocrine pancreas in alloxan-induced diabetic (Wistar) rats was firstly demonstrated by Noor and Ashcroft in 1989. The antiglycemic effect was significantly observed ($p<0.001$) with concomitant improvement of glucose tolerance and insulinaemia ($p<0.01$), after two-week treatment with the aqueous extract of *T. crispa* (4 g/l of drinking water). Moreover, the acute intravenous administration of the extract (50 mg/kg) also significantly increased plasma insulin levels when compared to controls ($p<0.05$) (Noor and Ashcroft, 1989).

Noor and co-workers went on to demonstrate *in-vitro* insulinotropic action of *T. crispa* using isolated human or rat islets of Langerhans and HIT-T15 cells. In static incubations with rat islets and HIT-T15 cells, the extract (at 0.01, 0.1 and 1.0 mg/ml) induced a significant dosage dependent stimulation of insulin secretion at basal (2 mmol/l glucose) and potentiation of 10 mmol/l glucose-stimulated insulin release. The insulino tropic effect was also evident in perifused human islets with basal medium (2 mmol/l glucose) containing 1 mg/ml of extract from *T. crispa*. The insulin secretory rates were rapidly returned to basal levels on the removal of either the extract or 10 mmol/l glucose (Noor H, et al, 1989).

The antiglycaemic properties of *T. crispa* is neither due to interference with intestinal glucose absorption nor enhanced peripheral uptake by adipose tissues. It's stimulation of insulin release is physiologic and probably via modulation of beta-cell Ca^{2+} concentration (Noor and Ashcroft, 1998).

In Thailand, the significant antidiabetic effects of both aqueous and solvent extracts from *T. crispa* were also demonstrated in alloxan-induced diabetic (Sprague-Dawley) rats using 500, 250 and 150 mg/kg BW of aqueous, ethanol and n-butanol fractions, respectively (Anulakanapakorn et al, 1998).

Antibacterial activity

The antibacterial activities of aqueous (AETT), ethanol (EETT) and chroloform (CETT) extracts of *T. crispa* against selected Gram positive (*Bacillus cereus*, *Staphyrococcus areus*, *Listeria monocytogenes*, *Steptococcus pneumoniae* and *Clostridium diphtheriae*) and Gram negative (*Shigella flexneri*, *Salmonella typhi*, *Klebsiella pneumoniae*, *Proteus vulgaris* and *Escherichia coli*) bacteria were determined by Zakaria ZA and co-workers using an *in-vitro* disc diffusion methods. Twenty microliters of the AETT extract at 50% of stock solution, was effective against *S. pneumoniae* and *C. diphtheriae* and *E. coli* while 25% of stock concentrations of EETT and CETT were effective against *S. pneumoniae*, *C. diphtheriae* and *S. flexneri*. In addition, 25% and 50% of stock solution of EETT and CETT were also effective against *S. aureus and E. coli*, *respectively* (Zakaria et al., 2006).

Antimalarial activity

Though the methanol extract of *T. crispa* at 2.5 mg/ml showed complete inhibition of Plasmodium falciparum at 72 h incubation, the potency is less than Piper sarmentosum and Andrographis panicuilata in the same study (Najib et al., 1999).

Bertani and co-workers (Bertani et al, 2005) have evaluated the anti-malarial effect of 35 traditional remedies used in Frence Guiana, prepared in their traditional form and screened for the schizonticidal activity *in-vitro* on *Plasmodium falciparum*, chloroquine resistant strain (W2) and *in-vivo* against *Plasmodium yoelii* rodent malaria. Five of these remedies including *T. crispa* were able to inhibit more than 50% of the parasitic growth *in-vivo* at 100 mg/kg.

Anti-inflammatory activity

Although oral administration of 50% methanol extract (10 mg/kg) inhibited carrageenin-induced foot pad edema by 38% in volume. The most significant with dose-dependent inhibitory action was in the n-butanol compared with the ethyl ether- or water-soluble fractions. Subcutaneous (s.c.) administration of n-butanol fraction (3 mg/kg) equivalents to 10 and 250 mg/kg of diphenhydramine and sulpyrine, respectively.

In addition, the i.v. administration also reduced LPS-induced fever in rabbits. This antagonistic effects equivalent to the i.p. administration of 100 and 1 mg/kg of sulpyrine and morphine hydrochloride, respectively (Higashino H, 1992).

Cardiotonic effects

The mild cardiotonic effect of two triterpenes, cycloeucalenol and cycloeucalenone isolated from *T. crispa* has been reported by Kongkathip and co-workers. Cycloeucalenol showed initial reduction and then slightly increased on right atrial contraction. Both cycloeucalenol and cycloeucalenone produced slight change on right atrial rate, an initial followed by sustained reduction on left atrial contraction (Kongkathip et al, 2002).

Inhibition of drug-metabolizing enzyme (cytochrome P450)

Among 30 samples of medicinal plant screening for the mechanism-based inhibition of cytochrome P450 3A4 (CYP3A4) and CYP2D6 using the erythromycin *N*-demethyla tion and dextromethorphan *O*-demethylation activities in human liver microsomes. The methanol extracts, of *T. crispa* showed over 30% increase of CYP3A4 inhibition, with no effect on CYP2D6 (Subehan et al, 2006).

Toxicological studies

Acute toxicity study

The highest oral dose of ethanolic extract of *T. crispa* stem (4.0 g/kg B.W.) equivalent to 28.95 g/kg BW powdered crude drug in mice did not produce any signs of acute toxicity (Chavalittumrong et al, 1997).

Chronic toxicity study

A six-month chronic toxicity study of 95% ethanolic extract of *T. crispa* stem in rats at the dosage of 0.02, 0.16 and 1.28 g/kg BW, equivalent to 1, 8 and 64

folds of the therapeutic doses, did not show any significant dose-related hematological changes. However, a significant decreased in body weight and alteration of liver and renal functions were observed in both male and female animals receiving the highest dose. The morphological changes in the liver of male rats include bile duct proliferation and focal hepatic cell hyperplasia. Hence, the authors suggested that prolonged use of high doses of *T. crispa* in humans should be avoided or discontinued immediately if signs of liver or renal toxicities occur while using *T. crispa* - containing herbal medicine (Chavalittumrong et al, 1997).

Clinical studies

The efficacy and safety of *T. crispa* in the treatment of 30 patients with type 2 diabetes of less than 5 years duration, was carried out at Lumpang hospital in the year 2000. Two capsules of 500 mg of crude drug were taken orally 3 times a day before meals for a period of six months.

No significant changes of fasting blood sugar (FBS) and HbA1c levels after finishing the course of therapy (p=0.597 and 0.387, respectively). Two patients withdrew from the study because of marked symptoms of hyperglycemia. No adverse effect was reported in one-third (33%) of patients (10 cases) while elevation of liver enzymes (SGOT rose to 212.78-269.83 unit /l and SGPT to 370.30-398.05 unit/l) without any symptoms of hepatitis were observed in 18 patients (67%) after 1-2 months of treatment. One patient developed marked jaundice and required admission for further management. In all cases, the SGOT and SGPT returned to normal level within one month after cessation of *T. crispa*. No significant changes in the levels of total cholesterol, triglycerides, BUN, creatinine, hematocrit and WBC are observed (Kanjanavong, 2000).

A prospective randomized double blind controlled study was conducted in diabetic clinic at Buddhachinaraj hospital during September, 1999 to October, 2000 (Sirigulsatien et al, 2001). The effectiveness and safety of *T. crispa* (crude drug preparation in capsule) were evaluated in 66 volunteer patients with type 2 diabetes who have normal renal and liver functions and FBS level between 126-250 mg/dL. The patients were divided into two groups. Group I patients (n = 35), were treated with regular medication (sulfonylurea and biguanide) and *T. crispa* (1 gram thrice daily for 6 months). Group II patients (n = 31), were treated with regular medication and a placebo.

At the end of study, FBS levels reduced significantly in both groups ($p=0.047$, 0.011 respectively) while no change was observed with HbA1c. Interestingly, the HDL levels in *T. crispa* group rose significantly after 6 months of therapy ($p<0.05$). Asymptomatic transient elevation of hepatic enzymes was observed in seven patients of *T. crispa* group. However, the enzyme levels returned to normal values without cessation of medication (Sirigulsatien et al, 2001).

Another randomized double blind placebo controlled trial was conducted at Siriraj hospital to determine the efficacy of *T. crispa* as additional treatment of patients with T2DM who did not respond to oral hypoglycemic agents and refused insulin injections (Sangsuwan et al, 2004).

Forty T2DM patients who had received an adequate dose of oral hypoglycemic agents for at least 2 months and still have HbA1c level greater than 8.5% were randomly allocated to receive either *T. crispa* powder in capsule (1 gram thrice daily) or placebo for 6 months. There were no significant changes in FBS, glycosylated hemoglobin and insulin levels among the patients within and between the groups. Two patients who received *T. crispa* showed marked elevation of liver enzymes that returned to normal after discontinuing the drug. In addition, significant weight reduction and cholesterol elevation in patients of the *T. crispa* group were observed. It is concluded that there is no supportive evidence to use *T. crispa* for additional therapy in T2DM patients who failed to respond to oral hyperglycemic agents. Risk of hepatic dysfunction should be aware in patients receiving *T. crispa* (Sangsuwan et al, 2004).

1.3 Aims of the study

The discrepancy on antiglycemic activity between the claims in ancient remedy, the *in-vitro* and *in-vivo* animal studies and the clinical efficacy studies of *T. crispa* for the treatment of T2DM patients prompted us to re-investigate scientifically the biological activity of *T. crispa* at both cellular and molecular levels.

Hence, the aims of this study are:

1.3.1 To verify the biological activity of crude extracts and potent fractions of *Tinospora crispa* on glucose transport using an *in-vitro* model of rat muscle cell-line, L6 myotubes.

1.3.2 To investigate its downstream activation pathways via the insulin signaling glucose transport and regulatory mechanism.

CHAPTER 2

RESEARCH METHODOLOGY

2.1 Materials

Reagents

Agarose	Sigma (Cat.No.A9539)
Anti-Actin antibody	Santa Cruze (Cat. No. Sc-8432)
Anti-ERK1 antibody	Santa Cruze (Cat. No. Sc-93)
Anti-ERK2 antibody	Santa Cruze (Cat. No. Sc-154)
Anti-p-ERK1/2	Santa Cruze (Cat. No. Sc-7383)
Anti-GLUT1 antibody	Santa Cruze (Cat. No. Sc-7903)
Anti-GLUT4 antibody	Santa Cruze (Cat. No. Sc-7938)
Anti-rabbit IgG HRP-linked Antibody	Cell Signaling Technology (Cat. No.7074)
Anti-mouse IgG HRP-linked Antibody	Cell Signaling Technology (Cat. No.7076)
Bio-Rad Protein assay	Bio-Rad (Cat. No. 500-0006)
Bovine serum albumin (BSA)	Bio-Rad (Cat.No. 500-0007)
Cycloheximide	Sigma (Cat.No. C1988)
Cytochalasin B	Sigma (Cat.No. C6762)
2-Deoxy-D-[2,6-^{3}H]glucose	Amersham Biosciences, UK
Deoxynucleotide Solution Set (dATP, dCTP, dGTP, and dTTP)	New England Biolabs (Cat. No. N0446S)
DMSO (dimethyl sulfoxide)	Sigma (Cat. No D2650)
DNA marker (1 kb plus DNA ladder)	Invitrogen/Life Technologies (Cat. No. 10787-018)
Fetal bovine serum (FBS)	Seromed, Biochrm KG, Germany
Horse serum (HS)	Seromed, Biochrm KG, Germany

L6 myoblast cells	ATCC (Cat.no.CRL1458)
Metformin	Sigma (Cat. No. D5053)
α-Minimum Essential medium (α-MEM)	GibCo, Invitrogen,UK
Penicillin/Streptomycin (10000 U/10000 μg/ml)	Seromed, Biochrm KG, Germany
PD98059 (MEK1 inhibitor)	Cell Signaling Technology (Cat. No.9900)
Pimer sets	Operon Biotechnologies, Germany
Prestained protein marker, broad range	Cell Signaling Technology (Cat. No.7720)
RIPA lysis buffer	Santa Cruze (Cat. No. Sc-24948)
RNase inhibitor RNaseOUT Recombinant Ribonuclease Inhibitor	Invitrogen/Life Technologies (Cat. No. 10777-019)
SB203580	Sigma (Cat.No. S8307)
Scintillation cocktail	Ecolume™ (Cat. No. 882470)
Silica-gel 60H	Merck, USA
Sulforhodamine B sodium salt	Sigma (Cat. No. S9012)
Taq DNA Polymerase with ThermoPol Buffer	New England (Cat. No. M0267S)
Trichloroacetic acid (TCA)	Sigma (Cat. No. T6399)
Trizma™base, SigmaUltra	Sigma (Cat. No. T6791)
TRIZOL™Reagent	Invitrogen/Life Technologies (Cat. No.5596-026)
Trypsin-EDTA (0.5%/0.2%)	Seromed, Biochrm KG, Germany
Wortmannin	Sigma (Cat. No. W1628)
UltraPure-DEPC-Treated Water	Invitrogen/Life Technologies (Cat. No.750023)

* Chemicals, solvents and other reagent were purchased from Sigma.

Materials

0.45-μm filter	Satorious, Germany
Centrifuge tubes	Corning™, US
Culture plates, dish	Costra,US
Scintillation vial	Sigma (Cat. No. V6880)
TLC plate (Silica gel 60 F254)	Merck (Cat. 1.05715.0001)
Glass homogenizer	Cowle, UK

2.2 Instruments

CO_2 incubator	SHEL-LAB, USA
Gel apparatus	Protean III Cell, Bio-Rad, USA
Horizontal electrophoresis apparatus	Hoefer Scientific Instruments, USA
Laminar flow hood	E.S.I. Flufrance, Model Supcris18, France
PCR Thermal Cycler	MyCycler Thermal Gradient Cycler, BIO-RAD LABORATORIES, USA
Phase-contrast microscopy.	Olympus Model CK2-TRC-3, Japan
Refrigerated Centrifuge	Hettich Universal 30RF, Germany
Scintillation counter	LS6000TA, Beckman, USA
Spectrophotometer	Shimazu Model UV-160A, Japan
Super Speed Centrifuge	Sorvall RC5Plus, Dupont, USA
Transfer apparatus	BioRad, USA

2.3 Preparation of *T. crispa* extracts (TC)

Preparation of *T. crispa* extracts was summarized in Figure 16.

2.3.1 Aqueous extracts (TCc)

A newly picked complete specimen of *T. crispa* was identified and collected. A voucher specimen (SKP 113 2002) was deposited in Department of Biology Herbarium at Prince of Songkla University, Thailand. Selected stems were washed, cut into small pieces and dried at 60°C. A hundred grams of the cut and dried stems were boiled in one litre of distilled water, and left to simmer for 24 hours. The boiling process was repeated again after the aqueous extract was collected. Both aqueous extracts were pooled and centrifuged at 800xg for 10 min before lyophilization and the resulting residue (8 ±2 g.) was stored at -70°C.

2.3.2 Methanol extracts

The 100 g. of cut dried stems were soaked and left to macerate in a solution of 95% methanol for 3-4 days at room temperature (RT) before collecting the solvent. The process was repeated three times and the collected extracts were evaporated at <45°C under reduced pressure. The six grams of crude methanolic extract was obtained and stored at -70°C until used.

2.3.3 Preliminary purification of aqueous extracts of *T. crispa*

The crude aqueous extract was sequentially fractionated with various concentrations of 90%, 85%, 80% and 70% ethanol in the ratio of 1:50 w/v and left at RT overnight. The suspension was then centrifuged at 800xg for 20 min and the supernatant collected. The collected fractions were evaporated at <45°C under reduced pressure. The fractionated extract was obtained as a, b, d, e, and f (2.29±0.14, 0.66±0.11, 0.53±0.22, 1.06±0.10 and 3.45±0.43 g. respectively) was stored at -70°C until ready for use.

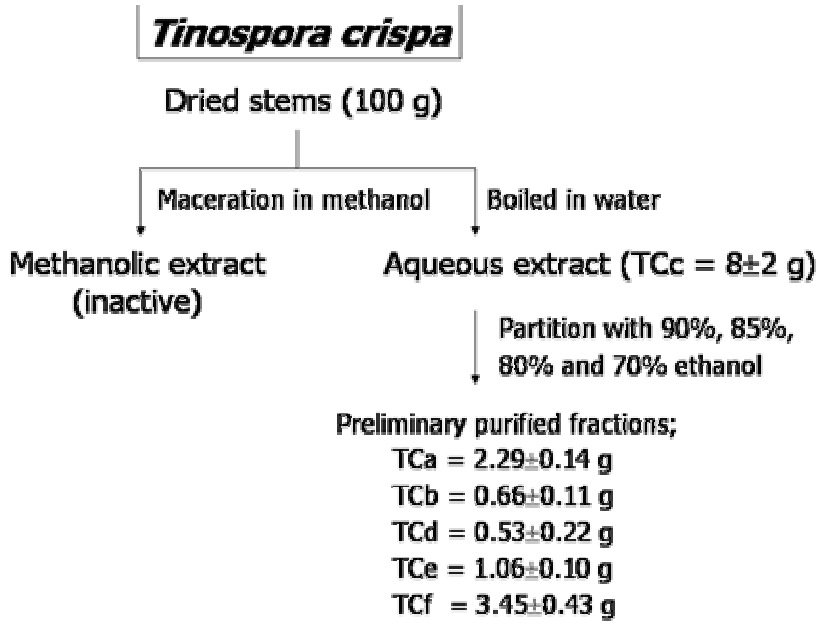

Figure 16. Extraction and partition of dried stem of *Tinospora crispa*.

2.3.4 Thin Layer Chromatography (TLC) Analysis of *Tinospora crispa*

TLC analysis of the compound was performed on the silica-gel plates (F254, 0.25 mm, Merck) in order to optimise the best eluent system for the chromatographic separation. Various combinations of ratios of the following solvents, *n*-hexane, chloroform and methanol was applied in mobile phases and visualised under UV light at 254 nm.

2.4 Analysis of glucose uptake in L6 myotube

It is widely recognized that cell culture systems offer a unique advantage for the study of the molecular mechanisms of the regulation of glucose transport. Myoblasts of the L6 cell line, originally derived from rat skeletal muscle (Yaffe, 1968), share many of the characteristics of the mature tissue. Moreover, L6 cells constitute a viable model for transport studied because they grown in monolayers of ready and even accessibility to substrates.

In this study, we used an optimised protocol to differentiate the L6 myoblasts into myotubes and the terminal myotubes were then used to measure glucose uptake. The

myoblast cells were derived from ATCC (CRL-1458) which exhibits a myoblast phenotype (*see* Figure 17A and Figure 17B) showing a myotubes phenotype. The myotubes were differentiated from the myoblast stage through their alignment and cell fusion up to the point of myotube formation. The percentage of the myotube formation was defined by phase-contrast microscopy as the percentage of nuclei present in multinucleated myotubes. Of the myoblasts that fused into myotubes, 80-90% were accepted for the study.

The glucose uptake assay using L6 myotubes is usually preformed between days 10 and 12 after the initiation of L6 myotube differentiation. The L6 myotubes were first incubated both with and without the extracts, Metformin and the inhibitors; cycloheximide, wortmannin and SB203580. Glucose uptake was measured using the radioactive 2-deoxyglucose that cannot be metabolised by the cells. The cells were washed in chilled phosphate-buffered saline (PBS) and after lysis used to measure the amount of 2-deoxyglucose taken up by the cells.

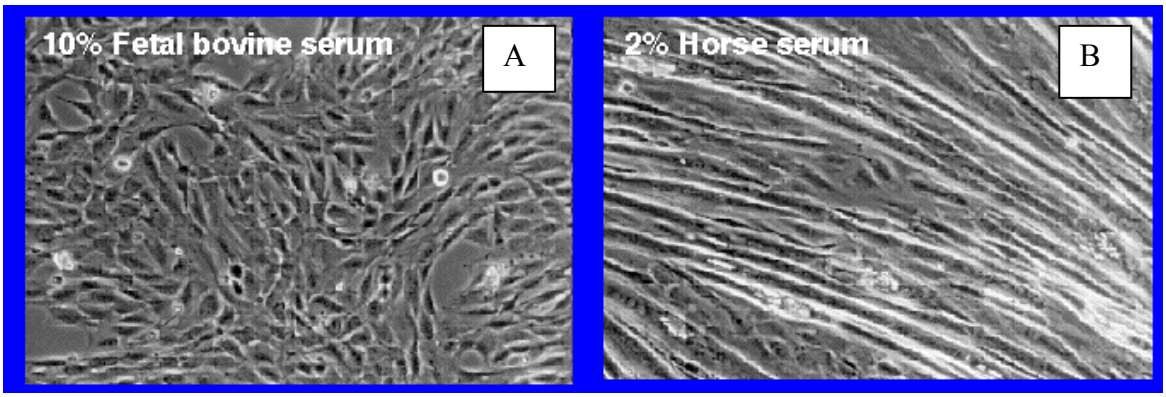

Figure 17. L6 myoblast cells were grown in α-MEM containing 10% FBS and 1% antibiotic solution (penicillin-streptomycin) in a 10-cm dish in an atmosphere of 5% CO_2 at 37°C (Figure 17A). L6 cells were rendered quiescent in α-MEM containing 2% FBS for 10-12 days to promote fusion into myotubes (Figure 17B).

2.4.1 Sub-culturing L6 myoblasts

1. Grew the L6 myoblasts in α-MEM complete medium containing 10% FBS until 80% confluent on a 10-cm dish at 37°C and 5% CO_2. All subsequent steps should be carried out under hood.

2. Washed the confluent cells twice with 10 ml sterile PBS.
3. Add 2.0 ml of trypsin-EDTA solution to the dish; gently swirled the dish to ensure that all the cells are detached.
4. Added 10 ml of the α-MEM complete medium containing 10% FBS and pipette the cells up and down gently to disaggregate the cells, so as to form a uniform suspension of the cells in the media.
5. Transfer the suspended cells into a 15-ml centrifuge tube and centrifuge at 1000 rpm (Hettich Universal 30RF, Germany) for five mins.
6. Discarded the supernatant and re-suspended the pellets with 30 ml of α-MEM complete medium containing 10% FBS.
7. Plated the cell suspension on three sterile 10-cm dish with 10 ml cell suspension each. Incubated at 37°C and 5% CO_2.
8. Replaced the media every two days.
9. Let the cells grow until they reach 80% confluent. This process usually takes four days.

2.4.2 Differentiation of L6 myoblasts

L6 is a skeletal muscle myoblast cell line originating in rats and which is capable of growing, fusing, and differentiating up to the point of formation of myotubes, in culture. The differentiation of myoblasts into fused myotubes is stimulated by cell contact and also by poorly known understood additional stimuli, such as lack of some growth factors present in the fetal calf serum. It is possible to promote fusion and differentiation by lowering the volume of fetal bovine serum in the medium from 10% to 2%. Other protocols have substituted horse serum for the bovine serum as shown below.

1. Placed seeded 15,000 cell/cm^2 of L6 myoblast cells into 24-well plates and grew in a differentiated medium (complete α-MEM complete medium containing 2% HS).
2. The media must be replaced every two days.
3. For the best results, use the L6 myotubes for experiments between day 10 and 15 after initiation of differentiation (>80% myotubes). L6 myotubes, after day 16 of post-differentiation initiation start to die and lose their phenotype.

2.4.3 [^{3}H]2-deoxy Glucose (2DG) uptake

The L6 skeletal muscle cell line has been identified as a suitable model to study glucose uptake (Klip et al, 1984). As is often observed in tissue culture, uptake rates in independent experiments may vary; yet excellent reproducibility is observed within each 24-multiwell plate of samples. Therefore, in all the experiments in which experimental and control samples were always present on the same plate each condition was tested at least three times. In total three or four similar plates were analysed following the method below.

1. L6 myotubes, grown in 24-well plates, were washed with sterile PBS and incubated both with and without the extracts, metformin and the inhibitors; cycloheximide, wortmannin SB203580 and PD98059 for each study.
2. They were then washed twice with PBS.
3. 500 µl of HBS-[^{3}H]2-deoxyglucose was added to each well and incubate for 10 min. Nonspecific uptake was determined in the presence of 10 µM cytochalasin B and subtracted from the total uptake.
4. After incubation for 10-min the cells were then washed three times with chilled PBS to stop the reaction.
5. 0.5 ml of 0.05 N NaOH solubilisation buffer was added to lyse the cells and left to stand at room temperature for 30 min.
6. Next, 0.5 ml of 0.05 N HCl was mixed in the cells by pipetting.
7. One ml of the solubilised cells was placed into a scintillation vial containing 4 ml of the scintillation cocktail and then counts the radioactivity. A further 1 ml solution of 0.05N NaOH/0.05N HCl in 4 ml of the scintillation cocktail as a blank, and 2 µl of 1 µCi/ml HBS-[^{3}H]2-deoxyglucose in a 1 ml solution of 0.05N NaOH/0.05 N HCl) in 4 ml of the scintillation cocktail to determine the total counts per minute (cpm).
8. The remaining lysate 10-20 µl was used to determine the protein concentration (we were using the BCA assay with BSA as standard, *see* **Protein Concentration Determination**).

9. Calculation of the Corrected cpm. From each group of the cells: by subtracting the cpm of the cytochalasin B-treated cells from that of the median cpm of the rest of the cells this gave the corrected cpm for that group.

2.4.4 [^{3}H]2-deoxy Glucose (2DG) uptake in the presence of specific inhibitors

Cultured L6 myotubes and 2DG uptake assay were carried out as 2.4.3. in the presence and absence of various specific inhibitors in order to determine its effects on TC-induced 2DG uptake in L6 myotubes. All inhibitors used in this thesis were summarized in Table 8.

Table 8. Concentration of used the specific inhibitors in 2DG uptake assay in L6 myotubes and adipocytes.

Specific inhibitors	Specific target	Concentration used	References
Cytochalasin-B	Cytoskeleton	10 μM	Klip et al, 1984
Wortmannin	PI3 kinase	1 μM	Okada et al, 1994
SB203580	p38MAPK	20 mM	Somwar et al, 2000 and Sweeney et al, 1999
Cycloheximide	Protein synthesis	3.5 μM	Klip et al, 1992
PD98059	MEK1/2	50 μM	Kimball et al, 1998

2.5 Protein concentration determination

Protein concentrations were determined using the dye-based Bradford assay method (Bradford, 1976). This assay is also referred to as the Bio-Rad assay after the name of the company that sells the test kit. Aliquots of samples (ranging from 10-20 μl) were diluted in water to 800 μl and 200 μl of the Bio-Rad-protein assay (Cat. No. 500-0006) was added. The extinction of the contained Serva Blue G dye was then measured after 20-30 min at 595 nm in a spectrophotometer. A standard curve for samples of known protein concentration was prepared in parallel to the assay to help assess the unknown protein

concentrations. Bovine serum albumin, BSA (Bio-Rad, Cat. No. 500-0007) was used as a standard and the following dilutions were used to generate the standard curve:

Standard	Protein/μg	μl BSA(200 μg/ml)
1	0	0
2	4	20
3	8	40
4	12	60
5	16	80
6	20	100

Table 9. Known protein concentrations used for the standard curve. Extinction was measured in plastic cuvettes. Polynomic regression was used for the calculation of unknown protein concentration of the sample.

2.6 Cytotoxicity test by sulforhodamine B assay

The Sulforhodamine-B (SRB) assay established by Skehan, 1990 is performed to determine cell survival. The assay is based on the uptake of the negatively charged pink amino-xanthine dye, the SRB, by basic amino acids of cellular proteins within the cells. The greater the number of cells, the greater the amount of dye is taken up. When cells are lysed after fixing, colorimetric measurement of the released dye will provide an estimate of the total protein mass related to the cell number.

The cytotoxic effect of the extracts on L6 myotubes was investigated using SRB assay. First, 4,500 cells/cm^2 L6 myoblasts were grown in 24-well plate. When the 80-90% of differentiated cells (myotubes) was shown, the cells were incubated for 24 h and 48 h with various concentrations of extracts. Cytotoxicity effect was performed using sulflorodamine B (SRB) assay according to the method described by Skehan, 1990 and Papazisis, 1997 with minor modifications.

Fixation step: the culture medium was aspirated and then 10% cold TCA was gently added into the wells. The plates were then left standing for 30 min at 4 °C

then washed five times with distilled water and left to dry at room temperature for at least 24 h.

SRB staining step: Sulforhodamine B (0.4% w/V) was added to each well and left at room temperature for 20 min. The SRB was removed and the plate washed five times with 1% acetic acid before air drying at room temperature for at least 24 h.

Solubilisation step: a plate with solubilised bound SRB and 10 mM unbuffered Tris-base solution was put into a plate and placed on a plate shaker for at least 10 min.

Absorbance reading step: the optical density of each well was determined at 540 nm (OD540) using a spectrophotometer. The mean OD540 of the untreated control wells represents 100% viability.

2.7 Determination of glucose transporter associated membrane proteins (plasma and micosomal vesicle membranes)

2.7.1 Preparation of RIPA cell lysates.

1. The total protein content of the L6 myotubes incubated with or without TC extracts for the specific conditions of each experiment was measured by washing three times with chilled PBS.
2. Cell lysates were prepared by adding RIPA buffer (sc-24948, Santa Cruz, CA) and the following steps were performed on a bed of ice and/or at 4°C using fresh chilled buffers.
   - First, 0.6 ml of RIPA buffer (sc-24948) was added to the monolayer cells on the 10-cm plate and then gently rocked for 15 minutes at 4°C. Adherent cells were then removed with a cell scraper and the resulting lysate transferred to a microcentrifuge tube.

- Next, the plate was washed with 0.3 ml of RIPA buffer and combined with the first lysate.
- The resulting solution was then incubated 30-60 minutes on a bed of ice.
- After centrifuging the cell lysate (10,000xg) at 4°C for 10 minutes the supernatant for protein assays was removed and the solid pellets discarded.

3. The total protein content of the supernatant was determined by the Bio-Rad protein assay (Bio-Rad, USA). Total protein lysates samples were stored at -20°C for daily use and -80°C for longer term use. Repeated freeze thaw cycles were avoided.

2.7.2 Separation of plasma and intracellular membranes.

The sub cellular fractionation of myotubes was carried out according to the method described by Yu B., 1999 with slight modifications.

1. The cells from the 10-cm dishes were gently scraped, centrifuge at low speed and then placed on ice. All the subsequent steps were carried out at 4°C.
2. The cells were suspended in homogenising buffer - 150 mM NaCl, 1 mM EGTA, 0.1 mM MgCl2, 10 mM HEPES pH 7.4 and 1:1,500 dilution of Proteases inhibitor cocktail (P 8340 Sigma, USA) and then homogenised using 20 strokes of glass homogeniser (Cowle, UK). This homogenisation was sufficient to almost complete cell breakage of the myotubes, adjudged by phase-contrast microscopy.
3. The resulting homogenate was centrifuged at 200g for five min. to remove nuclei and broken cells. The supernatant was then centrifuged at 16,000g for 15 min. to pelletise the plasma membrane (PMs) and collect the intracellular membrane (IMs) from the supernatant. Both the PMs and IMs were suspended in homogenising buffer. The protein concentration was determined using the Bio-Rad protein assay kit (Bio-Rad, USA) and kept frozen at -80°C.

2.7.3 Immunoblotting and quantification of GLUT1, GLUT4 proteins and ERK1/2 phosphorylation

2.7.3.1 Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) and electroblotting (electrotransfer to nitrocellulose membrane)

SDS-PAGE is a rapid qualifying and quantifiying method for proteins. This method separates proteins based on their molecular weights (Laemmli 1970). SDS binds to hydrophobic domains of proteins and disrupts their folded structure allowing them to exist in a stable state in solution. The resulting SDS-protein complex is proportional to the molecular weight of the protein. SDS-protein complexes all have a negative charge and can be size-separated; SDS-treatment masks the individual charge differences of proteins. During separation, SDS-protein complexes are attracted to the anode and separated by enforcement through the porous acrylamide gel. Usually, proteins are first concentrated on a stacking gel and later separated on a separating gel.

In this work, a separating gel of 7.5-10% acrylamide was used. Protein samples were combined with 5x Laemmli-buffer and heated at 95 °C for 10 min.

1. The samples to be run were first denatured in the sample buffer by heating to 95°C -100°C for five min.
2. The internal surfaces of the gel plates were cleaned with detergent or ethanol, dried then joined together with the gel plates to form a cassette, and clamped in a vertical position. The exact manner of forming the cassette will depend on the type of design being used.
3. The following were mixed in a 50-ml flask to form gels

	For 7.5% gels	**For 10% gels**
30% acrylamide gel (37.5:1)	2.5 ml	3.34 ml
1.5 M Tris-HCl pH 8.8	2.5 ml	2.5 ml
10% SDS	0.1 ml	0.1 ml
10% APS	0.1 ml	0.1 ml
TEMED	5 µl	5 µl
Distilled water	4.854 ml	4.0 ml

4. The flask was gently swirled to ensure even mixing. The addition of TEMED helped initiate the polymerization reaction and although it took about 15 min for the gel to set.
5. Using a Pasteur (or larger) pipette the separated gel mixture was transferred to the gel cassette by running the solution carefully down one edge between the glass plates. This solution was continuously added until it reached a position 1 cm from the bottom of the comb that formed the loading wells.
6. To ensure that the gel set with a smooth surface distilled water was very carefully run down one edge into the cassette using a Pasteur (or larger) pipette. The water spreads across the surface of the gel without serious mixing because of the density differences between water and the gel solution. Water was continuously added until a layer of about 2 mm existed on the top of the gel solution.
7. The gel was left to set. After setting, a clear refractive index change could be seen between the polymerised gel and overlaying water.
8. While the separated gel was setting the following stacking gel solution was prepared by mixing the following constituents in a 50- ml flask.

	For 3.7% gels
30% acrylamide gel (37.5:1)	0.161 ml
0.5 M Tris -HCl pH 6.8	1.25 ml
10% SDS	0.05 ml
10%APS	0.05 ml
TEMED	5 µl
Distilled water	3.029 ml

9. When the separating gel had set the overlaying water was poured off. Next the stacking gel solution was added to the gel cassette until the solution reached the cutaway edge of the gel plate. Placing the well-formed comb into this solution it was left to set, which took about 20 min.
10. After carefully removing the comb from the stacking gel any non polymerised acrylamide solution was rinsed out from the wells using an electrophoresis buffer. Any spacer was then removed from the bottom of the gel cassette, and

the cassette assembled into the electrophoresis tank. The top reservoir was filled with the electrophoresis buffer. An inspection was made of the top tank (reservoir?) to detect any leaks. Since no leaks were found the lower tank was also filled with the electrophoresis buffer, and then the whole apparatus tilted to dispel any bubbles caught under the gel.

11. Samples were then loaded onto the gel by placing a syringe needle through the buffer and locating it just above the bottom of the well and slowly delivering the sample into the well. All the wells were filled in this way with either standard or unknown, and a record made of the specific sample loaded.
12. A power pack was connected to the apparatus, and a constant 30 mA current passed through the gel. Continue Electrophoresis was continued until the bromophenol blue reached the bottom of the gel. This procedure took between one and two hours.
13. The final stage was to dismantle the gel apparatus by; prying open the gel plates, removing the gel , discarding the stacking gel, and placing the separating gel in a stain solution and placing the other one in transfer buffer for immunoblotting determination.
14. Lastly proteins were transferred from the gel to a nitrocellulose transfer membrane (Amersharm, UK) using an electroblotting apparatus (BioRad) and following the manufacturers recommended procedures at 100V and at 4°C for two hours. The membrane was then ready to proceed to the next step.

2.7.3.2 Immunodetection with antibodies to GLUT1, GLUT4 proteins and ERK1/2 phosphorylation

In immunoblotting or also called Western-blotting, SDS-PAGE separated proteins were transferred and immobilized on a matrix. Thereafter, monoclonal or polyclonal primary antibodies are added. Binding of primary antibodies to the antigen is visualized using horseradish peroxidase bound second antibodies.

In this work, wet blotting was performed using a nitrocellulose transfer membrane (Amersharm, UK)

1. First, non-specific binding was blocked by incubating a/the membrane(s) in 5% non-fat milk in tris-buffered saline (TBS) for 30-60 minutes at room temperature. Alternatively, the membrane may be blocked at 4°C overnight in a covered container, using 5% non-fat milk in tris-buffered saline (TBS).
2. Next, the blocked membranes were incubated overnight in a primary antibody solution (Table 10) diluted in TBS and then washed three times for five minutes each with TBST
3. The resultant membranes were then further incubated for 60 minutes at room temperature with horseradish peroxidase (HRP) conjugated secondary antibodies (Table 11) diluted in TBS, which were subsequently washed three times for five minutes each with TBST and once for five minutes with TBS.
4. The membranes were incubated with ECL reagents for five min. Excess reagent was drained off and the membrane then placed in clear plastic sandwich wrap.
5. The membranes could then be exposed to autoradiography film (BioMax MR-1 film).
6. The films were developed and measured by scanning densitometry using an automated gel doc system (Bioimagine System , Syngene, USA)

Table 10. Summary of used primary antibodies in western-Blot.

Antibody Against	Supplier	Dilution	Species
GLUT1	Sc-7903	1:1000	Rabbit polyclonal
GLUT4	Sc-7938	1:1000	Rabbit polyclonal
ERK1	Sc-93	1:2000	Rabbit polyclonal
ERK2	Sc-154	1:500	Rabbit polyclonal
p-ERK1/2	Sc-7383	1:1000	Mouse monoclonal
Actin	Sc-8432	1:1000	Mouse monoclonal

Table 11. Summary of used secondary antibodies in western-blot.

Secondary Antibody	Supplier	Dilution
Anti-rabbit IgG-HRP	#7074 cell signaling technology	1:2,000
Anti-mouse IgG-HRP	#7076 cell signaling technology	1:2,000

2.7.3.3 Quantitation of GLUT1 and GLUT4 proteins

The visualised bands of GLUT1 and GLUT4 proteins were relatively quantified by scanning densitometry using an automated gel doc system (Bioimaging System, Syngene, USA).

2.8 Quantification of the GLUT1 and GLUT4 gene expression by mRNA

2.8.1 Preparation of total RNA

Analysis of the RNA levels is a method used for gene expression studies. Isolation of the total full-length RNA is the first, and may be the most critical, step in analysing RNA levels. The total RNA extracted from eukaryotic RNA primarily contains ribosomal RNA (rRNA), transfer RNA (tRNA) and in a much lesser amount messenger RNA (mRNA). The quanidinium-isothicyanate method represents a powerful means for total RNA preparation (Laville and Auboeuf, et al. 1996), (Auboeuf and Vidal 1997), (Kohout and Rogers 1993). In this work, an optimised one step Guaninidinium isothiocyanat/Phenol method was used for the isolation of total RNA (TRIZOL^{TM}Reagent). In this method, simultaneous extraction is performed for removal of RNA, DNA and proteins. In this study isolation was performed by following the manufacturer's recommended instructions but with some minor modifications.

1. HOMOGENISATION

   By adding 1 ml of TRIZOL Reagent to a 100mm diameter dish, and passing the cell lysate several times through a pipette the cells were Lysed directly in a culture dish.
2. PHASE SEPARATION

The homogenized samples were incubated for five minutes at 15°C to 30°C to permit the complete dissociation of nucleoprotein complexes. 0.2 ml of chloroform per 1 ml of TRIZOL Reagent was then added and the sample tubes capped securely before shaking the tubes vigorously by hand for 15 seconds and incubating them at 15°C to 30°C for two to three minutes. Samples were then centrifuged at 12,000 rpm for 15 minutes at 2°C to 8°C. Following centrifuging, the mixture separated into a lower red, phenol-chloroform phase, an interphase, and a colorless upper aqueous phase. RNA remained exclusively in the aqueous phase. The volume of the aqueous phase was about 60% of the volume of TRIZOL Reagent used for homogenisation.

3. RNA PRECIPITATION

   The aqueous phase was transferred to a fresh tube. To precipitate the RNA from the aqueous phase isopropyl alcohol was mixed into the liquid using 0.5 ml of isopropyl alcohol per 1 ml of TRIZOL Reagent added in the initial homogenization. Samples were subsequently incubated at 15°C to 30°C for 10 minutes and centrifuged at no more than 12,000 rpm for 10 minutes at 2°C to 8°C. The RNA precipitate was invisible before centrifuging but formed a gel-like pellet on the side and bottom of the tube during the process.

4. RNA WASH

   The supernatant was then removed from the tube and the RNA pellets washed once with 75% ethanol, adding at least 1 ml of 75% ethanol per 1 ml of TRIZOL Reagent used for the initial homogenisation. The sample was mixed by vortexing and centrifuging at no more than 7,500 rpm for 5 minutes at 2°C to 8°C.

5. REDISSOLVING THE RNA

   At the end of the procedure the RNA pellets were air-dried for 5-10 minutes. It should be noted that because it is important not to let the RNA pellets dry completely as this will greatly decrease their solubility and also partially dissolved RNA samples have an A260/280 ratio < 1.6. As a result we dissolved RNA in RNase-free water by passing the solution a few times through a pipette tip and stored the product at -70°C.

6. Quantification of the RNA was made using absorption at 260 nm with a Shimazu Model UV-160A, Japan.

2.8.2 Quantitative reverse transcription-polymerase chain reaction (RT-PCR)

First-strand cDNA synthesis using M-MLV RT

A 24-μl reaction volume can be used for 1 μg of total RNA

1. The following components were added to a nuclease-free microcentrifuge tube:
    - 1 μl oligo (dT)12-18 (500 μg/ml)
    - 10 μl total RNA (0.1 μg/ul)
    - 1 μl 20 mM dNTP Mix (10 mM each dATP, dGTP, dCTP and dTTP at neutral pH)
    - 1 μl DTT (0.1 M)
    - 4.8 μl 5xbuffer
    - 0.5 μl M-MLV Reverse Transcriptase (200u/μl)
    - DEPC-Treated Water (+1u/ul RNaseOUT) to 24 μl
2. Then the contents of the tube were mixed gently and incubated for 60 minutes at 42°C.
3. After which the reaction was stopped by heating at 70°C for 15 minutes.

PCR condition for amplification of the GLUT1 and GLUT4 gene

1. Specific primers for GLUT1, GLUT4 and GAPDH were used for PCR amplification as follows in Table 12:

Table 12. Specific primers for GLUT1, GLUT4 and GAPDH genes

Gene	Sequences of PCR Primers	Size of PCR Products, bp	Gene Bank Accession No.
GLUT1	Sense (20 b, position 204) CCC TGC AGT TCG GCT ATA AC (Tm=62.45) Antisense (20 b, position 482) AGC ATC TCA AAG GAC TTG CC (Tm=60.4)	298	BC061873
GLUT4	Sense (20 b, position 1218) ATG TGT GGC TGT GCC ATC TT (Tm=60.4) Antisense (20 b, position 1608) GGT TTC ACC TCC TGC TCT AA (Tm=60.4)	410	D28561
GAPDH	Sense (24 b, position 995) CTA CCC ACG GCA AGT TCA ACG GCA (Tm=67.98) Antisense (24 b, position 1568) TCC AGG CGG CAT GTC AGA TCC ACA(Tm=67.98)	598	NM_017008

2. PCR reaction

Only 10% of the first-strand reaction was used (2 μl of the reaction from the previous page) for PCR because it has been found that adding larger amounts of the first-strand reaction may not increase amplification and may result in a decreased amount of PCR product.

1. The following were all added to a PCR reaction tube for a final reaction volume of 20 μl:

- 2 μl 10X PCR Buffer [200 mM Tris-HCl (pH 8.4), 500 mM KCl, 20 mM $MgSO_4$]
- 0.5 μl 40 mM dNTP Mix
- 1 μl amplification sense primer (10 μM)
- 1 μl amplification antisense primer 2 (10 μM)
- 0.2 μl Taq DNA polymerase (5 U/μl)
- 2 μl cDNA (from first-strand reaction)

- Distilled water added to 20 µl

2. The solution was mixed gently and heated the reaction to 94°C for 2 minutes to denature it.
3. Thirty-five cycles of PCR were performed. Annealing and extension conditions were primer and template dependent and had to be determined empirically.

Quantification of GLUT1 and GLUT4 gene expression

The obtained PCR products were electrophoresed on 2% agarose gels, stained with ethidium bromide and visualised under an UV light.

Relative quantification of the RT-PCR products were carried out in an automated gel doc system (Bioimaging System, Syngene, USA)

2.9 Statistical analysis

The results have been expressed as means ±SE. Statistical significance was tested with *student's t test* and differences were accepted as significant at the $p < 0.05$ level.

CHAPTER 3

RESULTS

3.1 Cytotoxicity effect of extracts' fractions of *T. crispa* (TC)

Since the methanol extract of TC required pure DMSO as a dissolving agent at 100 mg/ml. This has permitted the maximal concentration of 500 μg/ml (0.5%DMSO in cultured medium) to be tested in the bioassay.

The cytotoxic effects of crude methanol extract from TC and its vehicle (0.1% and 0.5% DMSO) were determined on L6 myotubues using sulforhodamine B (SRB) assay. As shown in Table 13, L6 myotubes are rather vulnerable to the DMSO even at the generally acceptable range of 0.5% concentration. This could also give the explanation why there was no dose related response in glucose uptake test between the dosages of 100 and 500 μg/ml. In addition, the enhancement of glucose uptake demonstrated by the maximal dose was rather low compared to the aqueous fraction (Table 14).

Therefore, only the crude extract of TC from the aqueous fraction (TCc) was used for further evaluation of cytotoxicity on L6 myotubes prior to the biological activity characterization of glucose transport in more details.

Table 13. Effects of methanol extract of *T. crispa* on cells viability (determined by SRB) and 2DG uptake in L6 myotubes. Results represent the mean ± SE of 3 independent experiments, run in triplicate.

Treatment condition	**Cell viability (% of control)**	**Glucose uptake (% of basal)**
Control	100	100
0.1% DMSO	nd	92.0 ± 9.0
0.5% DMSO	81.1 ± 4.3	77.0 ± 2.0
10 μg/ml of MeOH extract of TC (0.01% DMSO)	93.9 ± 4.0	80.0 ± 11.0
100 μg/ml of MeOH extract of TC (0.1% DMSO)	92.7 ± 2.6	130.5 ± 9.5
500 μg/ml of MeOH extract of TC (0.5% DMSO)	95.7 ± 8.4	130.7 ± 6.9
1 mg/ml of aqueous extract of TC	100.4 ± 0.6	132.6 ± 1.8
2 mg/ml of aqueous extract of TC	103.6 ± 0.6	146.9 ± 5.8
4 mg/ml of aqueous extract of TC	103.4 ± 2.7	173.7 ± 7.6
6 mg/ml of aqueous extract of TC	103.0 ± 1.1	205.5 ± 3.5

nd=not determine

Cell viability values from the SRB assay on L6 myotubes incubated with various concentrations of TCc in 2% horse serum media for 24 h were shown in Fig. 18. Cell viability was not affected by TCc extracts treatment up to <7 mg/ml.

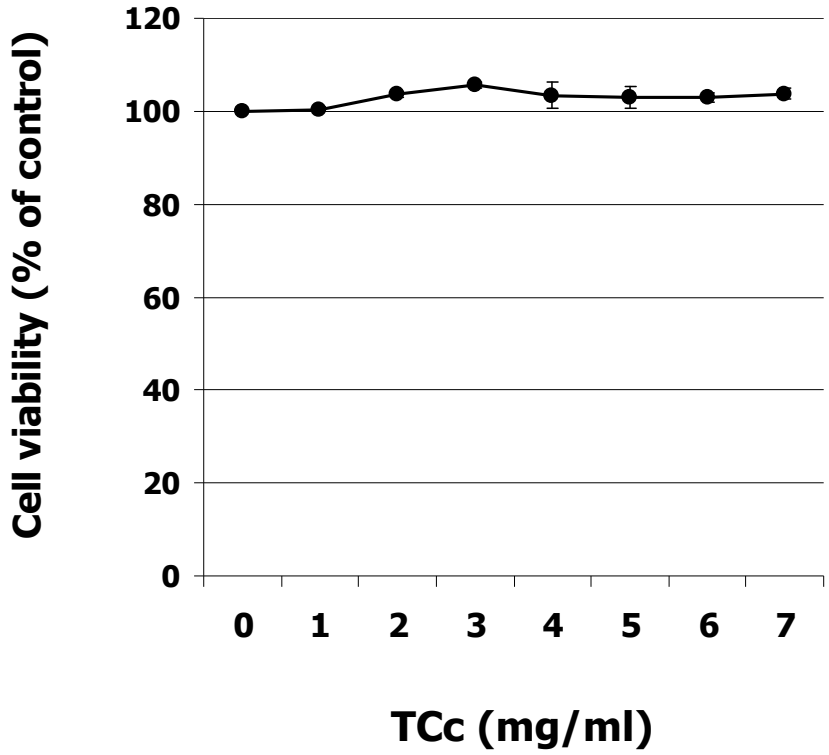

Figure 18. Effects of aqueous extract of *T. crispa* (TCc) on cell viability of L6 myotubes according to SRB assay. L6 myotubes were grown in 24-well plate and incubated with TC extract at various concentrations. After 24 h incubation, cell viability was performed by using SRB assay. Results represent mean ± SE of three independent assays carried out in triplicate.

3.2 Aqueous extract of TC (TCc) stimulates glucose uptake in L6 myotubes.

A preliminary study to evaluate the biological effects of various extracts from *T. crispa* on glucose transport in L6 myotubes was performed using 2-Deoxy-D-[2,6-^{3}H] glucose uptake assay. L6 myotubes were incubated with various concentrations of extracts for 24 h. The [^{3}H]2-deoxyglucose (2-DG) uptake was subsequently determined over a 10-minutes period. As shown in Table 14, only the aqueous extract of *T. crispa* (TCc) increased glucose uptake 1.5-2.0 fold above basal.

Table 14. The effect of *T. crispa* (TC) on glucose uptake. Results represent the mean of 5 independent experiments carried out in triplicate.

Extracts	**Glucose uptake (fold over basal)**
Low responded - methanol extract of TC	1.2-1.3
High responded - aqueous extract of TC (TCc)	1.5-2.0

3.2.1 Dose-dependent stimulation of 2-deoxyglucose (2-DG) uptake by aqueous extract of *T. crispa* (TCc)

The dose dependent effect of TCc on glucose uptake was shown in Fig. 19. The significant increased in glucose uptake was initially observed at 2 mg /ml of TCc (146.89 ± 5.84% above basal; $p<0.05$) with steadily increased in dose related manner, up to 6 mg/ml.

It should be noted that the non-carrier-mediated glucose transport determined by cytochalasin-B (CB) non-inhibitable uptake was less than 6.36±0.30% of total uptake and was not affected by the extract treatment. Moreover, TCc treatment had no significant effect on cell number or protein content in each well. There were no obvious differences in cell morphology after the treatment.

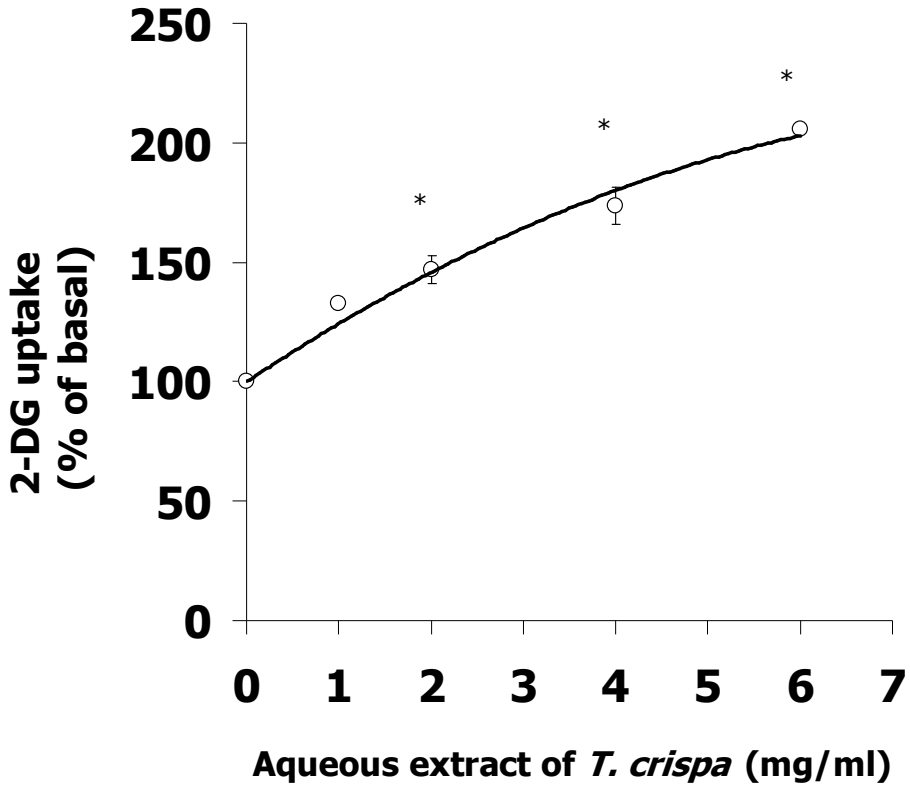

Figure 19. Dose-dependent stimulation of 2-deoxyglucose (2-DG) uptake by aqueous extract of *T. crispa*. L6 myotubes were grown in monolayers in 24-well plates, as indicated in *Materials and Methods*. Myotubes were incubated in α-MEM containing 2% horse serum, 5.5 mM glucose and various concentration of aqueous extract of *T. crispa*. at 37°C, for 24 h. At the end of the incubation, the medium was aspirated, the cells were rinsed twice in glucose-free HBS solution, and 2-DG uptake was determined in the presence of 1 μci/ml [^{3}H]2DG for 10 min, as described in *Materials and Methods*. Results represent the mean ± SE of 3-7 independent experiments carried out in triplicate. * $p<0.01$ compared to basal value.

3.2.2 Time-dependent stimulation of 2-deoxyglucose (2-DG) uptake by aqueous extract of *T. crispa* (TCc)

The effect of TCc (4 mg/ml) on the 2-DG uptake in L6 myotubes over 48-h time course was shown in Fig. 20, Coincubation of L6 myotubes with 4 mg/ml of TCc resulted in the stimulation of glucose uptake in time-dependent manner. TCc increased glucose uptake in L6 myotubes to the significant levels within 4 h with the maximal stimulation at 48 h (207.22±7.65 above basal; $p<0.05$) of incubation. The half maximal stimulation caused by this extract was observed at 24 h (166.47± 10.23 above basal; $p<0.05$). Therefore, 24-h incubation with the extract was used in all subsequent experiments.

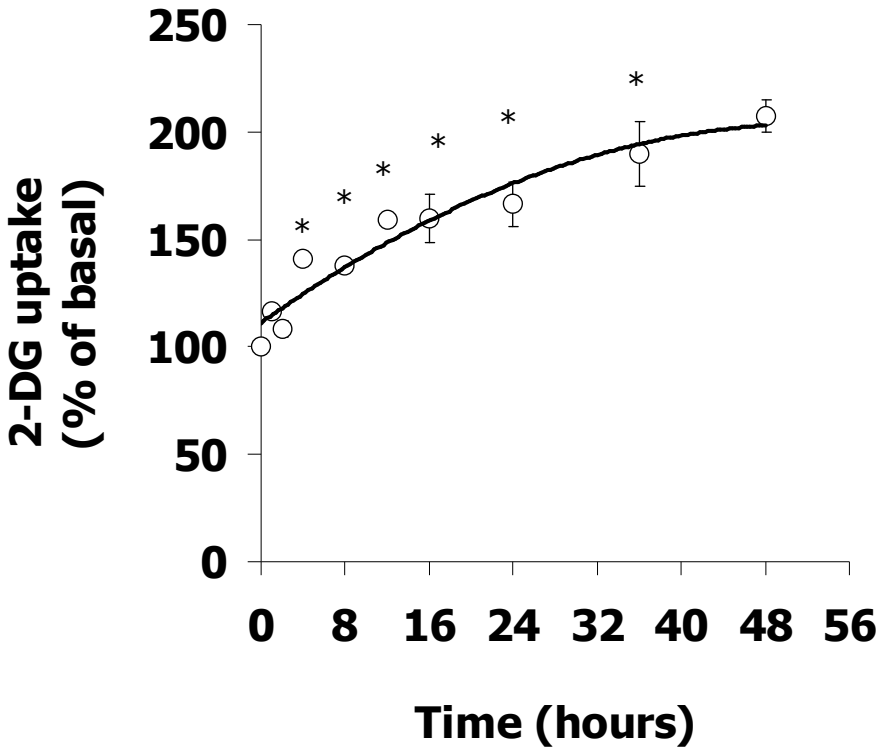

Figure 20. Time-dependent stimulation of 2-deoxyglucose (2-DG) uptake by aqueous extract of *T. crispa*. L6 myotubes were cultured and incubated, as described in Fig. 1, with 4 mg/ml of aqueous extract of *T. crispa* for 0-48 h. The cells were washed, and [^{3}H]2DG was measured. Results represent the mean ±SE of 3-7 independent experiments, carried out in triplicate. $^{*}p<0.01$ and $^{\dagger}p<0.001$ compared to basal value.

3.2.3 Metformin and aqueous extract of *T. Crispa* (TCc) stimulates 2-deoxyglucose (2-DG) uptake in L6 myotubes

The effects of prolonged (24 h) treatment with TCc on glucose transport in L6 myotubes, was compared with the known antiglycemic action of Metformin. As shown in Fig. 21, both TCc (4 mg/ml) and metformin (2 mM) significantly increased the 2-DG uptake (174.68±7.51% and 205.80±6.28% above basal, respectively, $p<0.05$). The enhancement of glucose transport activity was completely abolished by a cytoskeleton inhibitor, 10 μM of cytochalasin-B (remain ~6% compared to basal), suggesting an active process of glucose transport.

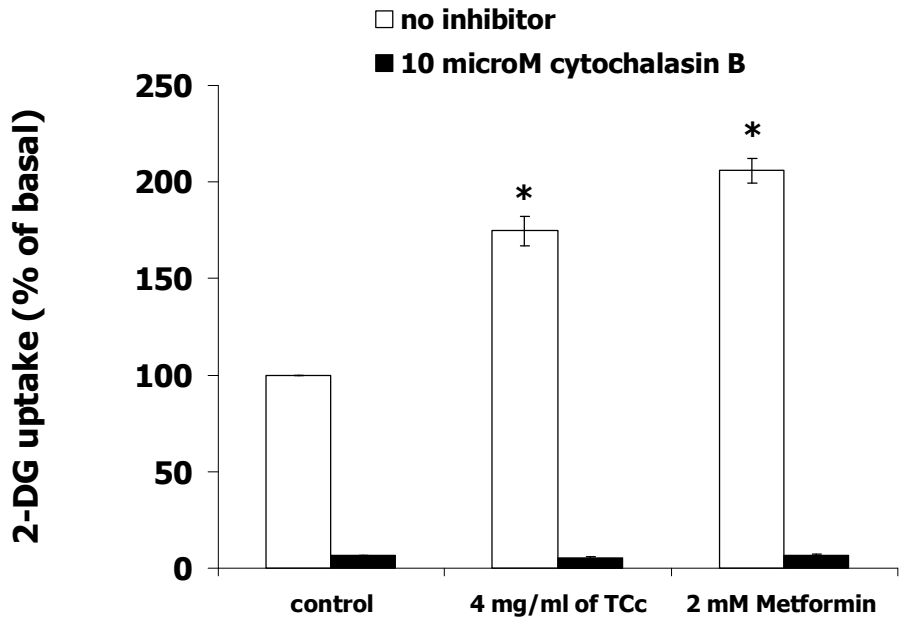

Figure 21. The effect of metformin and aqueous extract of *T. crispa* on glucose uptake. After the incubation of aqueous extract of *T. crispa* (4 mg/ml) and 2 mM Metformin for 24 h, the 2-DG uptake was measured. The open [□] and close [■] bars are the results of 2-DG uptake measured in the absence and presence of 10 μM cytochalasin-B representing the mean ± SE of 3-7 independent experiments carried out in triplicate. *$p<0.05$ compared to untreated control.

3.2.4 The effects of wortmannin and SB203580 on TCc-stimulated 2-DG uptake in L6 myotubes

The effects of TCc on the translocation and intrinsic activation of GLUT4 activity were evaluated in the presence and absence of wortmannin, a well known PI3-kinase inhibitor and a p38 MAPK inhibitor, SB203580. After 24-h co-cultured of TCc (4 mg/ml) with L6 myotubes, a specific kinase inhibitor either 1 μM wortmanin or 20 mM SB203580 was added for 30 min prior to the performance of 2-DG uptake assay. As shown in Table 15, the stimulation of glucose transport by TCc alone (59.97% above basal) was similar in cells incubated with TCc in the presence of 1 μM wortmannin (55.87% above basal) or 20 mM SB203580 (56.46% above basal). Therefore, both inhibitors did not prevent the stimulation of glucose transport by TCc.

Table 15. The effects of two specific kinase inhibitors on TCc-stimulated 2-DG uptake in L6 myotubes. Cells were 24-h co-cultured with 4 mg/ml of TCc in the absence and presence of wortmannin or SB203580 for 30-min prior to 2-DG glucose uptake. Results represent mean ± SE of 5 independent assays run in triplicate. $^{*}p<0.001$ compared to basal control.

Specific inhibitor	**2-Deoxyglucose uptake (percentage compared to basal)**		**% Stimulation by TCc**
	Basal control	**4 mg/ml of TCc**	
None (α-MEM alone)	100.00	159.97 ±4.71*	59.97
1 μM Wortmannin	83.83 ±0.65	139.70 ± 3.03	55.87
20 mM SB203580	98.64 ± 2.54	155.10 ±3.74	56.46

3.2.5 TCc-stimulated 2-DG uptake is inhibited by protein synthesis inhibitor, cycloheximide.

To assess whether the ongoing protein synthesis is required for the responses of *T. crispa*-induced glucose transport, L6 muscle cells were treated with a protein-synthesis inhibitor, 3.5μM cycloheximide (CHX), during the 24 h co-cultured with 4 mg/ml of TCc and 2 mM metformin. If TCc-mediated glucose transport is based on de novo biosynthesis of glucose transporter protein, cycloheximide should be able to abolish TCc-stimulated glucose transport.

As shown in Fig. 22, the presence of CHX alone decreased the basal glucose uptake to 64.62±11.47% of the control value. In the absence of 3.5μM CHX, both 4 mg/ml of TCc and 2 mM metformin significantly stimulated 2-DG uptake above the basal (159.97±4.71% and 163.89±5.19% of basal control, respectively). In the presence of 3.5μM CHX, the 2-DG uptake mediated by 2 mM metformin was significantly declined from 163.89±5.19% to 129.54±10.23%, $p<0.05$, while totally abolished the TCc-mediated glucose transport (159.97±4.71%, to 61.87±11.12%, $p<0.01$).

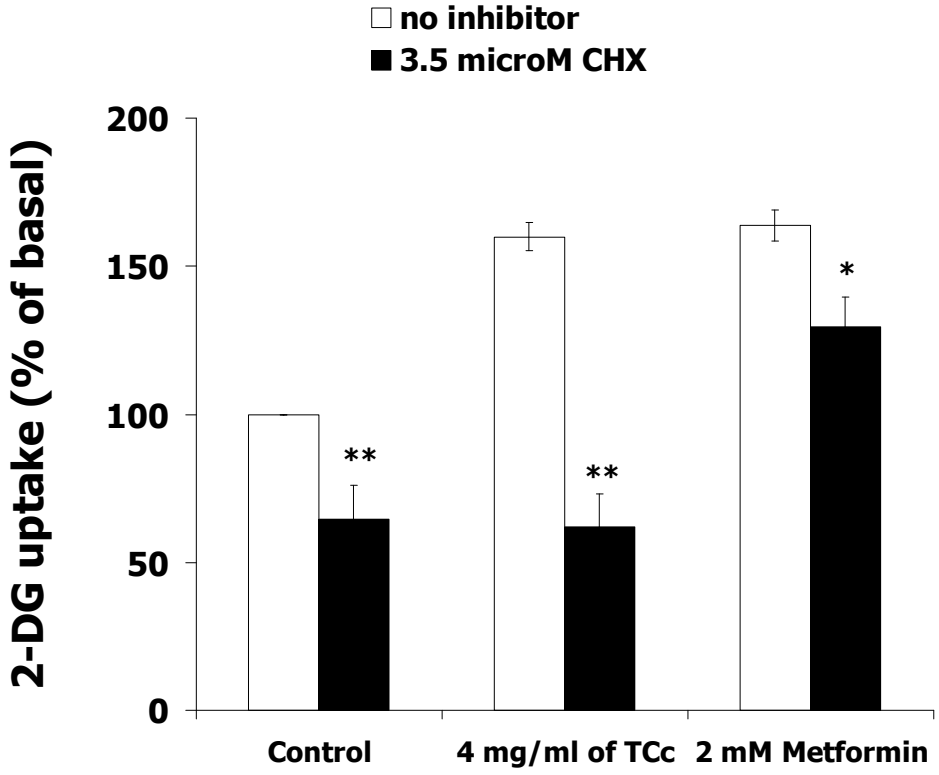

Figure 22. The effects of cycloheximide (CHX) on TCc and metformin- stimulated 2-DG uptake. L6 myotubes were co-cultured with either TCc (4 mg/ml) or Metformin (2 mM) in the presence [■] and absence [□] of 3.5 μM CHX for 24 h prior to the 10-min 2DG uptake. Results represent the mean ±SE of 3-7 independent carried out in triplicate *p<0.05, **p<0.01 compared to CHX-untreated cells.

3.3 TCc increases GLUT1 expression in L6 myotubes.

To verify that TCc-mediated 2-DG uptake via the increment of glucose transporter protein synthesis, the total amount of GLUT1 protein expression in various cultured conditions of L6 myotubes was quantified by Western blotting using a specific antibody against GLUT1 protein. The time dependent effects of TCc- (4 mg/ml) induced GLUT1 protein expression was clearly demonstrated in Fig. 23. The significant increased in GLUT1 protein expression mediated by TCc was initially observed at 4 h and increased steadily up to the experimental period of 24 h (2.63 ± 0.52 fold over the basal, n=3 at 24 h, $p<0.05$).

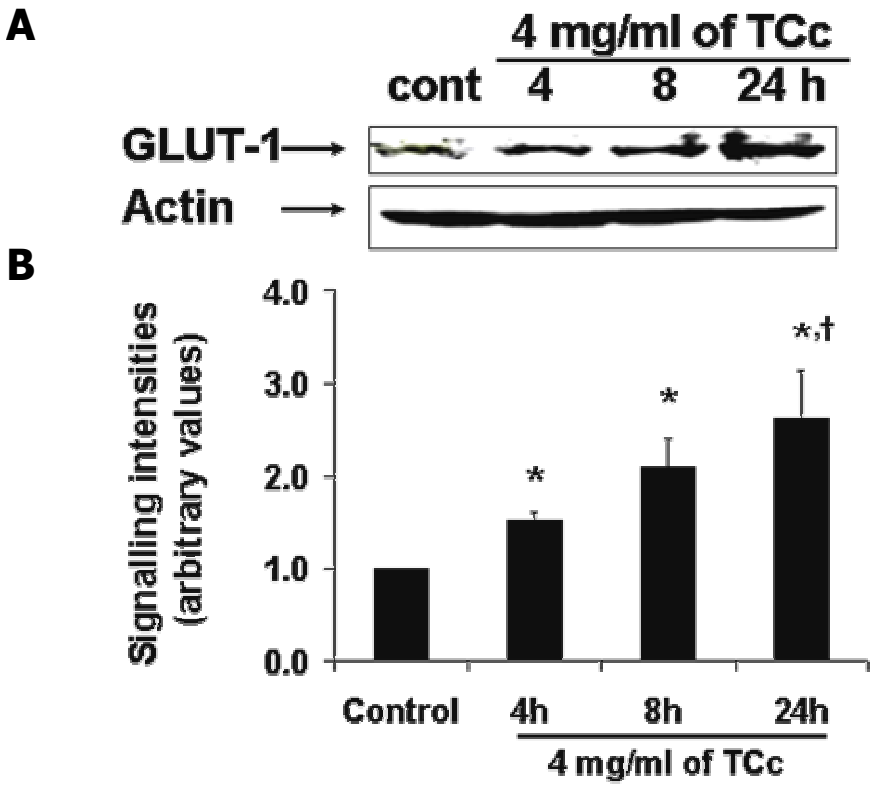

Figure 23. The effect of aqueous extract of TC (TCc) on GLUT1 content in L6 myotubes. L6 myotubes were treated with 4 mg/ml TCc for various time-points. At the end of incubation, total protein content of L6 myotubes were prepared as described in materials and methods and (A) immunoblotting of total cellular protein using anti-GLUT1 antibody. (B) Semi-quantify analysis of GLUT1 protein using scanning densitometry. Bars represent means±SE of 3 independent experiments. *,† $p<0.05$ compared to control and at 4 h-TCc-treated cells, respectively.

3.4 Effect of TCc on GLUT4 translocation in L6 myotubes.

However the PI3-kinase inhibitor (wortmannin) of the insulin-signaling pathway of glucose transport was not reduced the TCc-mediated glucose uptake (Table 15), we confirm this result using Western blot analysis. A differential quantification of soluble GLUT4 proteins on the plasma membrane (PMs) and intracellular (vesicular) membrane (IMs) fractions of TCc-treated L6 myotubes was evaluated. L6 myotubes were treated and non-treated with TC (4 mg/ml) at various time interval (4, 8, 24 h) and proceeded to membrane fractionation into PMs and IMs components. As shown in Fig. 24, the signal of GLUT4 protein in the PMs fraction was significantly increased with a corresponding decreased in the IMs fraction in 4-h TCc-treated cells. However, the signal of GLUT4 protein (PMs:IMs) ratio was slowly reversed with the prolong (8 and 24 h) incubation with TCc.

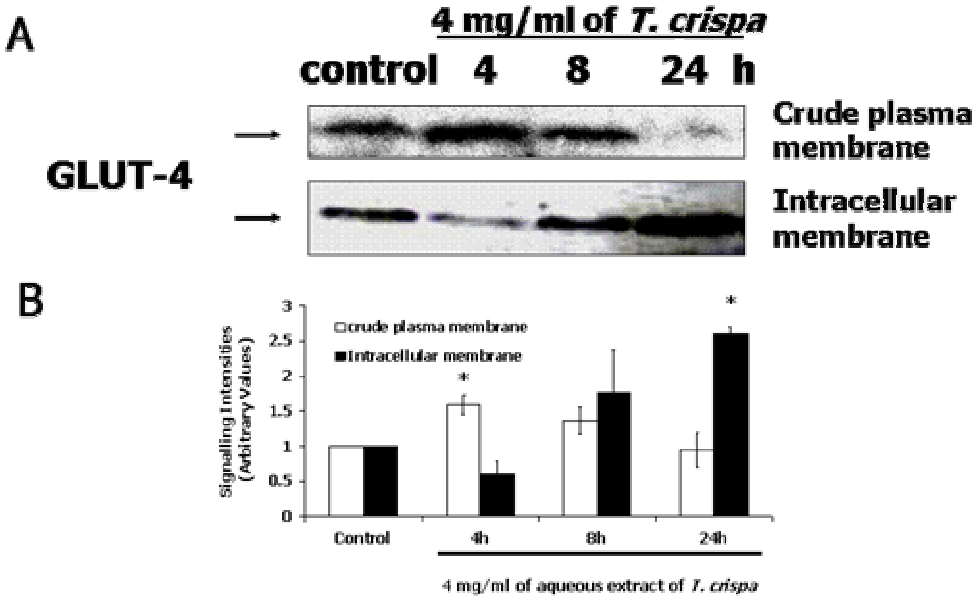

Figure 24. The effect of aqueous extract of *T. crispa* (TCc) on GLUT4 translocation in L6 myotubes. L6 myotubes were treated with 4 mg/ml of TCc for 4, 8 and 24 h. At the end of incubation, total protein content from L6 myotubes were prepared as described in materials and methods. (A) An immunoblot with anti-GLUT4 antibody. (B) Semiquantification of GLUT4 signal intensity in arbitrary unit using scanning densitometry. Bars represent the means±SE of 3 independent experiments.

3.5 Potent fraction of *T. crispa* (TCf) stimulates 2-DG uptake in L6 myotubes.

Preliminary data from all the above studies provide strong evidences of the antiglycemic property of the aqueous extract of *T. crispa* (TCc). The further step of purification was performed in order to identify the potent fractions or compounds that possess glucose transport enhancement activity in L6 myotubes. Obviously, this is not an easy task to tackle since the bioactive compounds stay in aqueous phase.

Using a stepwise partition with appropriate solvent and thin layer chromatography (TLC) tracking in combine with bioassay-guided approach, TCc was further purified into multiple fractions. All fractions were tested for the enhancement of active glucose transport by L6 myotubes. As shown in Fig. 25A one of these isolated fractions designated as TCf has exerted the enhancement pattern of 2-DG uptake similar to TCc with a 10-fold lower in dosage ($246.12 \pm 0.11\%$ above basal at 0.4 mg/ml, $p<0.001$).

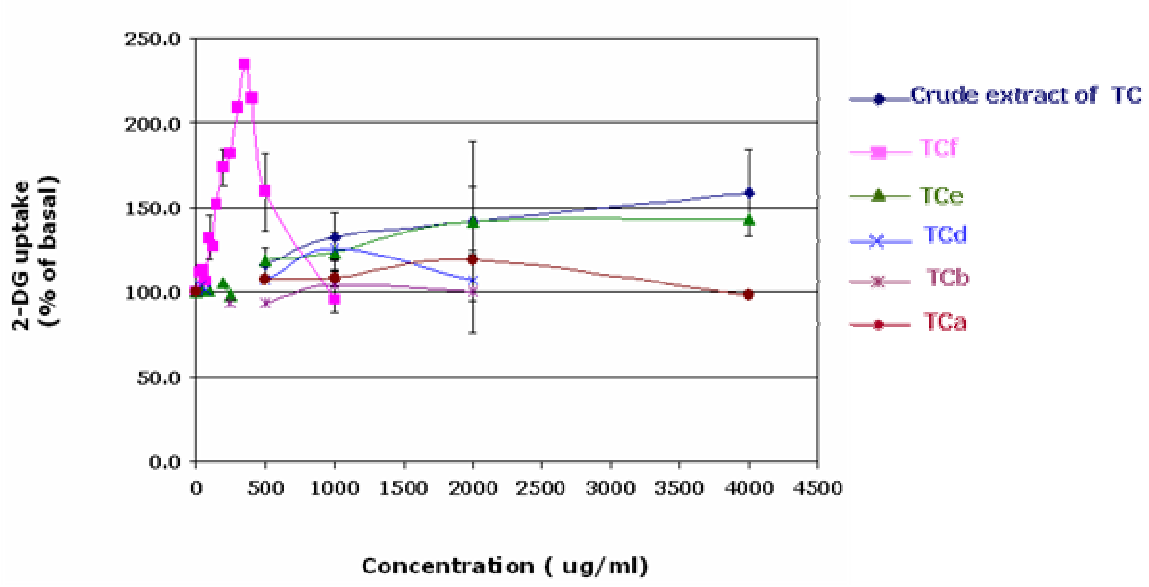

Figure 25A. Dose-dependent effect of preliminary purified fractions of *T. crispa* on [^{3}H]2DG uptake. L6 myotubes were grown in monolayers in 24-well plates, as indicated in *Materials and Methods*. Myotubes were incubated in α-MEM containing 2% horse serum, 5.5 mM glucose and various concentrations of extracts for 24 h. at 37°C. At the end of the incubation, the medium was aspirated, the cells were rinsed twice in glucose-free HBS solution, and 2-DG uptake was determined in the presence of 1 μci/ml [^{3}H]2DG for 10 min, as described in *Materials and Methods*. Results represent the mean ± SE of 3-7 independent experiments run in triplicate.

To investigate the main group of active compounds, TLC analysis of the compound was performed. Five fractions of crude extract; TCa, TCb, TCd, TCe and TCf were spotted near one end of the TLC plate. The plate is then dipped in to a suitable solvent; 10% methanol in chloroform as mobile phase in a sealed container. To analyze groups of active compound, spots were visualized under 254 nm UV light in order to detect the compounds containing unsaturated chromophores e.g. aromatic ring while the saturated molecules could be detected by heated the TLC plate after soaking in 40% sulfuric acid. As shown in Fig. 25B, TCf and TCe fractions showed the same TLC pattern under 254 nm UV light which these compounds were less polar than the other fractions. All spots of TCf fraction shown under UV light were seen in different color spots after heated with sulfuric acid. In addition, chemical analysis was indicated that TCf fraction contained at least two groups of active compounds; 1) terpenes which detected by p-Anisaldehyde spray: 0.5 ml p-anisaldehyde, 85 ml methanol, 10 ml acetic acid and 5 ml sulfuric acid and 2) alkaloids which detected by Dragendorff's reagent: 5 ml solution A, 5 ml solution B, 20 ml acetic acid and 70 ml water (sol. A: 1.7 g basic bismuth nitrate in 100 ml of water/acetic acid 4:1, Sol. B: 40 g potassium iodine in 100 ml water).

The cytotoxic effect of the semi-purified fraction of TCc (TCf) was determined at various concentrations ranging from 0.1-2.0 mg/ml for 24 and 48 h. As shown in Fig. 25C, TCf did not show any significant toxic effect on L6 myotubes at the concentration below 1 mg/ml.

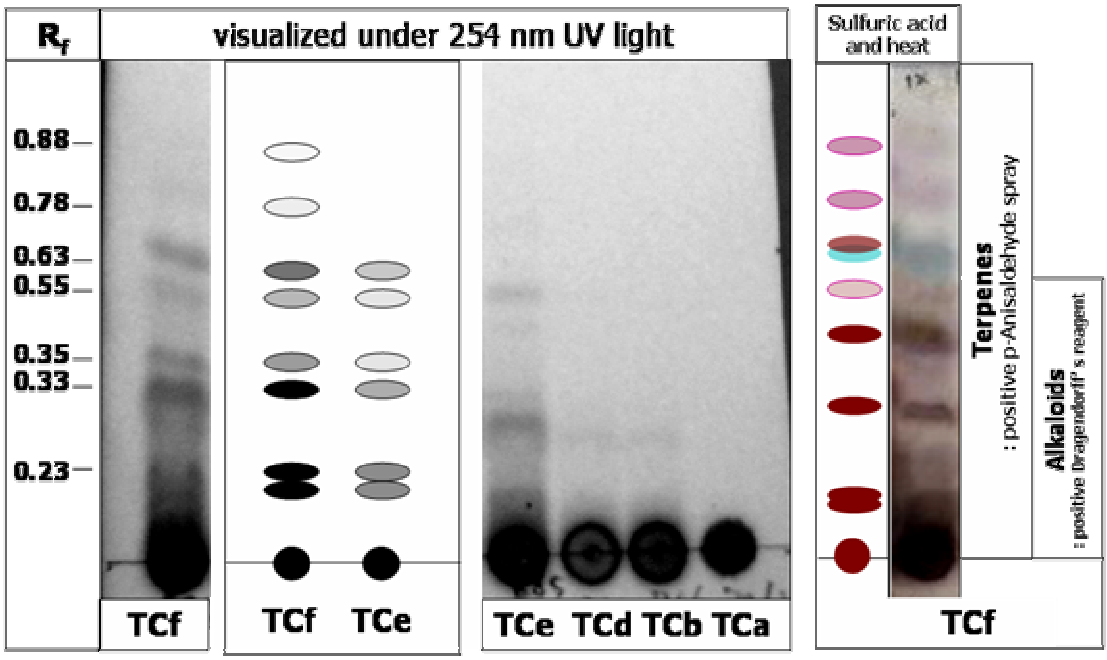

Figure 25B. Thin-layer chromatograms of preliminary purified-fractions of aqueous extract of *T. crispa*; TCa, TCb, TCd, TCe and TCf using chloroform: methanol (9:1) as mobile phase. The TLC plate was detected under 254 nm UV light and then heated after sulfuric acid soaking.

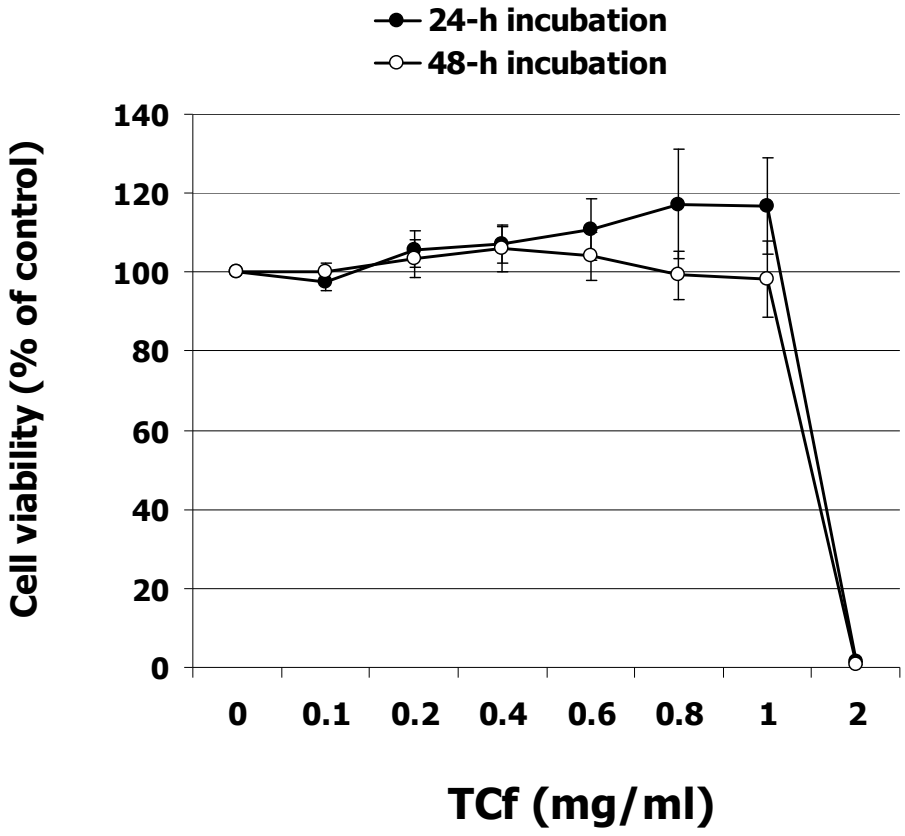

Figure 25C. Effects of potent fraction of TCc (TCf) on L6 myotube cells viability according to SRB assay. L6 myotubes were grown in 24-well plate and incubated with TCf extract at various concentrations for 24 and 48 h. After treatments, cell viability was performed by using SRB assay. Results represent mean ±SE of three independent assays in triplicate.

3.5.1 Dose-dependent effect of potent fraction of *T. crispa* (TCf) on 2-DG uptake.

The dose dependent activation of glucose transport was demonstrated on L6 myotubes using 100, 200, 300, 400, 500, 600, 700 and 1000 μg/ml of TCf (Fig. 26). The significant enhancement of glucose transport of TCf was initial observed at 100 μg/ml (128.63±6.87% above basal; $p<0.001$) and reached to the maximum at 600 μg/ml (182.25±8.78% above basal; $p<0.001$).

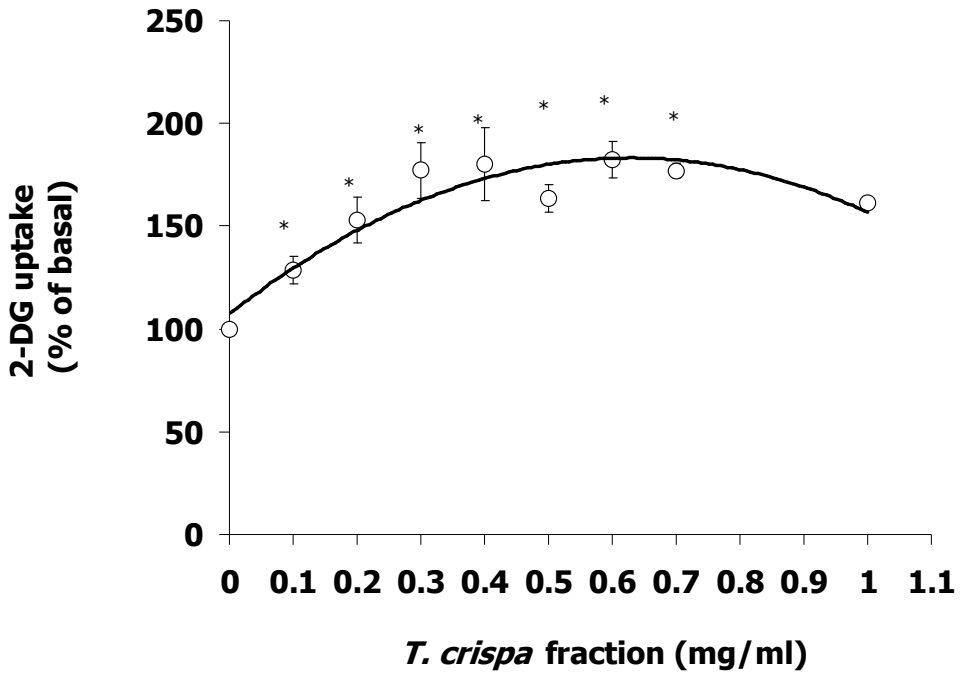

Figure 26. Dose-dependent effect of potent fraction of *T. crispa* (TCf) on [^{3}H]2DG uptake. L6 myotubes grown in monolayers were incubated in α-MEM containing 2% horse serum, 5.5 mM glucose with various concentration of TCf at 37°C for 24 h. At the end of the incubation, the medium was aspirated, the cells were rinsed twice in glucose-free HBS solution, and 2-DG uptake was determined in the presence of 1 μci/ml [^{3}H]2DG for 10 min, as described in *Materials and Methods*. Results represent mean ± SE of 3-7 independent assays carried out in triplicate. *$p<0.05$ vs basal.

3.5.2 Time-dependent effect of potent fraction of *T. crispa* (TCf) on 2-DG uptake.

L6 myotubes were co-cultured with either 0.4 mg/ml of TCf or 4 mg/ml of TCc at 4, 6, 8, and 24 h prior to the performance of 2-DG uptake assay. Both TCf and TCc stimulated 2-DG uptake of L6 myotubes in time dependent manner. The stronger enhancement was observed with TCf compared to TCc on corresponding time-points (Figure 27). In addition, no enhancement of 2-DG uptake was observed when cells were treated with TCf at the shorter time intervals *i.e.* from 30 min up to 3 h (data not shown).

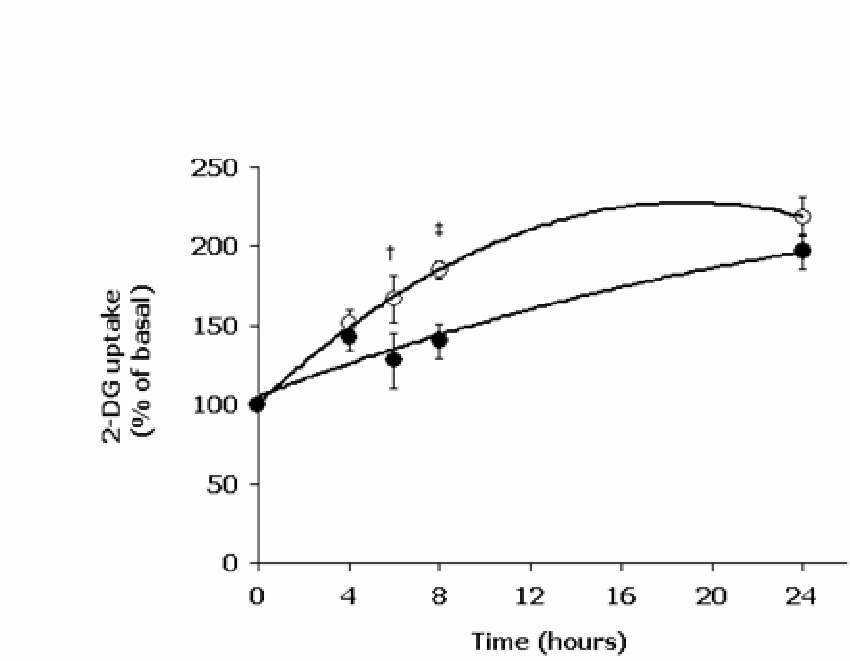

Figure 27. Time-dependent stimulation of 2 DG uptake by TCf compared to TCc. L6 myotubes were co-cultured with 0.4 and 4.0 mg/ml of TCf (○) and TCc (●) at 4-, 8- and -24-h prior to 2-DG uptake. Results represent mean ±SE of three independent experiments run in triplicate, $^{\dagger}p<0.01$ and $^{\ddagger}p<0.001$ compared to the corresponding time-points of TCc-treated value.

3.5.3 TCf-stimulated 2-DG uptake was inhibited by CHX (Cycloheximide; protein synthesis inhibitor) but not by wortmannin (PI3-kinase inhibitor) inhibits.

The experimental effects of specific inhibitors to PI3-kinase (1 μM wortmannin) and protein synthesis inhibitor (3.5 μM cycloheximide) on TC-induced 2-DG uptake in L6 myotubes were carried out simultaneously for comparison on both TCc and TCf fractions within the same assay. As shown in Fig. 28, the inhibitory profile previously observed on TCc was nicely reproduced in both TCc and TCf fractions.

In the 24-h co-cultured with CHX per se, decreased the basal glucose uptake to 45.66±1.66 % of the control value. TCc (4 mg/ml) and TCf (0.4 mg/ml) stimulated the 2-DG uptake to 155.0±4.00 % and 229.33±15.67 % of the control value $p<0.05$ and $p<0.01$, respectively, in the absence of CHX. The values of TCc- and TCf-stimulated 2-DG uptake were totally abolished to 85.0±6.02% and 83.66±3.66% of the basal control in the presence of protein synthesis inhibitor (3.5 μM CHX).

In contrast, wortmannin, a specific inhibitor to insulin-dependent GLUT4 translocation (PI3-kinase) pathway was not depress glucose transport by TCc and TCf in L6 myotubes (Fig. 28).

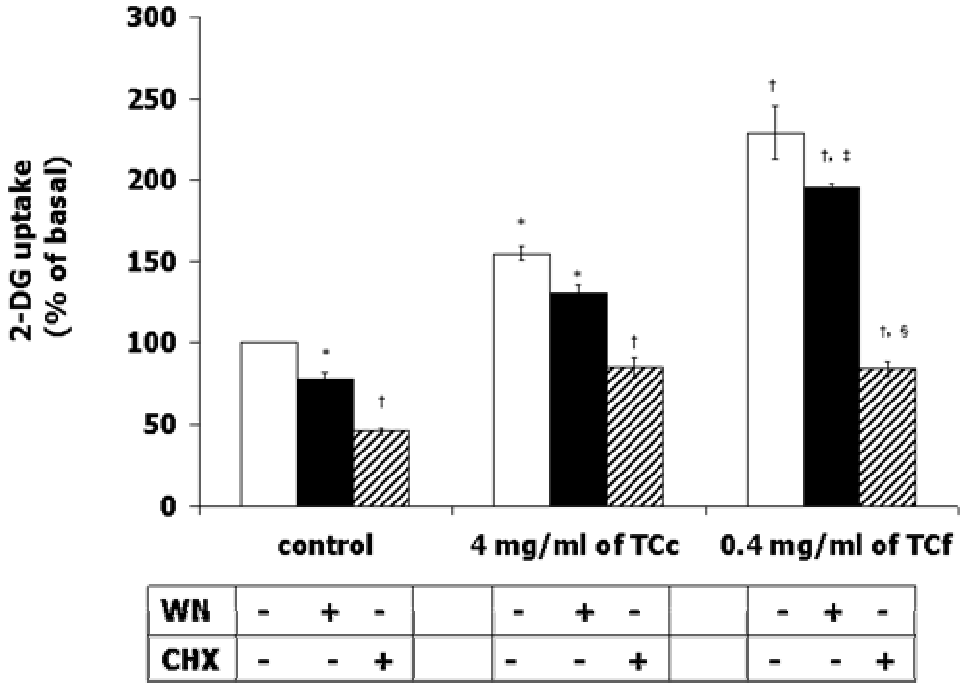

Figure 28. Effects of wortmannin (WN) and cyclohexemide (CHX) on TCc and TCf stimulated 2-DG uptake by L6 myotubes. Cells were co-cultured with TCc or TCf in the absence (□) and presence of either 1 μM wortmannin (■) or 3.5 μM CHX (▨) prior to glucose uptake. Results represent mean ± SE of three independent assays in triplicate. $^{*}p<0.05$, $^{\dagger}p<0.01$ compared to control in the absence and presence of WN and CHX, respectively. In the presence of TC_c and TC_f, $^{*}p<0.05$, $^{\dagger}p<0.01$ and ‡ $p<0.05$, $^{\S}p<0.001$ compared with the absence of the inhibitor, respectively.

3.6 TCf up-regulate glucose transporter gene (mRNA) expression

Our study indicates that TC enhances glucose uptake by increased both basal (GLUT1) and insulin-dependent (GLUT4) glucose transporter proteins expression. Therefore, we decided to investigate at which level the transporter proteins were up-regulated.

The effects of TCf on both GLUT1 and GLUT4 mRNA transcripts were demonstrated in Figure 29. TCf at 0.4 mg/ml significantly up-regulated the 298 bp of GLUT1 mRNA in L6 myotubes after a period of 4 h incubation, 1.29±0.06, 1.70±0.22 and 2.04±0.23 fold above basal at 4, 8 and 24 h, respectively. By contrast, no significant changes in the 410 bp of GLUT4 mRNA were observed through out the experimental period of 24 h.

It should be noted that the time-dependent effect of TCf on the GLUT1 mRNA levels is correspond with the significant increased in glucose uptake shown in Fig. 27.

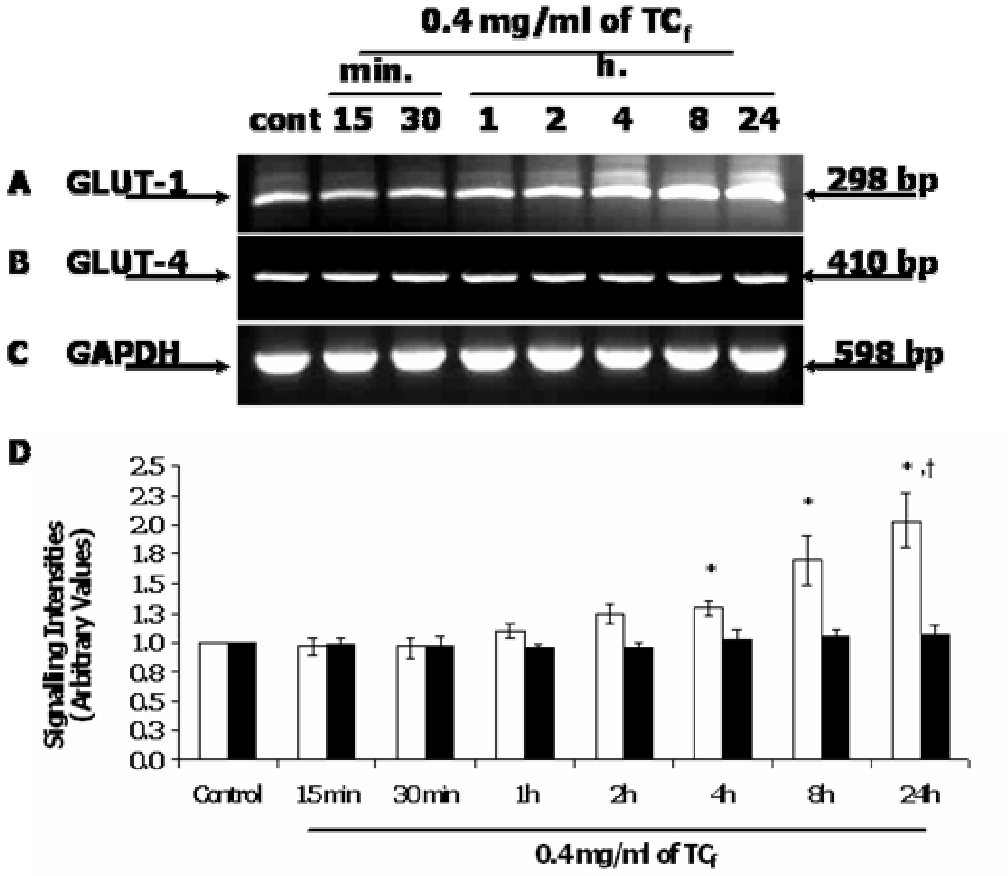

Figure 29. Effect of TCf on GLUT1 and GLUT4 transcript in L6 myotubes. L6 myotubes were incubated with TCf at 0.4 mg/ml for various time-interval prior to mRNA preparation with RT-PCR. Signals of GLUT1 and GLUT4 and GAPDH in 2% agarose gel were quantified in arbitrary unit. (A) The elevated levels of 298 bp GLUT1 mRNA transcript (B) The unchanged level of 410 bp GLUT4 mRNA. (C) An internal control of 598 bp GADPH transcripts. (D) Semi-quantificative analysis of GLUT1 and GLUT4 transcript using scanning densitometry. Open (□) and closed (■) bars represent means±SD of three independent experiments. *$p<0.05$ and $^{\dagger}p<0.05$ compared to untreated and 4-h TCf-treated cells, respectively.

3.7 The role of extracellular signal regulated kinase 1/2 (ERK1/2) on TCf- induced glucose uptake

One of the downstream signaling pathways activated by insulin receptor substrate (IRS) is the mitogen activated extracellular kinase (MEK1/2) and the extracellular signal regulated kinase (ERK 1/2) which lead to the activation of glucose transporter gene and protein expression with up-regulation of glucose transport. The use of specific inhibitor for the upstream kinases (MEK1/2), PD98059, will add up the information on TCf activation of glucose transport.

3.7.1 TCf increased glucose uptake and this effect is blocked by PD-98059.

To examine whether ERK1/2 are involved in the TCf-induced 2-DG uptake, a 24-h co-cultured of L6 myotubes with 0.4 mg/ml TCf in the presence and absence of 50 μM PD98059 was conducted for 2-DG uptake assay. As shown in Fig. 30, PD98059 significantly inhibited the TCf-induced 2-DG uptake, 166.95±11.09% and 127.99±6.51% in the absence and presence of 50 μM PD98059, respectively, $p<0.05$.

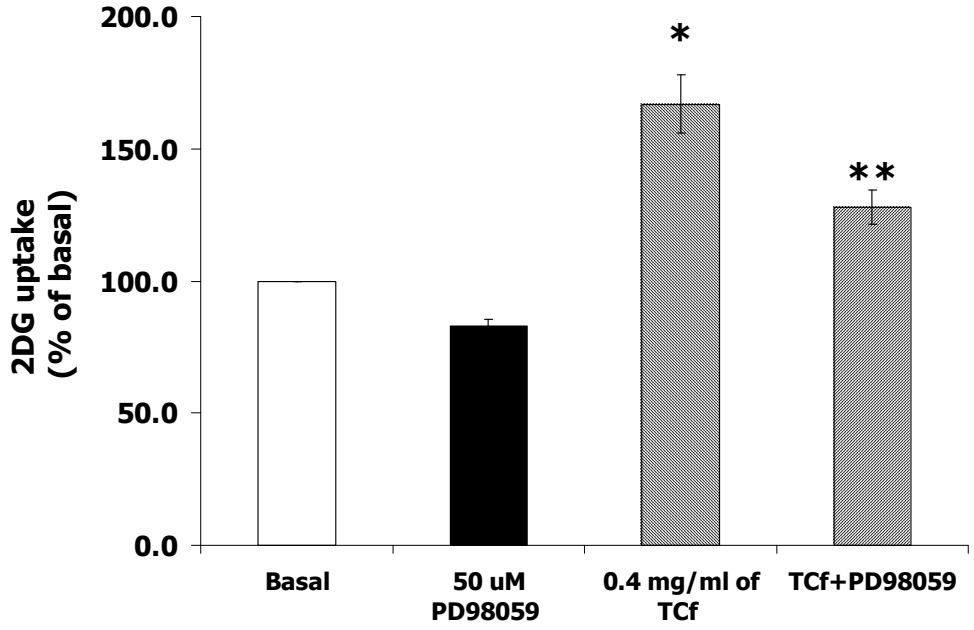

Figure 30. Effect of the MEK1 inhibitor (PD98059) on 2-deoxyglucose (2-DG) uptake in L6 myotubes. L6 cells were treated with 0.4 mg/ml of TCf in the presence and absence of 50 μM PD98059 for 24 h. Results represent the mean ±SE of 3-7 independent carried out in triplicate. *,** $p<0.05$ compared to TCf-untreated and TCf-treated cells, respectively.

3.7.2 TCf increases the phosphorylation of ERK1/2 in a time-dependent manner, and this effect is also blocked by PD-98059.

Since the TCf-induced glucose uptake is able to inhibit by PD98059, an inhibitor of MEK1/2 which lie in the upstream of ERK1/2 and GLUT1 mRNA expression. The phosphorylation study of MEK1/2 substrates, the ERK1/2, was carried out in various time intervals. As shown in Figure 31A, TCf at 0.4 mg/ml significantly phosphorylated ERK1/2 after 2-h incubation, $p<0.05$. In addition, the phosphorylation activity increased progressively in a time-dependent manner up to 24 h.

The TCf-induced phosphorylated activity on ERK1/2 was also significantly inhibited by 50 μM PD98059 (Fig. 31B).

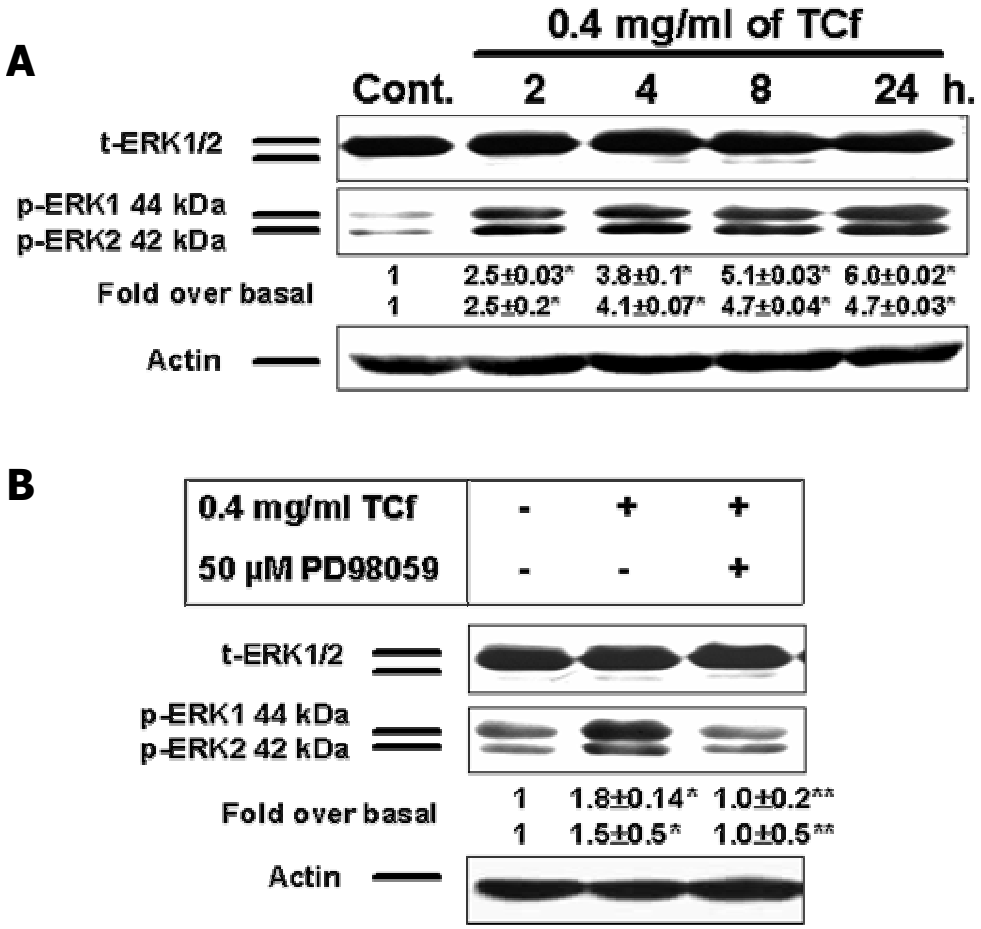

Figure 31. Effects of TCf on the activation of extracellular signal regulated kinase 1/2 (ERK1/2) in L6 myotubes. (A) Cells were treated with 0.4 mg/ml of TCf for various time intervals (0-24h) prior to determination of the phosphorylation activity on ERK1/2. (B) Cells were treated with 0.4 mg/ml of TCf in the presence or absence of 50 μM PD98059 for 24 h. and the phosphorylation of ERK1/2 were detected. The values represent mean ±SE of three independent experiments.

CHAPTER 4

DISCUSSION

4.1 Antidiabetic activity of *T. crispa*

The antiglycemic activity of *T. crispa* (TC) was firstly demonstrated in alloxan-induced diabetic (Wistar) rats in 1989 (Noor and Ashcroft, 1989). The oral administration of aqueous extract (4 g/L in drinking water) in moderately diabetic rats showed improvement of hyperglycemia, insulinaemia as well as glucose tolerance after 2-week of treatment. In addition, acute effect of TC on glucose and plasma insulin levels at various time intervals with bolus dosage of 5 and 50 mg/kg intravenously were also demonstrated in normal rats at basal and after a glucose load. No significant difference in the plasma glucose and insulin levels in treated and untreated rats with 5 mg/kg. However, plasma glucose and insulin levels in rats treated with 50 mg/kg were significantly lower and higher than the untreated animals, respectively. The authors mentioned in the discussion that the extract did not show a significant effect in severely diabetic animals (i.e. severely beta-cell damage) and suggested that the hypoglycemic effect of the TC extract is due to its ability to stimulate insulin release rather than extra-pancreatic action.

This group of investigators went on to demonstrate the insulinotropic action of TC in isolated human and rat islet of Langerhans as well as a beta cell-line, HIT-T15 using a static incubation and a perifusion system (Noor et al, 1989). Subsequently later, Noor and Ashcroft have demonstrated further that the antiglycemic effects of TC was neither due to interference with intestinal glucose absorption nor peripheral glucose uptake by primary isolated adipose tissues (Noor and Ashcroft, 1998).

Similary, another study to demonstrated the antiglycemic activity of TC using three ethanolic fractions soluble in ether (TC-2-1), n-butanol (TC-2-2) and water (TC-2-3) of TC in alloxan-induced diabetic rats (male, Sprague-Dawley) was reported in 1999 (Anulakanapakorn et al 1999). Although TC-2-1 showed no biological activity, the significant antiglycemic effect of TC-2-2 and TC-2-3 were demonstrated, again only in moderately severe diabetic rats as previously reported by Noor and Ashcroft 1989. In contrast, Anulakanapakorn and her colleagues failed to show a significant hypoglycemic

effect of TC in normal rats after the oral glucose load (OGTT) compared to sulfonylurea (200 mg/kg of Chlorpropamide).

These experimental studies did not totally convince us the antiglycemic activity of TC since there are several points in their studies that we would like to debate.

Firstly, in the in-vivo study, the improvement of blood sugar in alloxan-induced diabetic rats is based-on the number of remaining beta-cell recovered from the chemical (radicals) damage. The crude extract of TC as well as its ethanolic fractions contained many compounds with antioxidative property. None of the study has examined the number of islet of Langerhans in the pancreatic tissue at the end of their experiments. The improvement of hyperglycemia in TC-treated alloxan-induced diabetic rats could simply due to the beta-cell protection from radical damage by antioxidative property of TC in the TC-treated rats when compared to the untreated controls.

Secondly, in the in-vitro isolated beta-cell and HIT-T15 cells studies, the investigators used a high glucose solution (10 mM) as a vehicle for TC in both static incubation and perifusion models. Obviously, glucose is a well known stimulator of insulin release by beta-cells. It is hard to believe that the insulin release measurable in their assay were totally representing an insulinotropic effect of TC. Though its glucose enhancement (synergistic) effect of insulin release can not be excluded.

Thirdly, the exclusion of TC on peripheral glucose uptake by primary adipocyte isolate made by Noor et al 1989 is rather premature. Based on the data of our study, there is a delayed glucose uptake respond with a minimum of 4-h, by cultured L6 myotubes, since the activity required the activation of glucose transporter protein synthesis. Based on the review of literatures, muscle cell and adipocytes share similar response for glucose transporting activity. Therefore, the lack of glucose uptake response on 15-min co-incubation with TC can not rule out the peripheral effects of TC on glucose transporting activity in adipose tissues.

4.2 The molecular mechanistic actions of *T. crispa* on insulin signaling glucose transport pathway

The enhancement of glucose transport in muscle cells was regulated by two pathways; 1) insulin-dependent signaling pathway and 2) insulin-independent signaling pathway (Fig. 10). In insulin-dependent signaling pathway, insulin- stimulated glucose

transport was mediated via PI3 kinase-activated GLUT4 translocation and p38MAPK-activated GLUT4 intrinsic activity. In addition, Ras-MAPK-ERK1/2 pathway can activate GLUT1 and GLUT4 expression, resulting in the enhancement of glucose transport in muscle cells. Moreover, the important pathway which is insulin-independent and stimulated both translocation of GLUT4 and expression of GLUT1 and GLUT4 is AMPK signaling pathway. In this study, we focus to determine the antiglycemic effect of *T. crispa* on insulin- dependent signaling pathway.

4.2.1 *T. crispa* dose not activate GLUT4- translocation and intrinsic activity.

Acting as an inhibitor of lipid kinase PI3-kinase (Okada et al., 1994), wortmannin blocks the insulin-stimulated translocation of GLUT4 from its basal component to the plasma membrane (Le Marchand-Brustel et al., 1995 and Somwar et al., 2001). In the current study, wortmannin dose not reversed the *T. crispa* (TC)-stimulated 2-DG uptake when added after full stimulation by TC. Nevertheles, with additional data from the GLUT4 translocation study, GLUT4 protein of the PMs fraction was increased by TC at 4-h incubation. Thus, it seems likely that the translocation of GLUT4 is involved in the promotion of glucose uptake by aqueous extract of TC. Since in all other experiments in this study the effect of TC was tested in the presence of 2% horse serum, the effect of TC on GLUT4 translocation was also tested under this condition. In the presence of serum, culture medium was contained little amount of insulin and the effect on GLUT4 translocation may be produced by it. Surprisingly, incubation of cells with TC for 24 h stimulated GLUT4 protein in intracellular fraction. However, the GLUT4 mRNA level did not change when incubated with TC while the intracellular GLUT4 protein was increased by 24h-incubation of TC, suggesting that TC is involved in a posttranslational regulation such as translation or stabilization of GLUT4.

In this study, we suggest that TC was not involved in GLUT4 translocation. However, we cannot exclude the possibility that a small simultaneous change in glucose transporter intrinsic activity may have had contributed to these results. Therefore, we investigated next whether the activation of intrinsic activity of GLUT4 was involved in TC -stimulated glucose uptake. Amira Klip has reported that the inhibitor of p38 MAPK, SB203580 can prevent insulin-stimulated glucose uptake in 3T3-L1 adipocytes and L6 muscle cells (Somwar et al., 2000 and Sweeney et al., 1999). In the present study, the

effects of SB203580 on the TC-stimulated 2-DG uptake in L6 myotubes were examined by incubation with SB203580 and TC for 24 h, but no effects were observed. Therefore, we can conclude TC-enhanced glucose uptake was not mediated by the activation intrinsic activity of GLUT4.

The above mentioned results suggest that TC exposure dose not cause the translocation and the intrinsic activity of GLUT4. Similar to the previous study, it showed that metformin-stimulated glucose uptake in L6 muscle cells was not involved in GLUT4 translocation (Hundal et al., 1992). In contrast, TGZ induces GLUT4 translocation in L6 myotubes (Yonemitsu et al., 2001). They performed a GLUT4 translocation assay on membrane fractions from L6 myotubes. The cells were exposed or not exposed to TGZ (10^{-5} mol/l) for 24 h and then fractionated into membrane compartments, i.e., crude plasma membrane (CPM) and light microsomes (LMs). In the TGZ-treated myotubes, there was an increase in GLUT4 in the CPMs and a corresponding decrease of GLUT4 in the LMs, a result indicative of transporter translocation to the CPMs.

4.2.2 *T. crispa* activates glucose uptake via GLUT1 upregulation.

The onset of augmentation of the basal glucose transport by *T. crispa* (TC) was slow. This suggests that the effects may require the synthesis of new proteins that are involved in glucose transport especially GLUT1 and GLUT4 transporters in muscle cells. In examining this possibility, we incubated L6 cells together with 3.5 μM of cycloheximide, CHX; the protein synthesis inhibitor, and TC for 24 h. CHX can prevented the TC-induced 2-DG uptake. Furthermore, our results showed that TC did increase the expression of GLUT1 in L6 myotubes both in mRNA and protein levels. Thus, the increase of glucose uptake with time apparently reflects the ongoing synthesis of GLUT1 protein or a new protein that affects the GLUT1 protein expression. Therefore, the main mechanism of the enhancement effect by TC on glucose transport is involved in the activation of the GLUT1 expression. This up-regulation of the GLUT1 effect of TC is similar to standard drugs used in clinics, such as TGZ and metformin. Previous studies showed that TGZ increased glucose uptake by the activation of both GLUT1 and GLUT4 expressions (Ciaraldi et al, 1995). However, the results of some other previous studies differ from our current study, which demonstrates that the total of GLUT1 and GLUT4 contents remain unaltered in L6 myotubes after long-term treatment with metformin (Klip

et al, 1992). This group of investigators described that the ability of metformin to increase glucose uptake by skeletal muscle cells was mediated by specific changes in the distribution of existing GLUT1 transporters (Hundal et al, 1992).

Tsiani's investigations (Tsiani et al, 1995) into the mechanism of the stimulatory effects of sulfonylureas revealed that the increased glucose uptake was associated with a significant increase in total (1.7-fold) and plasma membrane GLUT1 protein (1.8-fold) without a change in internal membrane GLUT1 levels. They concluded that the stimulation of glucose uptake in L6 cells by gliclazide and glyburide was not associated with redistribution but, rather, with an increase in the total membrane content and plasma membrane levels of GLUT1, which was independent of protein synthesis. This data suggest novel action of SU to stabilize GLUT1 protein at the plasma membrane. However, our results for chronic TC action on glucose uptake are similar to our data; the mechanisms are distinct from this drug. In our present work, we found that CHX inhibit these actions, indicating that a new protein synthesis is required.

Berberine, an isoquinoline alkaloid isolated from some Chinese medicinal herbs such as *Coptidis Rhizoma* and *Cortex Phellodendri*, has shown antihyperglycemic effects (Yin et al, 2002, Leng et al, 2004 and Bian et al, 2006). Recently, it was reported that berberine at 5 μM increases glucose transport activity of 3T3-L1 adipocytes after a 6h incubation period by enhancing GLUT1 expression (Kim et al, 2007). These molecular mechanistic actions of berberine on glucose transport were also similar to *T. crispa*.

4.2.3 *T. crispa*-stimulation of GLUTl expression is mediated by ERK1/2 activation.

In insulin signaling, the Ras-MAPK pathway has primarily been associated with the regulation of mitogenesis (Denhardt, 1996). Related to GLUT1, it was reported that activation of RAS-MAPK pathway up-regulates GLUT1 expression, resulting in augmentation of glucose uptake in 3T3- L1 adipocytes. (Hausdorff et al, 1994 and 1995, Fingar and Birnbaum, 1994, Yamamoto et al, 2000). Since, this pathway is typically activated by growth factors and mitogens to alter transcription factor activity; we measured the expression levels of GLUT1 and GLUT4 in response to TC. No differences were found in the levels of GLUT4 whereas the amount of GLUT1 both at the mRNA and the protein levels were markedly increased in TC-treated L6 myotubes when compared with untreated cells. In addition, we showed that TC-stimulated glucose uptake and ERK1/2

phosphorylation were prevented by the MEK inhibitor, PD98059. Although we were not trying to determine relative contributions of the other gene expressions such as Ras or Raf-1 directly, these results showed that part of the stimulatory effect of TC on glucose uptake in L6 myotubes was mediated by ERK1/2, a component of the RAS-MAPK pathway (Denhardt, 1996).

PMA (Phorbol ester) is a potent activator of conventional and novel PKCs and increases the rate of glucose uptake in 3T3-L1 adipocytes (Bosch et al, 2003, Gibbs et al, 1991, Merrall et al, 1993, Nave et al, 1996). In 2004, Bosch and his co-workers reported that PMA treatment increased glucose uptake in 3T3-L1 adipocytes by two mechanisms: a MAPK-dependent increase in GLUT1 expression levels and a PKCλ-dependent translocation of GLUT1 towards the plasma membrane (Bosch et al, 2004). These report findings have shown that the mechanism for GLUT1 expression by the Ras-MAPK pathway agree with our report. However, it is interesting to note whether TC dose increase glucose transport into L6 myotubes by activated translocation of GLUT1 glucose transporters towards the plasma membrane.

In our present study, we propose that TC stimulates the RAS-MAPK pathway to promote GLUT1 expression in L6 myotubes (Fig. 32). Nevertheless, the inhibitory effects of PD98059 on TC-induced GLUT1 expression should be investigated further. Previous studies have shown that GLUT1 expression in both L6 myotubes and 3T3-L1 adipocytes is regulated by PI3/PKB (Akt)/mTOR/4E-BP1 (Taha et al, 1995 and 1999, Somwar et al, 1998). This would be an interesting area to further explore the effects of TC on GLUT1 expression. Because of the limitations of our present study, the inhibitory effects of PD98059 (MEK inhibitor) on TC-induced GLUT1 expression were not determined. If increasing GLUT1 levels are not completely blocked by this inhibitor, this suggests that there are several pathways of regulated GLUT1 expressions such as the PI3/PKB (Akt)/mTOR/4E-BP1 cascade.

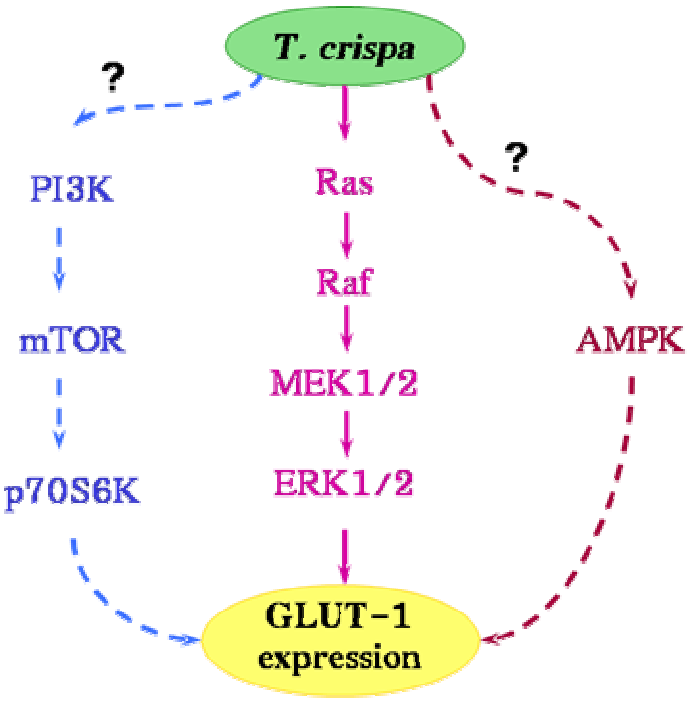

Figure 32. The proposed model for *T. crispa*-induce GLUT1 expression in L6 myotubes by two signaling pathways: Ras/Raf/MEK/MAPK pathway and PI3/PKB(Akt)/mTOR pathway.→ this present mechanism, --> predicted mechanisms.

In addition, several researchers have reported that the stimulation of AMPK activity is associated with the enhancement of GLUT1- glucose transport (Abbud et al, 2000, Barhes et al, 2002, Jing and Ismail-Beigi, 2006). The underlying mechanism shows that the enhanced response of glucose transport is mediated by the activation of GLUT1 pre-exiting in the plasma membrane without affecting the cell surface concentration of GLUT1. Collectively, the role of AMPK is critical in stimulation of glucose transport *via* GLUT1 activation. AMPK may be involved in TC-induced glucose uptake, leading to a conclusion that TC may augment the activity of GLUT1 to accelerate glucose transport by activating AMPK.

In our present study, we can verify that TC enhances basal glucose uptake in L6 myotubes, which is a result of increased GLUT1 expression *via* the ERK pathway. Our results are in agreement with other previous reports. In recent years, it has been found that berberine, a natural product, activates GLUT1- mediated glucose uptake in 3T3-L1 adipocytes *via* the ERK pathway and the AMPK pathway (Kim et al, 2007). TC also contains berberine (Bisset and Nwaiwu, 1983). Hence, this compound may play a

significant role on glucose transport in L6 myotubes. However, this herb also contains other various constituents such as alkaloids and phenolic compounds. Therefore, there should be future research about the effectiveness of these anti-diabetic components.

CHAPTER 5

CONCLUSION

As shown in figure 34, the propose model of *T. crispa*-enhanced glucose transport in L6 myotubes is mediated by up-regulation of GLUT1 protein. Glucose transport was accompanied by a significant increased in GLUT1 mRNA expression and phosphorylation of the extracellular signal regulated kinase (ERK1/2). In contrast, PI3-kinase-activated GLUT4 translocation and p38 MAPK-dependent stimulation of GLUT4 intrinsic activity had no real effect on *T. crispa* -stimulated glucose transport. (Fig. 34)

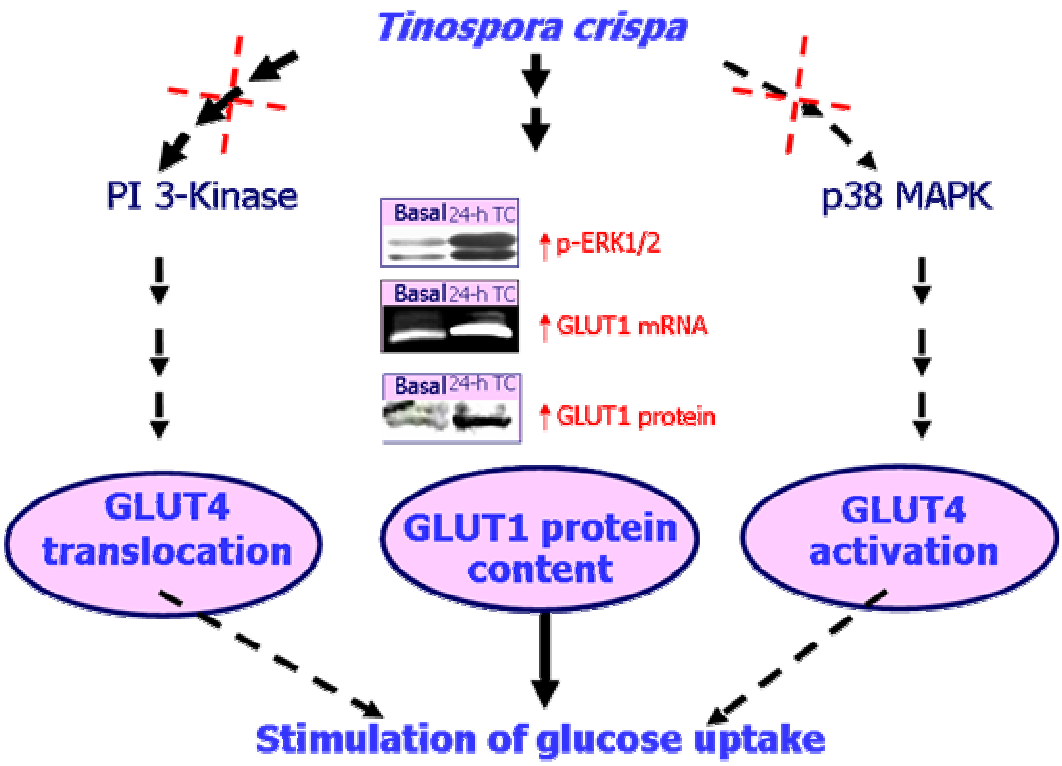

Figure 33. Proposed model of a-*Tinospora crispa* -stimulated glucose uptake in muscle cells. Stimulation of glucose transport by a-*Tinospora* is mediated by ERK1/2 phosphorylation-dependent regulation of GLUT1 expression.

By applying the techniques of modern science, we have moved a step closer in unraveling the mysteries of the traditional use of *T. criapa* and the mechanism (s) responsible for its role to the treatment of diabetes. Our findings demonstrate potential mechanisms for the traditional anti-diabetic action of *T. crispa* through the activation of GLUT1 and GLUT4 protein expression and up-regulation of GLUT1 mRNA via both insulin and non-insulin signaling pathways.

BIBLIOGRAPHY

Abbud W, Habinowski S, Zhang JZ, Kendrew J, Elkairi FS, Kemp BE, Witters LA, Ismail-Beigi F. 2000. Stimulation of AMP-activated protein kinase (AMPK) is associated with enhancement of Glut1-mediated glucose transport. Arch Biochem Biophys 380: 347-352.

Abel ED, Kaulbach HC, Tian R, Hopkins JC, Duffy J, Doetschman T, Minnemann T, Boers ME, Hadro E, Oberste- Berghaus C, Quist W, Lowell BB, Ingwall JS, Kahn BB. 1999. Cardiac hypertrophy with preserved contractile function after selective deletion of GLUT4 from the heart. J Clin Invest 104:1703-1714.

Abel ED, Peroni O, Kim JK, Kim YB, Boss O, Hadro E, Minnemann T, Shulman GI, Kahn BB. 2001. Adipose-selective targeting of the GLUT4 gene impairs insulin action in muscle and liver. Nature 409:729-733.

Ahmed I, Adeghate E, Sharma AK, Pallot DJ, Singh J. 1998. Effects of *Momordica charantia* fruit juice on islet morphology in the pancreas of streptozotocin diabetic rats. Diabetes Res Clin Pract 40: 145-151.

Ai H, Ihlemann J, Hellsten Y, Lauritzen HP, Hardie DG, Galbo H, Ploug T. 2002. Effect of fiber type and nutritional state on AICAR- and contraction-stimulated glucose transport in rat muscle. American Journal of Physiology 282: E1291-E1300.

Alarcon-Aguilara FJ, Roman-Ramos R, Perez-Gutierrez S, Aguilar-Contreras A, Contreras-Weber CC, Flores-Saenz JL. 1998. Study of the anti-hyperglycemic effect of plants used as antidiabetics. J Ethnopharmacol 61: 101-110.

Al-Hasani H, Kunamneni RK, Dawson K, Hinck CS, Muller- Wieland D, Cushman SW. 2002. Roles of the N- and C-termini of GLUT4 in endocytosis. J Cell Sci 115: 131-140.

Anandharajan R, Jaiganesh S, Shankernarayanan NP, Viswakarma RA, Balakrishnan A. 2006. In vitro glucose uptake activity of *Aegles marmelos* and *Syzygium cumini* by activation of Glut-4, PI3 kinase and PPARγ in L6 myotubes. Phytomedicine 13: 434-441.

Anandharajan R, Pathmanathan K, Shankernarayanan NP, Vishwakarma RA, Balakrishnan A. 2005. Upregulation of Glut-4 and PPARγ by an isoflavone from *Pterocarpus marsupium* on L6 myotubes: a possible mechanism of action. J Ethnopharmacol 97: 253-260.

Anulukanapakorn K, Pancharoen O, Basiddhi J. 1998. Hypoglycemic effect of *Tinospora crispa* (Linn.) Mier ex Hook f. & Thoms (Menispermaceae) in rats. J Bulletin of the department of medical sciences 41: 231-243.

Araki E, Lipes MA, Patti ME, Bruning JC, Haag B 3rd, Johnson RS, Kahn CR. 1994. Alternative pathway of insulin signaling in mice with targeted disruption of the IRS-1 gene. Nature 372: 186-190.

Auboeuf D, Vidal H. 1997. The use of the reverse transcription-competitive polymerase chain reaction to investigate the in vivo regulation of gene expression in small tissue samples. Anal Biochem 245: 141-148.

Bailey CJ, Day C. 1989. Traditional plant medicines as treatments for diabetes. Diabetes Care 12: 553-64.

Bak JF, Pedersen 0. 1991. Gliclazide and insulin action in human muscle. Diabetes Res Clin Prac 14561-14564.

Bak JF, Schmitz 0, Sorensen NS, Pedersen 0. 1989. Postreceptor effects of sulfonylurea on skeletal muscle glycogen synthase activity in type II diabetic patients. Diabetes 38:1343-1350.

Baldwin SA, Lienhard GE. 1989. Purification and reconstitution of glucose transporter from human erythrocytes. Methods Enzymol 174: 39-50.

Ballif BA, Blenis J. 2001. Molecular mechanisms mediating mammalian mitogen activated protein kinase (MAPK) kinase (MEK)-MAPK cell survival signals. Cell Growth Differ 12: 397-408.

Balon TW. 1999. Integrative biology of nitric oxide and exercise. Exerc Sport Sci Rev 27: 219-253.

Baodo R, Pardridge, WM. 1993. Glucose deprivation causes post transcriptional enhancement of brain capillary endothelial glucose transporter gene expression via GLUT1 mRNA stabilization. J Neurochem 60: 2290-2296.

Barnes K, Ingram JC, Porras OH, Barros LF, Hudson ER, Fryer LG, Foufelle F, Carling D, Hardie DG, Baldwin SA. 20002. Activation of GLUT1 by metabolic and

osmotic stress: potential involvement of AMP-activated protein kinase (AMPK). J Cell Sci 115: 2433-2442.

Baron AD, Laakso M, Brechtel G, Edelman SV. 1991. Reduced capacity and affinity of skeletal muscle for insulin-mediated glucose uptake in noninsulin-dependent diabetic subjects. Effects of insulin therapy. J Clin Invest 87: 1186-1194.

Baumann CA, Ribon V, Kanzaki M, Thurmond DC, Mora S, Shigematsu S, Bickel PE, Pessin JE, Saltiel AR. 2000. CAP defines a second signaling pathway required for insulin stimulated glucose transport. Nature 407: 202-207.

Baynes-Kenin CR. 2006. Introduction to diabetes mellitus. In Soumyanath, A eds. Traditional medicines for modern times: Antidiabetic plants. New York:Taylor and Francis press, p1-14.

Bell GI, Kayano T, Buse JB, Burant CF, Takeda J, Lin D, Fukumoto H, Seino S. 1990. Molecular biology of mammalian glucose transporters. Diabetes Care 13: 198-208.

Bertani S, Bourdy G, Landau I, Robinson JC, Esterre P, Deharo E. 2005. Evaluation of French Guiana traditional antimalarial remedies. J Etnopharmacol 98: 45-54.

Bian X, He L, Yang G. 2006. Synthesis and antihyperglycemic evaluation of various protoberberine derivatives.Bioorg Med Chem Lett 16: 1380-1383.

Bilan PJ, Mitsumoto Y, Maher F, Simpson IA, Klip A. 1992. Detection of the GLUT3 facilitative glucose transporter in rat L6 muscle cells: regulation by cellular differentiation, insulin and insulin-like growth factor-I. Biochem Biophys Res Commun 186: 1129-1137.

Birnbaum MJ. 1989. Identification of a novel gene encoding an insulin-responsive glucose transporter protein. Cell 57: 305-315.

Bisset NG, Nwaiwu J. 1983. Quaternary alkaloids of Tinospora species. Planta Medica. 48: 275-279.

Bjorntorp P, Berchtold P, Holm J, Larsson B. 1971. The glucose uptake of human adipose tissue in obesity. Eur J Clin Invest 1:480-485.

Bjorntorp P, Sjostrom L. 1978. Carbohydrate storage in man: speculations and some quantitative considerations. Metabolism 27: 1853-1865.

Bosch RR, Bazuine M, Span PN, Willems PH, Olthaar AJ, van Rennes H, Maassen JA, Tack CJ, Hermus AR, Sweep CG. 2004. Regulation of GLUT1-mediated

glucose uptake by PKCλ-PKCβ(II) interactions in 3T3-L1 adipocytes.Biochem J 384: 349-355.

Bosch RR, Bazuine M, Wake MM, Span PN, Olthaar AJ, Schrmann A, Maassen JA, Hermus AR, Willems PH, Sweep CG. 2003. Inhibition of protein kinase CβII increases glucose uptake in 3T3-L1 adipocytes through elevated expression of glucose transporter 1 at the plasma membrane. Mol Endocrinol 17: 1230-1239.

Boulton TG, Nye SH, Robbins DJ, Ip NY, Radziejewska E, Morgenbesser SD, DePinho RA, Panayotatos N, Cobb MH, Yancopoulos GD. 1991. ERKs: a family of protein serine/threonine kinases that are activated and tyrosine phosphorylated in response to insulin and NGF. Cell 65: 663-675.

Bradford MM. 1976. A rapid and sensitive method for the quantitaties of microgram quantities of proteins utilizing the principle of protein-dye binding. Ann Biochem 72: 247-254.

Bryant NJ, Govers R, James DE. 2002. Regulated transport of the glucose transporter GLUT4. Nat Rev Mol Cell Biol 3: 267-277.

Burant CF, Takeda E, Brot-Laroche E, Bell GI, Davidson NO. 1992. Fructose transporter in human spermatozoa and small intestine is GLUT5. J Biol Chem 267: 14523-14526.

Cairns MT, Alvarez J, Panico M, Gibbs AF, Morris HR, Chapman D, Baldwin SA. 1987. Investigation of the structure and function of the human erythrocyte glucose transporter by proteolytic dissection. Biochim Biophys Acta 905: 295-310.

Carayannopoulos MO, Chi MM, Cui Y, Pingsterhaus JM, McKnight RA, Mueckler M, Devaskar SU, Moley KH. 2000. GLUT8 is a glucose transporter responsible for insulin-stimulated glucose uptake in the blastocyst. Proc Natl Acad Sci U S A 20: 7313-7318.

Cavin, A., Aostettmann, K., Dyatmyko, W. and Poterat, O. 1998. Antioxidant and lipophilic constituents of *Tinospora crispa*. Planta Medica 64: 393-396.

Chang L, Chiang SH, Saltiel AR. 2004. Insulin signaling and the regulation of glucose transport. Mol Med 10: 65-71.

Charron MJ, Brosius FC 3rd, Alper SL, Lodish HF. 1989. A glucose transport protein expressed predominately in insulinresponsive tissues. Proc Natl Acad Sci USA 86:2535-2539.

Chavalittumrong P, Attawish A, Chuthaputti A, Chuntapet P. 1997. Toxicological study of crude extract of *Tinospora crispa* Mier ex Hook F.& Thoms. Thai Journal of Pharmaceutical Sciences 21: 199-210.

Cheeseman CI. 1993. GLUT2 is the transporter for fructose across the rat intestinal basolateral membrane. Gastroenterology 105: 1050-1056.

Chen HC, Bandyopadhyay G, Sajan MP, Kanoh Y, Standaert M, Farese RV Jr, Farese RV. 2002. Activation of the ERK pathway and atypical protein kinase C isoforms in exercise- and aminoimidazole-4-carboxamide-1-beta-Driboside (AICAR)-stimulated glucose transport. Journal of Biological Chemistry 277: 23554-23562.

Chen RH, Sarnecki C, Blenis J. 1992. Nuclear localization and regulation of erk- and rsk-encoded protein kinases. Mol Cell Biol 12: 915-927.

Chen Z, Gibson TB, Robinson F, Silvestro L, Pearson G, Xu B, Wright A, Vanderbilt C, Cobb MH. 2001. MAP kinases. Chem Rev 101: 2449-2476.

Chen Z, Heierhorst J, Mann RJ, Mitchelhill KI, Michell BJ, Witters LA, Lynch GS, Kemp BE, Stapleton D. 1999a. Expression of the AMP-activated protein kinase β1 and β2 subunits in skeletal muscle. FEBS Letters 460: 343-348.

Cheung PC, Salt IP, Davies SP, Hardie DG, Carling D. 2000. Characterization of AMP activated protein kinase γsubunit isoforms and their role in AMP binding. Biochemical Journal 346: 659-669.

Chiang SH, Baumann CA, Kanzaki M, Thurmond DC, Watson RT, Neudauer CL, Macara IG, Pessin JE, Saltiel AR. 2001. Insulin-stimulated GLUT4 translocation requires the CAPdependent activation of TC10. Nature 410: 944-948.

Ciaraldi TP, Huber Knudsen K, Hickman M, Olefsky JM. 1995. Regulation of glucose transport in cultured muscle cells by novel hypoglycemic agents. Metabolism 4:976-981.

Clancy BM, Harrison SA, Buxton JM, Czech MP. 1991. Protein synthesis inhibitors activate glucose transport without increasing plasma membrane glucose transporters in 3T3-L1 adipocytes. J Biol Chem 266: 10122-10130.

Cong LN, Chen H, Li Y, Zhou L, McGibbon MA, Taylor SI, Quon MJ. 1997. Physiological role of Akt in insulin-stimulated translocation of GLUT4 in transfected rat adipose cells. Mol Endocrinol 11: 1881-1890.

Cornelius P, Marlowe M, Lee MD, Pekala PH. 1990. The growth factor like effects of tumor necrosis factor-α: stimulation of glucose transport activity and induction of glucose transporter and immediate early gene expression. J Biol Chem 265: 20506-20516.

Corvera S, Czech MP. 1998. Direct targets of phosphoinositide 3-kinase products in membrane traffic and signal transduction. Trends Cell Biol 8: 442-446.

Coster AC, Govers R, James DE. 2004. Insulin stimulates the entry of GLUT4 into the endosomal recycling pathway by a quantal mechanism. Traffic 5:763-771.

Cummings E, Hundal HS, Wackerhage H, Hope M, Belle M, Adeghate E, Singh J. 2004. *Momordica charantia* fruit juice stimulates glucose and amino acid uptakes in L6 myotubes. Mol Cell Biochem 261: 99-104.

Cusin I, Terrettaz J, Rohner-Jeanrenaud F, Zarjevski N, Assimacopoulos-Jeannet F, Jeanrenaud B. 1990. Hyperinsulinemia increases the amount of GLUT4 mRNA in white adipose tissue and decreases that of muscles: a clue for increased fat depot and insulin resistance. Endocrinology 127: 3246-3248.

Day C, Cartwright T, Provost J, Baily CJ. 1990. Hypoglycaemic effect of *Momordica Charantia* extract. Planta Med 56: 426-429.

DeFronzo RA, Ferrannini E, Sato Y, Felig P, Wahren J. 1981. Synergistic interaction between exercise and insulin on peripheral glucose uptake. J Clin Invest 68:1468-1474.

DeFronzo RA, Ferrannini E. 1987. Regulation of hepatic glucose metabolism in humans. Diabetes Metab Rev 3: 415-459.

DeFronzo RA, Gunnarsson R, Bjorkman O, Olsson M, Wahren J. 1985. Effects of insulin on peripheral and splanchnic glucose metabolism in non-insulin dependent diabetes mellitus. J. Clin Invest 76: 149-155.

DeFronzo RA, RC Bonadonna, and E Ferrannini 1992. Pathogenesis of NIDDM. A balanced overview. Diabetes Care 15: 318-368.

DeFronzo RA. 1988. Lilly lecture 1987. The triumvirate: beta-cell, muscle, liver. A collusion responsible for NIDDM. Diabetes 37: 667-687.

Denhardt DT. 1996. Signal-transducing protein phosphorylation cascades mediated by Ras/Rho proteins in the mammalian cell: the potential for multiplex signalling. Biochem J 318: 729-747.

Doege H, Bocianski A, Joost HG, Schurmann A. 2000. Activity and genomic organization of human glucose transporter 9 (GLUT9), a novel member of the family of sugar-transport facilitators predominantly expressed in brain and leucocytes. Biochem J 350: 771-776.

Doege H, Bocianski A, Scheepers A, Axer H, Eckel J, Joost HG, Schurmann A. 2001. Characterization of human glucose transporter (GLUT) 11 (encoded by SLC2A11), a novel sugar-transport facilitator specifically expressed in heart and skeletal muscle. Biochem J 359 :443-449.

Dudek RW, Dohm GL, Holman GD, Cushman SW, Wilson CM. 1994. Glucose transporter localization in rat skeletal muscle. Autoradiographic study using ATB-[2- ^{3}H]BMPA photolabel. FEBS Lett 339: 205-208.

Dugani CB, Klip A. 2005. Glucose transporter 4: cycling, compartments and controversies. EMBO Rep 6: 1137-1142.

Durante PE, Mustard KJ, Park SH, Winder WW , Hardie DG. 2002. Effects of endurance training on activity and expression of AMP-activated protein kinase isoforms in rat muscles. American Journal of Physiology 283: E178-E186.

Eyers PA, van den IJssel P, Quinlan RA, Goedert M, Cohen P. 1999. Use of a drug-resistant mutant of stressactivated protein kinase 2a/p38 to validate the in vivo specificity of SB 203580. FEBS Lett 451: 191-196.

Farnsworth NR and Bunyapraphatsara N. 1992. *Tinospora crispa*. In: Thai medicinal plants: recommended for primary health care system. Prachachon Co.,Ltd. Bangkok, p244-245.

Fasshauer M, Klein J, Ueki K, Kriauciunas KM, Benito M, White MF, Kahn CR. 2000. Essential role of insulin receptor substrate- 2 in insulin stimulation of Glut4 translocation and glucose uptake in brown adipocytes. J Biol Chem 275:25494-25501.

Fernandes NP, Lagishetty CV, Panda VS, Naik SR. 2007. An experimental evaluation of the antidiabetic and antilipidemic properties of a standardized *Momordica charantia* fruit extract. BMC Complement Altern Med 24;7:29.

Fingar DC, Birnbaum MJ. 1994. A role for Raf-1 in the divergent signaling pathways mediating insulin-stimulated glucose transport. J Biol Chem 269: 10127-10132.

Flores-Riveros JR, McClenithan JC, Ezaki 0, Lane MD. 1993. Insulin down-regulates expression of the insulin-responsive glucose transporter (GLUT4) gene: effects on transcription and mRNA turnover. Proc Natl Sci. USA 90: 5122-5126.

Flrth RG, Bell PM, Rllza RA. 1986. Effects of tolazamide and exogenous insulin on. insulin action in patients with non-insulin-dependent diabetes mellitus. N Engl J Med 3141280-3141286.

Forman LL. 1991. Menispermaceae. In: Smitinand T, Larssen K eds. Flora of Thailand. Vol 5 Bangkok: Chutima press, p 300-365.

Friedman JE, Dohm GL, Legget-Frazier N, Elton CW, Tapscott EB, Pories WP, Caro JF. 1992. Restoration of insulin responsiveness in skeletal muscle or morbidly obese patients after weight loss. J Clin Invest 89: 701-705.

Friedman JE, Sherman WM, Reed MJ, Elton CW, Dohm GL. 1990. Exercise training increases glucose transporter protein GLUT4 in skeletal muscle of obese Zucker (faifa) rats. FEBS 268: 13-16.

Fruman DA, Meyers RE, Cantley LC. 1998. Phosphoinositide kinases. Annu Rev Biochem 67: 481-507.

Fryer LG, Foufelle F, Barnes K, Baldwin SA, Woods A, Carling, D. 2002. Characterization of the role of the AMP-activated protein kinase in the stimulation of glucose transport in skeletal muscle cells. Biochem J 363: 167-174.

Fujii N, Aschenbach WG, Musi N, Hirshman MF, Goodyear LJ. 2004. Regulation of glucose transport by the AMP-activated protein kinase. Proc Nutr Soc 63: 205-210.

Fujii N, Hayashi T, Hirshman MF, Smith JT, Habinowski SA, Kaijser L, Mu J, Ljungqvist O, Birnbaum MJ, Witters LA, Thorell A, Goodyear LJ. 2000. Exercise induces isoformspecific increase in 5'AMP-activated protein kinase activity in human skeletal muscle. Biochemical and Biophysical Research Communications 273: 1150-1155.

Fukuda N, Nakamura M, Yonemitsu M, Kimura T, Isobe R, Komori T, 1993. Studies on the constituents of the leaves of *Tinsospora tubercubata* 1.isolation and structure elucidation of 2 new furanoid diterpenes, tinotufolin-A and

tinotufolin-B. Liebiigs annalen der chemic 3: 925-927. Abstract form : SCI 1993.

Fukuda N, Yonemitsu M, Kimura T. 1983. Studies on the constituents of the stems of *Tinospora tuberculata* Beumee. I. N-trans-and N-cis Feruloyl tyramine, and a new phenolicglycoside, Tinotuberride. Chemical and Pharmaceutical Bulletin 31:156-161.

Fukuda N, Yonemitsu M, Kimura T. 1986. Studies on the constituents of the stems of *Tinospora tuberculata* Beumee. Chemical and Pharmaceutical Bulletin 34: 2868-2872.

Fukumoto H, Kayano T, Buse JB, Edwards Y, Pilch PF, Bell GI, Seino S. 1989. Cloning and characterization of the major insulin-responsive glucose transporter expressed in human skeletal muscle and other insulin-responsive tissues. J Biol Chem 264: 7776-7779.

Furtado LM, Poon V, Klip A. 2003. GLUT4 activation: thoughts on possible mechanisms. Acta Physiol Scand. 178: 287-296.

Garvey WT, Hardin D, Juhaszova M, Dominguez JH. 1993. Effects of diabetes on myocardial glucose transport system in rats: implications for diabetic cardiomyopathy. Am J Physiol 264: H837-H844.

Gibbs EM, Calderhead DM, Holman GD, Gould GW. 1991. Phorbol ester only partially mimics the effects of insulin on glucose transport and glucose-transporter distribution in 3T3-L1 adipocytes. Biochem J 275: 145-50.

Goodyear LJ, Hirshman MF, Valyou PM, Horton ES. 1992. Glucose transporter number, function, and subcellular distribution in rat skeletal muscle after exercise training. Diabetes 41: 1091-1099.

Govers R, Coster AC, James DE. 2004. Insulin increases cell surface GLUT4 levels by dose dependently discharging GLUT4 into a cell surface recycling pathway. Mol Cell Biol 24: 6456-6466.

Gross SD, Schwab MS, Lewellyn AL, Maller JL. 1999. Induction of metaphase arrest in cleaving *Xenopus embryos* by the protein kinase p90Rsk. Science 286: 1365-1367.

Grover JK, Yadav S, Vats V. 2002. Medicinal plants of India with anti-diabetic potential. J Ethnopharmacol 81: 81-100.

Habib T, Hejna JA, Moses RE, Decker SJ. 1998. Growth factors and insulin stimulate tyrosine phosphorylation of the 51C/SHIP2 protein. J Biol Chem 273: 18605-18609.

Han Q, Leng J, Bian D, Mahanivong C, CarpenterKA, Pan ZK, Han J, Huang S. 2002. Rac1-MKK3-p38-MAPKAPK2 pathway promotes urokinase plasminogen activator mRNA stability in invasive breast cancer cells. J Biol Chem 277: 48379-48385.

Hardie DG, Carling D, Carlson M. 1998. The AMP-activated/ SNF1 protein kinase subfamily: metabolic sensors of the eukaryotic cell? Annual Review of Biochemistry 67: 821-855.

Hausdorff SF, Bennett AM, Neel BG, Birnbaum MJ. 1995. Different signaling roles of SHPTP2 in insulin-induced GLUT1 expression and GLUT4 translocation. J Biol Chem 270: 12965-12968.

Hausdorff SF, Fingar DC, Morioka K, Garza LA, Whiteman EL, Summers SA, Birnbaum MJ. 1999. Identification of wortmannin-sensitive targets in 3T3-L1 adipocytes. Dissociation of insulin stimulated glucose uptake and GLUT4 translocation. J Biol Chem 274: 24677-24684.

Hausdorff SF, Frangioni JV, Birnbaum MJ. 1994. Role of $p21^{ras}$ in insulin-stimulated glucose transport in 3T3-L1 adipocytes. J Biol Chem 269: 21391-21394.

Hediger MA, Coady MJ, Ikeda TS, Wright EM. 1987. Expression cloning and cDNA sequencing of the Na^+/glucose co-transporter. Nature 330: 379-381.

Hediger MA, Kanai Y, You G, Nussberger S. 1995. Mammalian ion-coupled solute transporters. Journal of Physiology 482: 7S-17S.

Hediger MA, Turk E, Wright EM. 1989. Homology of the human intestinal Na^+/glucose and Escherichia coli Na^+/praline cotransporters. Proceedings of the National Academy of Sciences USA 86: 5748-5752.

Higashino H, Suzuki A, Tanaka Y, Pootakham K, 1992. Inhibitory effects of Siames *Tinospora crispa* extract on the carrageenin-induced foot pad edema in rats (the 1^{st} report). Nippon Yakurigaku Zasshi 100: 339-344.

Hiraki Y, Rosen 0M, Birnbaum MJ. 1988. Growth factors rapidly induce expression for the glucose transporter gene. J Biol Chem 263: 13655-13662.

Hirshman MF, Horton ES. 1990. Glyburide increases insulin sensitivity and responsiveness in peripheral tissues of the rat as determined by the glucose clamp technique. Endocrinology 126: 2407- 2412.

Holman GD, Kozka IJ, Clark AE, Flower CJ, Saltis J, Habberfield AD, Simpson IA, Cushman SW. 1990. Cell surface labeling of glucose transporter isoform GLUT4 by bis-mannose photolabel. Correlation with stimulation of glucose transport in rat adipose cells by insulin and phorbol ester. J Biol Chem 265: 18172-18179.

Holman GD, Rees WD. 1987. Photolabelling of the hexose transporter at external and internal sites: fragmentation patterns and evidence for a conformational change. Biochim Biophys Acta 897: 395-405.

Holmes BF, Kurth-Kraczek EJ, Winder WW. 1999. Chronic activation of 5'-AMP activated protein kinase increases GLUT-4, hexokinase, and glycogen in muscle. J Appl Physiol 87: 1990-1995.

Hook FT. 1975. Menispermaceae. In: Hooker JD, ed. Flora of Brithis India Vol. 1: Ranunculaceae to Sapindaceae. Ashford: L.Reeve & Co. Ltd, p 672.

Huang C, Thirone AC, Huang X, Klip A. 2005. Differential contribution of insulin receptor substrates 1 versus 2 to insulin signaling and glucose uptake in L6 myotubes. J Biol Chem 280: 19426-19435.

Hundal HS, Ahmed A, Guma A, Mitsumoto Y, Marette A, Rennie MJ, Klip A. 1992. Biochemical and immunocytochemical localization of the GLUT5 glucose transporter in human skeletal muscle. Biochem J 286: 339-343.

Hundal HS, Ramlal T, Reyes R, Leiter LA, Klip A. 1992. Cellular mechanism of metformin action involves glucose transporter translocation from an intracellular pool to the plasma membrane in L6 muscle cells. Endocrinology 131:1165-1173.

International Diabetes Federation, Diabetes Atlas. Article online. 2006. Available from: http://www.eatlas.idf.org/media/ Accessed Jan 18, 2008

Ishiki M, Klip A. 2005. Minireview: recent developments in the regulation of glucose transporter-4 traffic: new signals, locations, and partners. Endocrinology 146: 5071-5078.

James DE, Hiken J, Lawrence JC. 1989. Isoproterenol stimulates phosphorylation of the insulin-regulatable glucose transporter in rat adipocytes. Proc. Natl Acad. Sci. USA 86: 8368-8372.

JeBailey L, Rudich A, Huang X, Di Ciano-Oliveira C, Kapus A, Klip A. 2004. Skeletal muscle cells and adipocytes differ in their reliance on TC10 and Rac for insulin-induced actin remodeling. Mol Endocrinol 18: 359-72.

Jing M, Ismail-Beigi F. 2006. Role of 5′-AMP-activated protein kinase in stimulation of glucose transport in response to inhibition of oxidative phosphorylation. Am J Physiol Cell Physiol 290: C484- C491.

Joost HG, Bell GI, Best JD, Birnbaum MJ, Charron MJ, Chen YT, Doege H, James DE, Lodish HF, Moley KH, Moley JF, Mueckler M, Rogers S, Schurmann A, Seino S, Thorens B. 2002. Nomenclature of the GLUT/SLC2A family of sugar/polyol transport facilitators. American Journal of Physiology 282: E974-E976.

Joost HG, Thorens B. 2001. The extended GLUT-family of sugar/polyol transport facilitators: nomenclature, sequence characteristics, and potential function of its novel members (review). Molecular Membrane Biology 18: 247-256.

Joost HG, Weber TM, Cushman SW, Simpson IA. 1986. Insulin-stimulated glucose transport in rat adipose cells. Modulation of transporter intrinsic activity by isoproterenol and adenosine. J Biol Chem 261: 10033-10036.

Kaestner KH, Christy RJ, McLenithan JC, Braiterman LT, Cornelius P, Pekala PH, Lane MD. 1989. Sequence, tissue distribution, and differential expression of mRNA for a putative insulin-responsive glucose transporter in mouse 3T3-L1 adipocytes. Proc Natl Acad Sci USA 86: 3150-3154.

Kahn BB, Horton ES, Cushman SW. 1987. Mechanism for enhanced glucose transport response to insulin in adipose cells from chronically hyperinsulinemic rats. J Clin Invest 79: 853-858.

Kahn BB, Rosen AS, Bak JF, Anderson PH, Damsbo P, Lund S, Pederson 0. 1992. Expression of GLUT1 and GLUT4 glucose transporters in skeletal muscle of humans with insulin dependent diabetes mellitus: regulatory effects of metabolic factors. J. Clin. Endocrin. Mer 74: 1101-1109.

Kahn CR. 1994. Banting lecture. Insulin action, diabetogenes, and the cause of type II diabetes. Diabetes 43: 1066-1084.

Kahn SE. 2003. The relative contributions of insulin resistance and β-cell dysfunction to the pathophysiology of type 2 diabetes. Diabetologia 46: 3-19.

Kanai Y, Lee WS, You G, Brown D, Hediger MA. 1994. The human kidney low affinity Na^+/glucose cotransporter SGLT2. Delineation of the major renal reabsorptive mechanism for D-glucose. Journal of Clinical Investigation 93: 397-404.

Kanjanavong C. 2000. Efficacy and safety of *Tinospora crispa* for treatment of diabetes mellitus. The bulletin of Lumpang hospital 21: 1-7.

Karnieli E, Zarnowski MJ, Hissin PJ, Simpson IA, Salans LB, Cushman SW. 1981. Insulin-stimulated translocation of glucose transport systems in the isolated rat adipose cell. Time course, reversal, insulin concentration dependency, and relationship to glucose transport activity. J Biol Chem 256: 4772-4777.

Karylowski O, Zeigerer A, Cohen A, McGraw TE. 2004. GLUT4 is retained by an intracellular cycle of vesicle formation and fusion with endosomes. Mol Biol Cell 15: 870-882.

Kasahara T, Kasahara M. 1996. Expression of the rat GLUT1 glucose transporter in the yeast *Saccharomyces cerevisiae*. Biochem J 315:177-182.

Kasahara T, Kasahara M. 1997. Characterization of rat Glut4 glucose transporter expressed in the yeast *Saccharomyces cerevisiae*: comparison with Glut1 glucose transporter. Biochim Biophys Acta 1324: 111-119.

Kayano T, Burant CF, Fukumoto H, Gould GW, Fan YS, Eddy RL, Byers MG, Shows TB, Seino S, Bell GI. 1990. Human facilitative glucose transporters. Isolation, functional characterization, and gene localization of cDNAs encoding an isoform (GLUT5) expressed in small intestine, kidney, muscle, and adipose tissue and an unusual glucose transporter pseudogene-like sequence (GLUT6). J Biol Chem 265:13276-13282.

Kayano T, Fukumoto H, Eddy RL, Fan YS, Byers MG, Shows TB, Bell GI. 1988. Evidence for a family of human glucose transporter-like proteins. Sequence and gene localization of a protein expressed in fetal skeletal muscle and other tissues. J Biol Chem 263: 15245-15248.

Kemp BE, Mitchelhill KI, Stapleton D, Michell BJ, Chen ZP, Witters LA. 1999. Dealing with energy demand: the AMPactivated protein kinase. Trends in Biochemical Sciences 24: 22-25.

Kim SH, Shin EJ, Kim ED, Bayaraa T, Frost SC, Hyun CK. 2007. Berberine activates GLUT1-mediated glucose uptake in 3T3-L1 adipocytes. Biol Pharm Bull 30: 2120-2125.

Kimball SR, Horetsky RL, Jefferson LS. 1998. Signal transduction pathways involved in the regulation of protein synthesis by insulin in L6 myoblasts.Am J Physiol: 274: C221-C228.

Kitagawa T, Masumi A, Akamatsu Y. 1991. Transforming growth factor-β1 stimulates glucose uptake and the expression of glucose transporter mRNA in quiescent Swiss mouse 3T3 cells. J Biol Chem 266: 18066- 18071.

Kitagawa T, Tanaka M, Akamatsu Y. 1989. Regulation of glucose transport activity and expression of glucose transporter mRNA by serum, growth factors and phorbol ester in quiescent mouse fibroblasts. Biochim Biophys Acta 980: 100-108.

Klip A, Guma A, Ramlal T, Bilan PJ, Lam L, Leiter LA. 1992. Stimulation of hexose transport by metformin in L6 muscle cells in culture. Endocrinology 130: 2535-2544.

Klip A, Li G, Logan WJ. 1984. Induction of sugar uptake response to insulin by serum depletion in fusing L6 myoblasts. Am J Physiol 247: E291-E296.

Klip A, Marette A. 1992. Acute and chronic signals controlling glucose transport in skeletal muscle. J Cell Biochem 48: 51-60.

Kohn AD, Barthel A, Kovacina KS, Boge A, Wallach B, Summers SA, Birnbaum MJ, Scott PH, Lawrence JC Jr, Roth RA. 1998. Construction and characterization of a conditionally active version of the serine/threonine kinase Akt. J Biol Chem 273: 11937-11943.

Kohn AD, Summers SA, Birnbaum MJ, Roth RA. 1996. Expression of a constitutively active Akt Ser/Thr kinase in 3T3-L1 adipocytes stimulates glucose uptake and glucose transporter 4 translocation. J Biol Chem 271: 31372-31378.

Kohno M, Pouyssegur J. 2003. Pharmacological inhibitors of the ERK signaling pathway: application as anticancer drugs. Prog Cell Cycle Res 5: 219-224.

Kohout TA, Rogers TB. 1993. Use of a PCR-based method to characterize protein kinase C isoform expression in cardiac cells. Am J Physiol 264: C1350-C1359.

Koivisto UM, Martinez-Valdez H, Bilan PJ, Burdette E, Ramlal T, Klip A. 1991. Differential regulation of the GLUT1 and GLUT4 glucose transport systems by

glucose and insulin in L6 muscle cells in culture. J Biol Chem 266: 2615-2621.

Kolterman OG, Gray RS, Shapiro G, Scarlett J, Griffin J, Olefsky JM. 1984. The acute and chronic effects of sulfonylurea therapy in type II diabetic subjects. Diabetes 33: 346-354.

Kongkathip N, Dhumma-upakorn P, Kongkathip B, Chawananoraset K, Sangchomkaeo P, Hatthakitpanichakul S. 2002. Study on cardiac contractility of cycloeucalenol and cycloeucalenone isolated from *Tinospora crispa*. J Etnopharmacol 83:95-99.

Kumar S, Jiang MS, Adams JL, Lee JC. 1999. Pyridinylimidazole compound SB 203580 inhibits the activity but not the activation of p38 mitogen-activated protein kinase. Biochem Biophys Res Commun 263: 825-831.

Kurth-Kraczek EJ, Hirshman MF, Goodyear LJ, Winder WW. 1999. 5' AMP-activated protein kinase activation causes GLUT4 translocation in skeletal muscle. Diabetes 48: 1667-1671.

Kyriakis JM, Avruch J. 2001. Mammalian mitogenactivated protein kinase signal transduction pathways activated by stress and inflammation. Physiol Rev 81: 807-869.

Laemmli UK. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227: 680-685.

Laville M, Auboeuf D, Khalfallah Y, Vega N, Riou JP, Vidal H. 1996. Acute regulation by insulin of phosphatidylinositol-3-kinase, Rad, Glut 4, and lipoprotein lipase mRNA levels in human muscle. J Clin Invest 98: 43-49.

Lawrence JC Jr, Piper RC, Robinson LJ, James DE. 1992. GLUT4 facilitates insulin stimulation and cAMP-mediated inhibition of glucose transport. Proc Natl Acad Sci USA 89: 3493-3497.

Lazar DF, Wiese RJ, Brady MJ, Mastick CC, Waters SB, Yamauchi K, Pessin JE, Cuatrecasas P, Saltiel AR. 1995. Mitogen-activated protein kinase kinase inhibition does not block the stimulation of glucose utilization by insulin. J Biol Chem 270: 20801-20807.

Le Marchand-Brustel Y, Gautier N, Cormont M, Van Obberghen E. 1995. Wortmannin inhibits the action of insulin but not that of okadaic acid in skeletal muscle: comparison with fat cells. Endocrinology 136: 3564-3570.

Lee JC, Laydon LT, McDonnell PC, Gallagher TF,Kumar S, Green D, McNulty D, Blumenthal MJ, Heys JR, Landvatter SW, and et al. 1994. A protein kinase involved in the regulation of inflammatory cytokine biosynthesis. Nature 372: 739-746.

Leng SH, Lu FE, Xu LJ. 2004. Therapeutic effects of berberine in impaired glucose tolerance rats and its influence on insulin secretion. Acta Pharmacol Sin 25: 496-502.

Lenormand P, Sardet C, Pages G, Allemain GL, Brunet A, Pouyssegur J. 1993. Growth factors induce nuclear translocation of MAP kinases ($p42^{mapk}$ and $p44^{mapk}$) but not of their activator MAP kinase kinase ($p45^{mapkk}$) in fibroblasts. J Cell Biol 122: 1079-1088.

Lewis TS, Shapiro PS, Ahn NG. 1998. Signal transduction through MAP kinase cascades. Adv. Cancer Res. 74: 49-139.

Li D, Randhawa VK, Patel N, Hayashi M, Klip A. 2001. Hyperosmolarity reduces GLUT4 endocytosis and increases its exocytosis from a VAMP2- independent pool in L6 muscle cells. J Biol Chem 276: 22883-22891.

Liu J, Kimura A, Baumann CA, Saltiel AR. 2002. APS facilitates c-Cbl tyrosine phosphorylation and GLUT4 translocation in response to insulin in 3T3-L1 adipocytes. Mol Cell Biol 22: 3599-3609.

Livingstone C, James DE, Rice JE, Hanpeter D, Gould GW. 1996. Compartment ablation analysis of the insulin-responsive glucose transporter (GLUT4) in 3T3-L1 adipocytes. Biochem J 315: 487-495.

Ludvik B, Nolan JJ, Roberts A, Baloga J, Joyce M, Bell JM, Olefsky JM. 1997. Evidence for decreased splanchnic glucose uptake after oral glucose administration in non-nsulin-dependent diabetes mellitus. J Clin Invest 100: 2354-2361.

Maher F, Harrison LC. 1990. Stabilization of glucose transporter mRNA by insulin/IGF-1 and glucose deprivation. Biochem. Biophys Rex Commun 171: 210-215.

Maher F, Harrison LC. 1991. Stimulation of glucose transport (GLUT1) mRNA and protein expression by inhibitors of glycosylation. Biochem Biophys Actu 1089: 27-32.

Maher F, Vannucci SJ, Simpson IA. 1994. Glucose transporter proteins in brain. FASEB J 8: 1003-1011.

Malide D, Dwyer NK, Blanchette-Mackie EJ, Cushman SW. 1997. Immunocytochemical evidence that GLUT4 resides in a specialized translocation postendosomal VAMP2 positive compartment in rat adipose cells in the absence of insulin. J Histochem Cytochem 45: 1083-1096.

Marette A, Burdett E, Douen A, Vranic M, Klip A. 1992a. Insulin induces the translocation of GLUT-4 glucose transporters from a unique intracellular organelle to transverse tubules in rat skeletal muscle. Diabetes 41: 1562-1569.

Marette A, Richardson JM, Ramlal T, Balson TW, Vranic M, Pessin JF, Klip A. 1992b. Abundance, localization and insulin induced translocation of glucose transporters in the red and white muscle of the rat hindlimb. Am J Physiol 263: C443-C453.

Martin BC, Warram JH, Krolewski AS, Bergman RN, Soeldner JS, Kahn CR. 1992. Role of glucose and insulin resistance in development of type 2 diabetes mellitus: results of a 25-year follow-up study. Lancet 340:925-929.

Martin S, Tellam J, Livingstone C, Slot JW, Gould GW, James DE. 1996. The glucose transporter (GLUT-4) and vesicle-associated membrane protein-2 (VAMP-2) are segregated from recycling endosomes in insulin-sensitive cells. J Cell Biol 134:625-635.

Martin SS, Haruta T, Morris AJ, Klippel A, Williams LT, Olefsky JM. 1996. Activated phosphatidylinositol 3-kinase is sufficient to mediate actin rearrangement and GLUT4 translocation in 3T3-L1 adipocytes. J Biol Chem 271: 17605-17608.

Mauvais-Jarvis F, Ueki K, Fruman DA, Hirshman MF, Sakamoto K, Goodyear LJ, Iannacone M, Accili D, Cantley LC, Kahn CR. 2002. Reduced expression of the murine p85α subunit of phosphoinositide 3-kinase improves insulin signaling and ameliorates diabetes. J Clin Invest 109: 141-149.

McGee SL, Hargreaves M. 2006. Exercise and skeletal muscle glucose transporter 4 expression: molecular mechanisms. Clin Exp Pharmacol Physiol 33: 395-399.

McVie-Wylie AJ, Lamson DR, Chen YT. 2001. Molecular cloning of a novel member of the GLUT family of transporters, SLC2a10 (GLUT10), localized on chromosome 20q13.1: a candidate gene for NIDDM susceptibility. Genomics 15: 113-117.

Merrall NW, Wakelam MJ, Plevin R, Gould GW. 1993. Insulin and platelet-derived growth factor acutely stimulate glucose transport in 3T3-L1 fibroblasts independently of protein kinase C. Biochim Biophys Acta 1177: 191-198.

Mitra P, Zheng X, Czech MP. 2004. RNAi-based analysis of CAP, Cbl, and CrkII function in the regulation of GLUT4 by insulin. J Biol Chem 279: 37431-37435.

Molero JC, Whitehead JP, Meerloo T, James DE. 2001. Nocodazole inhibits insulin stimulated glucose transport in 3T3-L1 adipocytes via a microtubule-independent mechanism. J Biol Chem 276: 43829-43835.

Mora A, Komander D, van Aalten DM, Alessi DR. 2004. PDK1, the master regulator of AGC kinase signal transduction. Semin Cell Dev Biol 15: 161-170.

Morris DI, Robbins JD, Ruoho AE, Sutkowski EM, Seamon KB. 1991. Forskolin photoaffinity labels with specificity for adenylyl cyclase and the glucose transporter. J Biol Chem 266: 13377-13384.

Mountjoy KG, Flier JS. 1990. Vanadate regulates glucose transporter GLUT1 expression in NIH 3T3-Ll mouse fibroblasts. Endocrinology 127: 2025-2034.

Mueckler M, Caruso C, Baldwin SA, Panico M, Blench I, Morris HR, Allard WJ, Lienhard GE, Lodish HF. 1985. Sequence and structure of a human glucose transporter. Science 229: 941-945.

Mueckler M. 1994. Facilitative glucose transporters. European Journal of Biochemistry 219: 713-725.

Mueller-Oerlinghausen B, Ngamwathana W, Kanchanapee P. 1971. Investigation into Thai medicinal plants said to cure diabetes. J Med Assoc Thai 54: 105-112.

Murakoshi, I. et al. 1993. Monoligan and diterpene glycosides from the stems of *Tinospora rumphil* Borel. Thai Journal Phamaceutical Sciences 17:33-37.

Musi N, Goodyear LJ. 2003. AMP-activated protein kinase and muscle glucose uptake. Acta Physiol Scand 178: 337-345.

Musi N, Hayashi T, Fujii N, Hirshman MF, Witters LA, Goodyear LJ. 2001a. AMP activated protein kinase activity and glucose uptake in rat skeletal muscle. American Journal of Physiology 280: E677-E684.

Najib NA, Rahman N, Furuta T, Kojima S, Takane K, Ali MM. 1999. Antimalarial activity of extracts of Malaysian medicinal plants. J Etnopharmacol 64: 249-254.

Nave BT, Siddle K, Shepherd PR. 1996. Phorbol esters stimulate phosphatidylinositol 3,4,5-trisphosphate production in 3T3-L1 adipocytes: implications for stimulation of glucose transport. Biochem J 318: 203-205.

Neufer PD, Dohm GL. 1993. Exercise induces a transient increase in transcription of the GLUT4 gene in skeletal muscle. Am J Physiof 265: C1597-C1603.

Neufer PD, Shinebarger MH, Dohm GL. 1992. Effect of training and detraining on skeletal muscle glucose transporter (GLUT4) content in rats. Curt J Physiol Pharmacol 70: 1286-1290.

Noor H, Ashcroft SJ., 1989. Antidiabetic effects of *Tinospora crispa* in rats. J Ethnopharmacol 27: 149-161.

Noor H, Hammonds P, Sutton R, Ashcroft SJ, 1989. The hypoglycaemic and insulinotropic activity of *Tinospora crispa*: studies with human and rat islets and HIT-T15 B cells. Diabetologia 32: 354-359.

Noor H, Ashcroft SJ. 1998. Pharmacological characterisation of the antihyperglycaemic properties of *Tinospora crispa* extract. J Ethnopharmacol 62: 7-13.

Ojuka EO, Nolte LA, Holloszy JO. 2000. Increased expression of GLUT-4 and hexokinase in rat epitrochlearis muscles exposed to AICAR in vitro. J Appl Physiol 88: 1072-1075.

Okada T, Kawano Y, Sakakibara T, Hazeki O, Ui M. 1994. Essential role of phosphatidylinositol 3-kinase in insulin-induced glucose transport and antilipolysis in rat adipocytes: studies with a selective inhibitor wortmannin. J Biol Chem 269: 3568-3573.

Okada T, Sakuma L, Fukui Y, Hazeki O, Ui M. 1994. Blockage of chemotactic peptide-induced stimulation of neutrophils by wortmannin as a result of selective inhibition of phosphatidylinositol 3-kinase. J Biol Chem 269: 3563-3567.

Pachaly P, Adnan AZ, Will G. 1992. NMR-Assignments of N-Acylaporphine Alkaloids from *Tinospora crispa*. Planta Med 58: 184-187.

Papazisis KT, Geromichalos GD, Dimitriadis KA, Kortsaris AH. 1997. Optimization of the sulforhodamine B colorimetric assay. J Immunol Methods 208: 151-158.

Peltz SW, Brewer G, Bernstein P, Hart PA, Ross J. 1991. Regulation of mRNA turnover in eukaryotic cells. Crit Rev Euk Gene Express 1: 99-126.

Peltz SW, Jacobson A. 1992. mRNA stability: in trans-it. Current Opin Cell Biol 4: 979-983.

Pereira LO, Lancha AH Jr. 2004. Effect of insulin and contraction up on glucose transport in skeletal muscle. Prog Biophys Mol Biol 84: 1-27.

Pesesse X, Deleu S, De Smedt F, Drayer L, Erneux C. 1997. Identification of a second SH2-domain-containing protein closely related to the phosphatidylinositol polyphosphate 5-phosphatase SHIP. Biochem Biophys Res Commun 239: 697-700.

Peungvicha P, Temsiririrkkul R, Prasain JK, Tezuka Y, Kadota S, Thirawarapan SS, Watanabe H. 1998a. 4-Hydroxybenzoic acid: a hypoglycemic constituent of aqueous extract of *Pandanus odorus* root. J Ethnopharmacol 62: 79-84.

Peungvicha P, Thirawarapan SS, Watanabe H. 1998b. Possible mechanism of hypoglycemic effect of 4-hydroxybenzoic acid, a constituent of *Pandanus odorus* root. Jpn J Pharmacol 78: 395-398.

Phay JE, Hussain HB, Moley JF. 2000. Cloning and expression analysis of a novel member of the facilitative glucose transporter family, SLC2A9 (GLUT9). Genomics 66: 217-220.

Piper RC, Hess LJ, James DE. 1991. Differential sorting of two glucose transporters expressed in insulin-sensitive cells. Am J Physiol 260: C570-C580.

Piper RC, James DE, Slot JW, Puri C, Lawrence JC Jr. 1993. GLUT4 phosphorylation and inhibition of glucose transport by dibutyryl cAMP. J Biol Chem 268: 16557 -16563.

Ploug T, Stallknecht BM, Pederson O, Kahn BB, Ohkuwa T, Vinten J, Galbo H. 1990. Effect of endurance training on glucose transporter capacity and glucose transporter expression in rat skeletal muscle. Am J Physiol 259: E778-E786.

Porte Jr D. 1991. Banting lecture 1990. β-cells in type II diabetes mellitus. Diabetes 40: 166-180.

Pouyssegur J, Volmat V, Lenormand P. 2002. Fidelity and spatiotemporal control in MAP kinase (ERKs) signalling. Biochem Pharmacol 64: 755-763.

Randhawa VK, Thong FS, Lim DY, Li D, Garg RR, Rudge R, Galli T, Rudich A, Klip A. 2004. Insulin and hypertonicity recruit GLUT4 to the plasma membrane of muscle cells by using N-ethylmaleimide-sensitive factor-dependent SNARE mechanisms but different v SNAREs: role of TI-VAMP. Mol Biol Cell 15: 5565-5573.

Reaven GM. 1991. Insulin resistance, hyperinsulinemia, hypertriglyceridemia, and hypertension. Parallels between human disease and rodent models. Diabetes Care 14:195-202.

Ribon V, Printen JA, Hoffman NG, Kay BK, Saltiel AR. 1998. A Novel, multifunctional c-Cbl binding protein in insulin receptor signaling in 3T3-L1 Adipocytes. Mol Cell Biol 18: 872-879.

Ribon V, Saltiel AR. 1997. Insulin stimulates tyrosine phosphorylation of the proto oncogene product of c-Cbl in 3T3-L1 adipocytes. Biochem J 324: 839-45.

Robinson LJ, Pang S, Harris DS, Heuser J, James DE. 1992. Translocation of the glucose transporter (GLUT4) to the cell surface in permeabilized 3T3-L1 adipocytes: effects of ATP, insulin and GTPγS and localization of GLUT4 to clathrin lattices. J Cell Biol 117: 1181-1196.

Rogers S, Macheda ML, Docherty SE, Carty MD, Henderson MA, Soeller WC, Gibbs EM, James DE, Best JD. 2002. Identification of a novel glucose transporter-like protein GLUT-12. Am J Physiol Endocrinol Metab 282: E733-738.

Rollins BJ, Morrison ED, Usher P, Flier JS. 1988. Platelet-derived growth factor regulates glucose transporter expression. J Biol Chem 263: 16523-16526.

Ros-Baro A, Lopez-Iglesias C, Peiro S, Bellido D, Palacin M, Zorzano A, Camps M. 2001. Lipid rafts are required for GLUT4 internalization in adipose cells. Proc Natl Acad Sci USA 98: 12050-12055.

Rudich A, Klip A. 2003. Push/pull mechanisms of GLUT4 traffic in muscle cells. Acta Physiol Scand 178: 297-308.

Russell RR III, Bergeron R, Shulman GI, Young LH. 1999. Translocation of myocardial GLUT-4 and increased glucose uptake through activation of AMPK by AICAR. Am J Physiol 277: H643-H649.

Sakamoto K, Goodyear LJ 2002. Invited review: intracellular signaling in contracting skeletal muscle. J Appl Physiol 93: 369-383.

Salt I, Celler JW, Hawley SA, Prescott A, Woods A, Carling D, Hardie DG. 1998. AMP activated protein kinase: greater AMP dependence, and preferential nuclear localization, of complexes containing the α2 isoform. Biochemical Journal 334: 177-187.

Saltiel AR, Kahn CR. 2001. Insulin signalling and the regulation of glucose and lipid metabolism. Nature 414:799-806.

Saltiel AR, Olefsky JM. 1996. Thiazolidinediones in the treatment of insulin resistance and type II diabetes. Diabetes 45: 1661-1669.

Saltiel AR, Pessin JE. 2002. Insulin signaling pathways in time and space. Trends Cell Biol 12: 65-71.

Sandouk T, Reda D, Hoffman C. 1993. The antidiabetic agent pioglitzone increases expression of glucose transporters in 3T3-F442A cells by increasing mRNA transcript stability. Endocrinology 133: 352-359.

Sangsuwan C, Udompanthurak S, Vannasaeng S, Thamlikitkul V, 2004. Randomized controlled trial of *Tinospora crispa* for additional therapy in patients with type 2 diabetes mellitus. J Med Assoc Thai 87: 543-546.

Sarbassov DD, Guertin DA, Ali SM, Sabatini DM. 2005. Phosphorylation and regulation of Akt/PKB by the rictor-mTOR complex. Science 307: 1098-1101.

Satoh S, Nishimura H, Clark AE, Kozka IJ, Vannucci SJ, Simpson IA, Quon MJ, Cushman SW, Holman GD. 1993. Use of bismannose photolabel to elucidate insulin-regulated GLUT4 subcellular trafficking kinetics in rat adipose cells. Evidence that exocytosis is a critical site of hormone action. J Biol Chem 268: 17820-17829.

Schurmann A, Monden I, Joost HG, Keller K. 1992. Subcellular distribution and activity of glucose transporter isoforms GLUT1 and GLUT4 transiently expressed in COS-7 cells. Biochim Biophys Acta 1131: 245-252.

Sevilla L, Tomas E, Munoz P, Guma A, Fischer Y, Thomas J, Ruiz-Montasell B, Testar X, Palacin M, Blasi J, Zorzano A. 1997. Characterization of two distinct intracellular GLUT4 membrane populations in muscle fiber. Differential protein composition and sensitivity to insulin. Endocrinology 138: 3006-3015.

Sharma AK, Ahmed I, Tadaygon M, Penery AS, Aloamaka P, Absood G, Pallot DJ. 1996. The beneficial effects of *Momordica charantia* fruit juice on streptozotocin-induced diabetes and hypertension in rats. Int J Diabetes 4: 29-38.

Sharma PM, Egawa K, Huang Y, Martin JL, Huvar I, Boss GR, Olefsky JM. 1998. Inhibition of phosphatidylinositol 3-kinase activity by adenovirus-mediated gene transfer and its effect on insulin action. J Biol Chem 273: 18528-18537.

Shepherd PR. 2005. Mechanisms regulating phosphoinositide 3-kinase signaling in insulin-sensitive tissues. Acta Physiol Scand 183: 3-12.

Shetty M, Ismail-Beigi N, Loeb JN, Ismail-Beigi F. 1993. Induction of GLUT1 mRNA in response to inhibition of oxidative phosphorylation. Am J Physiol 265: Cl224- C1229.

Shewan AM, van Dam EM, Martin S, Luen TB, Hong W, Bryant NJ, James DE. 2003. GLUT4 recycles via a trans-Golgi network (TGN) subdomain enriched in syntaxins 6 and 16 but not TGN38: involvement of an acidic targeting motif. Mol Biol Cell 14: 973-986.

Shulman GI. 2000. Cellular mechanisms of insulin resistance. J Clin Invest 106: 171-176.

Sinha A. 1960. Chemical examination of the mucilage from the leaves of *Tinospora crispa*. Indian J Appl Chem 32: 87-90.

Sirigulsatien V, Bunsit J, Bunjob M, Tachadomrongsin Y. 2001. Effectiveness and safety of *Tinospora crispa* for diabetes mellitus treatment. Buddhachinaraj medical journal 18: 87-94.

Skehan P, Storeng R, Scudiero D, Monks A, McMahon J, Vistica D, Warren JT, Bokesch H, Kenney S, Boyd MR.1990. New colorimetric cytotoxicity assay for anticancer-drug screening. J Natl Cancer Inst 82: 1107-1112.

Slot JW, Geuze HJ, Gigengack S, Lienhard GE, James DE. 1991. Immunolocalization of the insulin regulatable glucose transporter in brown adipose tissue of the rat. J Cell Biol 113: 123-135.

Somwar R, Kim DY, Sweeney G, Huang C, Niu W, Lador C, Ramlal T, Klip A. 2001a. GLUT4 translocation precedes the stimulation of glucose uptake by insulin in muscle cells: potential activation of GLUT4 via p38 mitogen-activated protein kinase. Biochem J 359: 639-649.

Somwar R, Koterski S, Sweeney G, Sciotti R, Djuric S, Berg C, Trevillyan J, Scherer PE, Rondinone CM, Klip A. 2002. A dominant-negative p38 MAPK mutant and novel selective inhibitors of p38 MAPK reduce insulin-stimulated glucose uptake in 3T3-L1 adipocytes without affecting GLUT4 translocation. J Biol Chem 277:50386-50395.

Somwar R, Niu W, Kim DY, Sweeney G, Randhawa VK, Huang C, Ramlal T, Klip A. 2001b. Differential effects of phosphatidylinositol 3-kinase inhibition on intracellular signals regulating GLUT4 translocation and glucose transport. J Biol Chem 276: 46079-4608.

Somwar R, Perreault M, Kapur S, Taha C, Sweeney G, Ramlal T, Kim DY, Keen J, Cote CH, Klip A & Marette A. 2000. Activation of p38 mitogen-activated protein kinase αand β by insulin and contraction in rat skeletal muscle: potential role in the stimulation of glucose transport. Diabetes 49: 1794-1800.

Somwar R, Sumitani S, Taha C, Sweeney G, Klip A. 1998. Temporal activation of p70 S6 kinase and Akt1 by insulin: PI 3-kinase-dependent and -independent mechanisms. Am J Physiol 275: E618-E625.

Stapleton D, Mitchelhill KI, Gao G, Widmer J, Michell BJ, The T, House CM, Fernandez CS, Cox T, Witters LA, Kemp BE. 1996. Mammalian AMP-activated protein kinase subfamily. J Biol Chem 271: 611-614.

Stephens J, Pekala PH. 1992. Transcriptional repression of the CiEBP and GLUT4 genes in 3T3-Ll acipocytes by tumor necrosis factor-α: regulation is coordinate and independent of protein synthesis. J Biol Chem 267: 13580-13584.

Stephens JM, Carter BZ, Pekala PH, Malter JS. 1992. Tumor necrosis factor-α induced glucose transporter (GLUT1) mRNA stabilization in 3T3-Ll preadipocytes. J Biol Chem 267: 8336-8341.

Strack V, Hennige AM, Krutzfeldt J, Bossenmaier B, Klein HH, Kellerer M, Lammers R, Haring HU. 2000. Serine residues 994 and 1023/25 are important for insulin receptor kinase inhibition by protein kinase C isoforms beta2 and theta. Diabetologia 43: 443-449.

Stumpel F, Jungerman K. 1997. Sensing by intrahepatic muscarinic nerves of a portal arterial glucose concentration gradient as a signal for insulin dependent glucose uptake in the perfused rat liver. FEBS Lett 406: 119-122.

Subehan, Usia T, Iwata H, Kadota S, Tezuka Y. 2006. Mechanism-based inhibition of CYP3A4 and CYP2D6 by Indonesian medicinal plants. J Ethnopharmacol 105: 449-455.

Sweeney G, Keen J, Somwar R, Konrad D, Garg R, Klip A. 2001. High leptin levels acutely inhibit insulin stimulated glucose uptake without affecting glucose transporter 4 translocation in L6 rat skeletal muscle cells Endocrinology 142: 4806-4812.

Sweeney G, Somwar R, Ramlal T, Volchuk A, Ueyama A, Klip A. 1999. An inhibitor of p38 mitogen-activated protein kinase prevents insulin-stimulated glucose

transport but not glucose transporter translocation in 3T3-L1 adipocytes and L6 myotubes. J Biol Chem 274: 10071-10078.

Taha C, Liu Z, Jin J, Al-Hasani H, Sonenberg N, Klip A. 1999. Opposite translational control of GLUT1 and GLUT4 glucose transporter mRNAs in response to insulin. Role of mammalian target of rapamycin, protein kinase b, and phosphatidylinositol 3-kinase in GLUT1 mRNA translation. J Biol Chem 274: 33085-33091.

Taha C, Mitsumoto Y, Liu Z, Skolnik EY, Klip A. 1995. The insulin-dependent biosynthesis of GLUT1 and GLUT3 glucose transporters in L6 muscle cells is mediated by distinct pathways. Roles of $p21^{ras}$ and pp70 S6 kinase. J Biol Chem 270: 24678-24681.

Tebbey PW, McGowan KM, Stephens JM, Buttke TM, Pekala PH. 1994. Arachidonic acid down regulates the insulin-dependent glucose transporter gene (GLUT4) in 3T3-Ll adipocytes by inhibiting transcription and enhancing mRNA turnover. J Biol Chem 269: 639-644.

Terada S, Goto M, Kato M, Kawanaka K, Shimokawa T, Tabata I. 2002. Effects of low intensity prolonged exercise on PGC-1 mRNA expression in rat epitrochlearis muscle. Biochem Biophys Res Commun 296: 350-354.

Terauchi Y, Tsuji Y, Satoh S, Minoura H, Murakami K, Okuno A, Inukai K, Asano T, Kaburagi Y, Ueki K, Nakajima H, Hanafusa T, Matsuzawa Y, Sekihara H, Yin Y, Barrett JC, Oda H, Ishikawa T, Akanuma Y, Komuro I, Suzuki M, Yamamura K, Kodama T, Suzuki H, Yamamura K, Kodama T, Suzuki H, Koyasu S, Aizawa S, Tobe K, Fukui Y, Yazaki Y, Kadowaki T. 1999. Increased insulin sensitivity and hypoglycaemia in mice lacking the p85 alpha subunit of phosphoinositide 3-kinase. Nat Genet 21: 230-235.

The Expert Committee on the Diagnosis and Classification of Diabetes Mellitus. 2002. Report of the Expert Committee on the Diagnosis and Classification of Diabetes Mellitus. Diabetes Care 25: S5-S20.

Thirone AC, Carvalheira JB, Hirata AE, Velloso LA, Saad MJ. 2004. Regulation of Cbl-associated protein/Cbl pathway in muscle and adipose tissues of two animal models of insulin resistance. Endocrinology 145: 281-293.

Thorens B. 1993. Facilitated glucose transporters in epitherial cells. Annu Rev Physiol 55:591-608.

Thornton C, Snowden MA, Carling D. 1998. Identification of a novel AMP-activated protein kinase β subunit isoform that is highly expressed in skeletal muscle. J Biol Chem 273: 12443-12450.

Torok D, Patel N, Jebailey L, Thong FS, Randhawa VK, Klip A, Rudich A. 2004. Insulin but not PDGF relies on actin remodeling and on VAMP2 for GLUT4 translocation in myoblasts. J Cell Sci 117: 5447-5455.

Tsiani E, Ramlal T, Leiter LA, Klip A, Fantus IG. 1995. Stimulation of glucose uptake and increased plasma membrane content of glucose transporters in L6 skeletal muscle cells by the sulfonylureas gliclazide and glyburide. Endocrinology 136: 2505-2512.

Ueki K, Fruman DA, Yballe CM, Fasshauer M, Klein J, Asano T, Cantley LC, Kahn CR. 2003. Positive and negative roles of p85 alpha and p85 β regulatory subunits of phosphoinositide 3-kinase in insulin signaling. J Biol Chem 278: 48453-48466.

Uldry M, Ibberson M, Horisberger JD, Chatton JY, Riederer BM, Thorens B. 2001. Identification of a mammalian H(+)-myo-inositol symporter expressed predominantly in the brain. EMBO J 20: 4467-4477.

Uldry M, Ibberson M, Hosokawa M, Thorens B. 2002. GLUT2 is a high affinity glucosamine transporter. FEBS Lett 524: 199-203.

Van Obberghen E, Baron V, Delahaye L, Emanuelli B, Filippa N, Giorgetti-Peraldi S, Lebrun P, Mothe-Satney I, Peraldi P, Rocchi S, Sawka-Verhelle D, Tartare-Deckert S, Giudicelli J. 2001. Surfing the insulin signaling web. Eur J Clin Invest 31: 966-977.

Virkamaki A, Ueki K, Kahn CR. 1999. Protein-protein interaction in insulin signaling and the molecular mechanisms of insulin resistance. J Clin Invest 103: 931-943.

Volchuk A, Mitsumoto Y, He L, Liu Z, Habermann E, Trimble W, Klip A. 1994. Expression of vesicle-associated membrane protein 2 (VAMP-2)/synaptobrevin II and cellubrevin in rat skeletal muscle and in a muscle cell line. Biochem J 304: 139-145.

Waddell ID, Zomerschoe AG, Voice MW, Burchell A.1992. Cloning and expression of a hepatic microsomal glucose transport protein. Comparison with liver plasma-membrane glucose-transport protein GLUT 2. Biochem J 286: 173-177.

Wadley GD, Bruce CR, Konstantopoulos N, Macaulay SL, Howlett KF, Hawley JA, Cameron-Smith D. 2004. The effect of insulin and exercise on c-Cbl protein abundance and phosphorylation in insulin-resistant skeletal muscle in lean and obese Zucker rats. Diabetologia 47: 412-419.

Wadzinski BE, Shanahan MF, Clark RB, Ruoho AE.1988. Identification of the glucose transporter in mammalian cell membranes with a 125I-forskolin photoaffinity label. Biochem J 255: 983-990.

Walker PS, Ramlal T, Sarabia V, Koivisto UM, Bilan PJ, Pessin JE, Klip A. 1990. Glucose transport activity in L6 muscle cells is regulated by the coordinate control of subcellular glucose transporter distribution, biosynthesis and mRNA transcription. J Biol Chem 265: 1516-1523.

Wallberg-Henriksson H. 1987. Glucose transport into skeletal muscle. Influence of contractile activity, insulin, catecholamines, and diabetes mellitus. Acta Physiol Scand 564: 1-80.

Wang Q, Somwar R, Bilan PJ, Liu Z, Jin J, Woodgett JR, Klip A. 1999. Protein kinase B/Akt participates in GLUT4 translocation by insulin in L6 myoblasts. Mol Cell Biol 19: 4008-4018.

Warden SM, Richardson C, O'Donnell JJ, Stapleton D, Kemp BE, Witters LA. 2001. Post-translational modifications of the β-1 subunit of AMP-activated protein kinase affect enzyme activity and cellular localization. Biochemical Journal 354: 275-283.

Wardzala LJ, Hirshman M, Pofcher E, Horton D, Mean PM, Cushman SW, Horton ES. 1985. Regulation of glucose utilization in adipose cells and muscle following long-term experimental hyperinsulinemia in the rat. J Clin Invest 76: 460-469.

Watford M, Goodridge AG. 2000. Regulation of fuel utilization. In Biochemical and Physiological Aspects of Human Nutrition. Edited by: Stipanuk MH. Philadelphia, PA: W.B. Saunders Company : 384-407.

Watson RT, Kanzaki M, Pessin JE. 2004. Regulated membrane trafficking of the insulin responsive glucose transporter 4 in adipocytes. Endocr Rev 25: 177-204.

Watson RT, Shigematsu S, Chiang SH, Mora S, Kanzaki M, Macara IG, Saltiel AR, Pessin JE. 2001. Lipid raft microdomain compartmentalization of TC10 is

required for insulin signaling and GLUT4 translocation. J Cell Biol 154: 829-840.

Wells RG, Pajor AM, Kanai Y, Turk E, Wright EM, Hediger MA. 1992. Cloning of a human kidney cDNA with similarity to the sodium-glucose cotransporter. American Journal of Physiology 263: F459-F465.

Weyer C, Bogardus C, Mott DM, Pratley RE. 1999. The natural history of insulin secretory dysfunction and insulin resistance in the pathogenesis of type 2 diabetes mellitus. J Clin Invest 104:787-794.

White MF. 2002. IRS proteins and the common path to diabetes. Am J Physiol Endocrinol Metab 283: E413-E422.

Winder WW and Hardie DG. 1999. AMP-activated protein kinase, a metabolic master switch: possible roles in type 2 diabetes. American Journal of Physiology 277: E1-E10.

Witters LA. 2001. The blooming of the French lilac. J Clin Invest 108: 1105-1107.

Wright EM. 1993. The intestinal Na^+/glucose cotransporter. Annu Rev Physiol 55:575-589.

Wright EM. 2001. Renal Na^+-glucose cotransporters. American Journal of Physiology 280: F10-F18.

Wu X, Freeze HH. 2002. GLUT14, a duplicon of GLUT3, is specifically expressed in testis as alternative splice forms. Genomics. 80: 553-557.

Xia Y, Makris C, Su B, Li E, Yang J, Nemerow GR, Karin M. 2000. MEK kinase 1 is critically required for c-Jun N-terminal kinase activation by proinflammatory stimuli and growth factor-induced cell migration. Proc. Natl. Acad. Sci. USA 97: 5243-5248.

Yaffe D. 1968. Retention of differentiation potentialities during prolonged cultivation of myogenic cells. Proc Natl Acad Sci USA. 61: 477-483.

Yamamoto Y, Yoshimasa Y, Koh M, Suga J, Masuzaki H, Ogawa Y, Hosoda K, Nishimura H, Watanabe Y, Inoue G, Nakao K. 2000. Constitutively active mitogen-activated protein kinase kinase increases GLUT1 expression and recruits both GLUT1 and GLUT4 at the cell surface in 3T3-L1 adipocytes. Diabetes 49: 332-339.

Yamauchi T, Tobe K, Tamemoto H, Ueki K, Kaburagi Y, Yamamoto-Honda R, Takahashi Y, Yoshizawa F, Aizawa S, Akanuma Y, Sonenberg N, Yazaki Y,

Kadowaki T. 1996. Insulin signalling and insulin actions in the muscles and livers of insulin-resistant, insulin receptor substrate 1-deficient mice. Mol Cell Biol 16: 3074-3084.

Yang J, Clarke JF, Ester CJ, Young PW, Kasuga M, Holman GD. 1996. Phosphatidylinositol 3-kinase acts at an intracellular membrane site to enhance GLUT4 exocytosis in 3T3-L1 cells. Biochem J 313: 125-132.

Yin J, Hu R, Chen M, Tang J, Li F, Yang Y, Chen J. 2002. Effects of berberine on glucose metabolism in vitro. Metabolism 51: 1439-1443.

Yonemitsu S, Nishimura H, Shintani M, Inoue R, Yamamoto Y, Masuzaki H, Ogawa Y, Hosoda K, Inoue G, Hayashi T, Nakao K. 2001. Troglitazone induces GLUT4 translocation in L6 myotubes. Diabetes 50: 1093-1101.

Yu B, Poirier LA, Nagy LE. 1999. Mobilization of GLUT-4 from intracellular vesicles by insulin and K(+) depolarization in cultured H9c2 myotubes. Am J Physiol 277: E259-E267.

Yujiri T, Sather S, Fanger GR, Johnson GL. 1998. Role of MEKK1 in cell survival and activation of JNK and ERK pathways defined by targeted gene disruption. Science 282: 1911-1914.

Zakaria ZA, Mat Jais AM, Henie EFP, Zaiton H, Somchit MN, Sulaiman MR, Faizal FO. 2006. The in vitro Antibacterial Activity of *Tinospora crispa* Extracts. J Bio Sci 6: 398-401.

Zeigerer A, Lampson MA, Karylowski O, Sabatini DD, Adesnik M, Ren M, McGraw TE. 2002. GLUT4 retention in adipocytes requires two intracellular insulin-regulated transport steps. Mol Biol Cell 13: 2421-2435.

Zheng D, MacLean PS, Pohnert SC, Knight JB, Olson AL, Winder WW, Dohm GL. 2001. Regulation of muscle GLUT-4 transcription by AMP-activated protein kinase. J Appl Physiol 91: 1073-1083.

Zisman A, Peroni OD, Abel ED, Michael MD, Mauvais-Jarvis F, Lowell BB, Wojtaszewski JF, Hirshman MF, Virkamaki A, Goodyear LJ, Kahn CR, Kahn BB. 2000. Targeted disruption of the glucose transporter 4 selectively in muscle causes insulin resistance and glucose intolerance. Nat Med 6: 924-928.

Zottola RJ, Cloherty EK, Coderre PE, Hansen A, Hebert DN, Carruthers A. 1995. Glucose transporter function is controlled by transporter oligomeric structure. A

single, intramolecular disulfide promotes GLUT1 tetramerization. Biochemistry 34: 9734-9747.

APPENDIX

1. Analysis of glucose uptake in L6 myotube

1.1 L6 myoblast culture medium

α-Minimum Essential medium (α-MEM) with 5.5 mM glucose and, L-glutamine containing 10% Fetal bovine serum (FBS), 2.2 g sodium bicarbonate, 100 U penicillin and streptomycin 100 μg/ml. Sterile filter through a 0.45-μm filter.

1.2 Differentiation medium

α-Minimum Essential medium (α-MEM) with 5.5 mM glucose and, L-glutamine containing 2% Horse serum (HS), 2.2 g sodium bicarbonate, 100 U penicillin and streptomycin 100 μg/ml. Sterile filter through a 0.45-μm filter.

1.3 1X PBS

Dissolved 8 g NaCl, 0.2 g KCl, 1.15 g Na_2HPO_4 and 0.2 g KH_2PO_4 in 800 ml distilled water. Adjust the pH to 7.4 with HCl. Add water to 1 L.

1.4 0.05% trypsin, 0.02% EDTA

0.05 g trypsin, 0.02 g EDTA (disodium salt), dissolved in 1 L sterile distilled water. Sterile filtered, 50 ml aliquots. Store at -20°C.

1.5 HEPES-Buffer Saline (HBS)

Dissolved 8.0 g NaCl (140 mM), 0.15 g $CaCl_2.2H_2O$ (1 mM), 0.38 g KCl (5 mM), 0.62 g MgSO4.7H2O (2.5 mM) and 4.75 g HEPES-Na (20 mM) in 800 ml. Adjust pH to 7.4 with HCl. Add water to 1 L.

1.6 2-Deoxy-D-[2,6-^{3}H]glucose (Amersham Biosciences) stock

1.0 mCi/ml store at -20°C.

1.7 Metformin (Sigma) stock

100 mM (0.0165 g in 1 ml sterile distilled water). This stock can be store for 6 month at 2-8 °C.

1.8 Cytochalasin B (Sigma) stock

10 mM (1 mg in 208.5 μl DMSO). Store at -20°C

1.9 Cycloheximide (Sigma) stock

10 mM (2.8 mg in 1 ml DMSO). This solution can be stored up to month at -20°C.

1.10 Wortmannin (Sigma) stock

5 mM (1 mg in 466.8 µl DMSO). This solution can be stored up to month at -20°C.

1.11 SB203580 (Sigma) stock

20 mM This solution can be stored up to month at -20°C.

1.12 PD98059 (Cell Signaling Technology) stock

50 mM, reconstitute 1.5 mg in 112 µl in DMSO. Store in aliquots at -20°C.

1.13 HBS-[^{3}H]2-deoxyglucose label

1 µCi/ml final concentration. Prepare prior to use.

1.14 0.05N NaOH solubilization buffer

Make 2.0 g NaOH up to 1 liter with distilled water; prepare fresh.

1.15 0.05 N HCl solution

Concentrated HCl has a molarity of approximately 11.6. HCl is a gas, which is soluble in water and which comes in the form of concentrated reagent grade HCl. This solution is approximately 36-38% (w/v) HCl. To make a 1 N solution, add 86 ml of concentrated HCl to 800 ml of water and dilute to a final volume of 1 L. For 0.05 N, dilute the 1 N by a factor of 20.

2. Cytotoxicity test by sulforhodamine B Assay

2.1 10% (w/v) TCA (Trichloroacetic acid)

Dissolved 10 g TCA in 100 ml distilled water. Store at 4°C.

2.2 0.4%(w/v) Sulforhodamine B solution

Dissolved 0.4 g in 100 ml of 1% acetic acid. Store at 4°C.

2.3 1% acetic acid

Add 1 ml glacial acetic acid in 99 ml distilled water.

2.4 10 mM Tris-base solution

Dissolved 1.2 g in 1 L distilled water.

3. Preparation of RIPA cell lysates

RIPA lysis buffer: 10 mM Tris-HCl [pH 8.0], 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 0.004% sodium azide, 1 mM phenylmethylsulfonyl fluoride, and 0.1 mM Na_3VO_4, Proteinase inhibitor cocktail

4. Separation of plasma and intracellular membranes

4.1 Homogenizing buffer

Dissolved 8.765 g NaCl (150 mM), 0.38 g EGTA (1 mM), 0.0203 g $MgCl_2$ (0.1 mM), 2.383 g HEPES (10 mM) in 800 ml water. Adjust pH to 7.4 with HCl. Add water to 1 L.

4.2 1:1,500 dilution of Proteases inhibitor cocktail (P 8340 Sigma, USA)

5. Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) and Electroblotting (electrotransfer to nitrocellulose membrane)

5.1 Stock acrylamide solution

30% acrylamide, 0.8%bis-acrylamide (Bio-Rad, USA) or make 1 L by make 300 g acrylamide (toxic!), 8 g N′N′-bis-methylene-acrylamide in H_2O, store in brown bottle at 4°C.

5.2 Buffer for acrylamide gel preparation

- 1.5 M Tris-HCl, pH 8.8: 90.83 g Tris base (MW=121.1), 400 ml H_20, adjust to pH 8.8 with conc. HCl, add H_2O to give 500 ml, store at 4°C.
- 0.5 M Tris-HCl, pH 6.8 : 37.82 g Tris base (MW=121.1), 200 ml H_2O, adjust pH with conc. HCl, add water to give 250 ml, store at 4°C.

5.3 10% Ammonium persulfate (APS).

Dissolved ammonium persulfate (sigma) 1.0 g in 10 ml of sterile distilled water. Make fresh.

5.4 10% SDS (Sodium Dodecyl Sulfate)

Dissolved 100.0 g SDS (Electrophoresis-grade) in 800 ml of water and stir, then fill up to 1 L. Do not autoclave. Wear masks and gloves when weighing-Wipe down area when done-SDS disperses easily.

5.5 Electrophoresis buffer

6 g Tris (MW=121.1, 50 mM), 28.8 g glycine (MW=70.05, 380 mM), and 1 g SDS. Make up to 1 L. with water. No pH adjustment is necessary.

5.6 4x Laemmli buffer

8 ml of 5x sample buffer (31.25 ml 2 M Tris-HCl, pH 6.8, 25.0 ml glycerol, 25.0 ml β-mercaptoethanol 10 g SDS, 50mg bromophenol blue, add water to 100 ml) , 0.1234 g DTT. Make up to 10 ml.

5.7 Protein stain

0.1% Coomassie brilliant blue R250 in 50% methanol, 10% glacial acetic acid. Dissolve the dye in the methanol and water component first, and then add the acetic acid. Filter the final solution through Whatman No. 1 filter paper if necessary.

5.8 Destain (10% methanol and 7% acetic acid)

Add 10 ml of methanol and 7 ml of acetic acid in 83 ml water.

5.9 Transfer buffer

Dissolved 3.03 g Tris (MW=121.1, 25 mM), 14.4 g Glycine (MW=70.05, 190 mM), 200 ml methanol (20%) in 800 ml water and made up to 1 L. No pH adjustment is necessary.

6. Immunodetection with antibodies to GLUT1, GLUT4 proteins and phosphorylation of ERK1/2

6.1 Tris-buffered saline (TBS)

Dissolved 8 g NaCl (137 mM), 20 ml of 1 M Tris-HCl pH 7.6 (20 mM) in 800 ml water and made up to 1 L.

6.2 5% non-fat milk

5 g non-fat dry milk in 95 ml TBS

6.3 TBS-tween (TBST)

Mix 1 ml Tween 20 in 1 L of TBS.

7. Quantification of GLUT1 and GLUT4 gene expression by mRNA

7.1 10X TBE electrophoresis buffer

Dissolved 108 g Tris Base (890 mM), 55 g Boric Acid (890mM), 20 mL 0.5 M EDTA in 1.0 L Water.

7.2 Ethidium bromide (0.5 μg/ml)

8. TLC staining reagent

8.1 p-Anisaldehyde reagent

Mix 0.5 ml p-anisaldehyde, 85 ml methanol, 10 ml acetic acid and 5 ml sulfuric acid and kept at 4°C for one week.

8.2 Dragendorff's reagent

Mix 5 ml solution A, 5 ml solution B, 20 ml acetic acid and 70 ml water (sol. A: 1.7 g basic bismuth nitrate in 100 ml of water/acetic acid 4:1, Sol. B: 40 g potassium iodine in 100 ml water) and kept at 4°C for one week.

VITAE

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List of Publication and Proceeding

Assanangkornchai S, Noi-pha K, Saunders JB, Ratanachaiyavong S. 2003. Aldehyde dehydrogenase 2 genotypes, alcohol flushing symptoms and drinking patterns in Thai men. *Psychiatry Res*. 118: 9-17.

Abstract

Suvina Ratanachaiyavong and Kusumarn Noipha. 2007. Semi-purified fraction from aqueous extract of *Tinospora crispa* (TCf) enhanced muscle glucose uptake, up-regulated AMPK and GLUT1 mRNA expression. Online proceeding of The 33rd Congress on Science and Technology of Thailand. Available from: http://www.scisoc.or.th/stt/33/sec_h/paper/stt33_H_H0015.pdf Accessed January, 2008.

กุสุมาลย์ น้อยผา, สุวิณา รัตนชัยวงศ์. 2550. การออกฤทธิ์การพาน้ำตาลกลูโคสเข้าสู่เซลล์กล้ามเนื้อของพืชผัก 12 ชนิด โดยเปรียบเทียบกับยาเมทฟอร์มิน. Journal of Thai Traditional & Alternative Medicine. Vol. 5 Suppl. (2) May-August: 67.

Kusumarn Noipha, Juntipa Purintrapiban, Suvina Ratanachaiyavong. 2005. The enhancement of active glucose transport by *Tinospora crispa* in L6 myotubes. In The 1st International Conference on Natural Products for Health Health and Beauty from local wisdom to global marketplace,17th -21st October 2005, Maha Sarakham, Thailand : program & abstracts / organized by Mahasarakham University (Reference Book, ISBN 9479655931), page 153.

Kusumarn Noipha, Juntipa Purintrapiban, Suvina Ratanachaiyavong. 2004. The *Tinospora crispa* enhances active glucose transport in L6 myotubes. Songklanagarind Medical Journal. Vol. 22 Suppl. (1) August: 53.

List of Presentation

Suvina Ratanachaiyavong and Kusumarn Noipha. 2007. *Tinospora crispa* (BO-RA-PED) enhanced muscle glucose uptake by up-regulation of glucose transporter proteins and pathways. Oral presentation at The Annual Meeting Endocrine Society of Thailand 2007. November 1-2, 2007. Bangkok, Thailand.

Kusumarn Noipha, Suvina Ratanachaiyavong. 2007. *T. crispa* activated Extracellular signal-Regulated Kinase 1/2 (ERK1/2) in CHO-IR cells. Oral presentation at The 23rd Annual Academic Meeting, Faculty of Medicine, Prince of Songkla University. August 15-17, 2007. Songkhla, Thailand.

Kusumarn Noipha, Juntipa Purintrapiban, Suvina Ratanachaiyavong. 2007. The Antiglycemic Effect of *Tinospora crispa* (Miers ex Hook. f. & Thom): A bioactivity study at both cellular and molecular levels. Oral presentation at The 7th National Grad Research Conference. Prince of Songkla University. April 4-5, 2007. Suratthani, Thailand.

Kusumarn Noipha. 2006. Glucose transporter (GLUT) proteins; 1. Classification, function and tissue distribution, 2. Activation and expression. Mini-talk on The 1st BMS's Teaching Platform "Diabetes and Antiglycemic Activity of Medicinal Plants" Faculty of Medicine, Prince of Songkla University. May 22-26, 2006. Songkhla, Thailand.

Kusumarn Noipha, Juntipa Purintrapiban, Suvina Ratanachaiyavong. 2004. *Tinospora crispa* enhances active glucose transport in L6 myotubes. Oral presentation at The Annual Meeting Endocrine Society of Thailand 2004. November 4-5, 2004. Bangkok, Thailand.

List of Award

Servier Award 2007 for Distinguished Clinical Research in Diabetology from The Endocrine Society of Thailand Servier (Thailand) Ltd. On "Suvina Ratanachaiyavong and Kusumarn Noipha. 2007. *Tinospora crispa* (BO-RA-PED) enhanced muscle glucose uptake by up-regulation of glucose transporter proteins and pathways" Oral

presentation at The Annual Meeting Endocrine Society of Thailand 2007. November 1-2, 2007. Bangkok, Thailand.

รางวัลการนำเสนอผลงานด้วยวาจาประเภทดีเด่น กลุ่มวิชาวิทยาศาสตร์สุขภาพ จากการประชุมเสนอผลงานวิจัยระดับบัณฑิตศึกษาแห่งชาติ (grad-research) ครั้งที่ 7 ประจำปี 2550 จัดโดยบัณฑิตวิทยาลัย มหาวิทยาลัยสงขลานครินทร์ ดังรายละเอียด "Kusumarn Noipha, Juntipa Purintrapiban, Suvina Ratanachaiyavong. 2007. The Antiglycemic Effect of *Tinospora crispa* (Miers ex Hook. f. & Thom): A bioactivity study at both cellular and molecular levels. Oral presentation at The 7th National Grad Research Conference. Prince of Songkla University. April 4-5, 2007. Suratthani, Thailand."

รางวัลการนำเสนอผลงานโปสเตอร์ประเภทดีเด่น จากการประชุมวิชาการนานาชาติ ครั้งที่ 1 เรื่อง ผลิตภัณฑ์ธรรมชาติเพื่อสุขภาพและความงาม จัดโดย มหาวิทยาลัยมหาสารคาม ในปี 2548 ดังรายละเอียด "Kusumarn Noipha, Juntipa Purintrapiban, Suvina Ratanachaiyavong. 2005. The enhancement of active glucose transport by *Tinospora crispa* in L6 myotubes. Poster presentation at The 1st International Conference on Natural Products for Health and Beauty. Takasila Hotel. October 17-21, 2005. Maha Sarakham, Thailand."

รางวัลรองชนะเลิศอันดับหนึ่ง ผลงานประเภท Oral presentation จากการประชุมวิชาการประจำปี คณะแพทยศาสตร์ ครั้งที่ 20 ให้โดยคณะแพทยศาสตร์ มหาวิทยาลัยสงขลานครินทร์ ดังรายละเอียด "Kusumarn Noipha, Juntipa Purintrapiban, Suvina Ratanachaiyavong. 2004. The *Tinospora crispa* enhances active glucose transport in L6 myotubes. Oral presentation at The 20th Annual Academic Meeting, Faculty of Medicine, Prince of Songkla University. August 18-20, 2004. Songkhla, Thailand."