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LAPORAN AKHIR PENYELIDIKAN

TAJUK PROJEK: **STUDY OF THE OF ANTIDIABETIC ACTION OF CINNAMON EXTRACT ON CELL CULTURE OF 3T3-L1 ADIPOCYTES**

Saya **MOHAMAD ROJI BIN SARMIDI**

(HURUF BESAR)

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STUDY OF THE OF ANTIDIABETIC ACTION OF CINNAMON EXTRACT ON CELL CULTURE OF 3T3-L1 ADIPOCYTES

(KAJIAN TINDAKAN ANTIDIABETES DARIPADA EKSTRAK CINNAMON KEPADA KULTUR SEL 3T3-L1 ADIPOS)

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2005

ACKNOWLEDGEMENTS

Authors would like to thank Bioprocess Department technicians, En. Yaakop Sabudin, Puan Siti Zalita, En. Nur Muhammad and En. Abdul Malek. Thanks are also due to Sultanah Zanariah Library for enabling us access the literature and facilitating interlibrary loan.

ABSTRACT

Insulin binds to the extracellular α–subunit of the insulin receptor, and induces a conformational change in the kinase domain of the trans membrane β-subunit resulting in activation of insulin receptor tyrosine kinase, an essential step for the downstream insulin signalling events. In type 2 diabetes, part of the insulin resistance is due to inability of insulin to activate the receptor kinase activity. The activity of cinnamtannin B1 on phosphorylation of insulin receptor was analyzed using western blot technique. Cinnamtannin B1 stimulated phosphorylation of insulin receptor β-subunit. There was no phosphorylation of insulin receptor observed in 3T3-L1 preadipocytes. The activity of cinnamtannin B1 in stimulating phosphorylation was inhibited by wortmannin and cytochalasin B. In contrast, sodium orthovanadate stimulated phosphorylation of insulin receptor.

ABSTRAK

Insulin mengikat pada ekstraselular reseptor insulin α-subunit, dan merangsangkan suatu perubahan konformasi dalam domain kinase trans membran β-subunit mengaktifkan reseptor insulin tyrosine kinase, yang merupakan satu langkah penting untuk bahagian hiliran pengisyaratan insulin. Sebagian daripada rintangan insulin dalam diabetes jenis 2 adalah disebabkan oleh ketidakmampuan insulin untuk mengaktifkan aktiviti reseptor kinase. Aktiviti cinnamtannin B1 atas fosforilasi reseptor insulin dianalisis menggunakan teknik western blot. Didapati cinnamtannin B1 merangsangkan reseptor insulin β-subunit. Walaubagaimanapun tiada sebarang fosforilasi reseptor insulin berlaku dalam 3T3-L1 preadipos. Aktiviti perangsangan fosforilasi oleh cinnamtannin B1 adalah disekat oleh wortmannin dan cytochalasin B. Sebailknya, sodium orthovadate merangsangkan fosforilasi reseptor insulin.

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LIST OF SYMBOLS

Mr	-	Molecular weight

LIST OF ABBREVIATIONS

$CaCl_2$	-	calcium chloride
C/EBP	-	C/AAT Enhancer Binding Protein
COSY	-	Correlated Spectroscopy
DMEM	-	Dulbecco's Modified Eagle Medium
ELISA	-	Enzyme-linked Immunosorbent Assay
FBS	-	foetal bovine serum
GLUT-4	-	glucose transporter-4
HEPES	-	(N-[2-Hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid])
IDDM	-	Insulin-Dependent Diabetes Mellitus
IGF-I	-	Insulin-Like Growth Factor I
IgG	-	immunoglobulin G
IR	-	insulin receptor
IRS	-	insulin receptor substrate
kDa	-	kiloDalton
KRPH	-	Krebs Ringer Phosphate Hepes
L	-	Liter
lit	-	literature
M	-	molar
MAPK	-	mitogen-activated protein kinase
mL	-	milliliter
µg	-	microgram
mM	-	millimolar
Na_2HPO_4	-	disodium hydrogen phosphate
Na_3VO_4	-	sodium orthovanadate
NIDDM	-	Non-Insulin-Dependent Diabetes Mellitus
nM	-	nanomolar

PBS	-	phosphate buffered saline
PI3-K	-	phosphatidylinositol 3-kinase
PPARγ	-	peroxisome proliferator-activated receptor gamma
rel. int.	-	relative intensity
SDS-PAGE	-	sodium dodecylsulfate-polyacrylamide gel

CHAPTER 1

INTRODUCTION

1.1 General

Type 2 diabetes (non-insulin-dependent diabetes mellitus) is a chronic metabolic disease that results from defects in insulin secretion and insulin receptor kinase. Investigation of novel small active molecule that can potentiate insulin action or having a similar action as insulin is important in the treatment of diabetes. World ethnobotanical information on medicinal plants reports almost 800 plants used in the treatment of diabetes mellitus. However, only a small number of them have been studied thoroughly (Alarcon-Aguilar *et al.*, 1998).

Cell line provides a continuous source of large numbers of cells necessary for study of proliferation and differentiation. The 3T3-L1 cell line is selected for this study because it plays an important role in lipid storage and glucose homeostasis. 3T3-L1 adipocytes have been used extensively to study the regulation of such as glucose transporters, cell proliferation and insulin signaling. During differentiation, 3T3-L1 cells experience a 20-fold increase in the number of insulin receptors and acquire the ability to utilize glucose in response to insulin (Frost and Lane, 1985). The most frequently employed adipocytes cell lines are 3T3-F442A and 3T3-L1. They were clonally isolated from Swiss 3T3 cells derived from disaggregated 17-to19-day mouse embryos (Green and Kehinde, 1975 and 1976).

1.2 Plants in Type 2 Diabetes Treatment

The plant extracts and its product play an important role in treating many symptoms. Pioneering studies on the active constituents of *Podophyllum peltatum* followed by the discovery and development of the antileukemic agents, vinblastine and vincristine from *Catharantus roseus* provided convincing evidence that plants could be sources of novel and potential chemotherapeutic agents (Baker *et al.*, 1995).

Imparl-Radosevich *et al.* (1998), Jarvill-Taylor *et al.* (2001), Anderson *et al.* (2004) and Pszczola (2001) have introduced method to evaluate plants compound for antihyperglycemia activity. The plant used is cinnamon and suggested to contain a novel phenolic polymer. The compound stimulated phosphorylation insulin receptor and enhance glucose uptake in 3T3-L1 adipocytes. Khan *et al.* (2003) reported the effect of *Cinnamomum cassia* on the diabetes patients. The results of their study demonstrate that intake of 1, 3, or 6 g of cinnamon per-day reduces serum glucose, triglyceride, LDL cholesterol, and total cholesterol in people with type 2 diabetes. They suggested that the inclusion of cinnamon in the diet of people with type 2 diabetes reduces the risk factors associated with diabetes and cardiovascular diseases.

There are several bioactive plant extracts that have been studied for antidiabetic agent. A bioactive compound from Chinese plant *Lithospermum erythrorhizon* stimulates glucose uptake in 3T3-L1 adipocytes (Kamei *et al.*, 2002). An extract from *Lagerstroemia speciosa* has insulin like glucose uptake stimulatory effect (Liu *et al.*, 2001). In addition, an antidiabetic fungal metabolite from culture broth of *Pseudomassaria* sp. was discovered as an insulin agonist and showed to be highly effective in animal models diabetes (Qureshi *et al.*, 2000).

1.3 Diabetes Mellitus

According to International Diabetes Federation, currently more than 194 million people with diabetes worldwide and the epidemiological estimates that by 2025 there will be 333 million diabetes sufferers. It will be almost twice as many sufferers as today, and has become a serious public health problem, particularly in developed countries. This will be predominantly individuals with type 2 diabetes (Vessby, 2000; Seidell, 2000; Kim *et al.*, 2001; Barrett, 2004).

Type 2 diabetes mellitus is an increasingly common disorder of carbohydrate and lipid metabolism (Nadler and Attie, 2001). Two important characteristics of this disease are insulin resistance, the failure of peripheral tissues; including liver, muscle, and adipose tissue, to respond to physiologic doses of insulin, and failure of pancreatic β-cells to properly secrete insulin in response to elevated blood glucose levels. Obesity is a significant risk factor for the development of type 2 diabetes mellitus. An extremely lean and lipoatrophic models have revealed a similar predisposition to developing diabetes. Although it may seem paradoxical that both increased adiposity and severely reduced fat mass cause diabetes, a common pathophysiologic process in fat may be responsible for the predisposition to develop hyperglycemia in both conditions (Kim *et al.*, 2001; Nadler and Attie, 2001).

Broadhurst (1997) proposed the major causative factors for non-insulin dependent-diabetes mellitus (NIDDM) involving obesity and overfatness; carbohydrate and fat over nutrition; lack of polyunsaturated fatty acids (PUFA) in plasma membranes and unbalanced triglyceride intake; chromium deficiency; and lack of soluble fiber and relevant beneficial phytochemicals.

NIDDM is a complex disease that is currently thought to be influenced by more than a single gene or environmental factor. Although the relative contribution of genetic and environmental factors to the development of NIDDM differs among individuals, patients generally have two common metabolic abnormalities: insulin resistance and defects in glucose-stimulated insulin secretion, which lead to disease state (Fig.1.1).

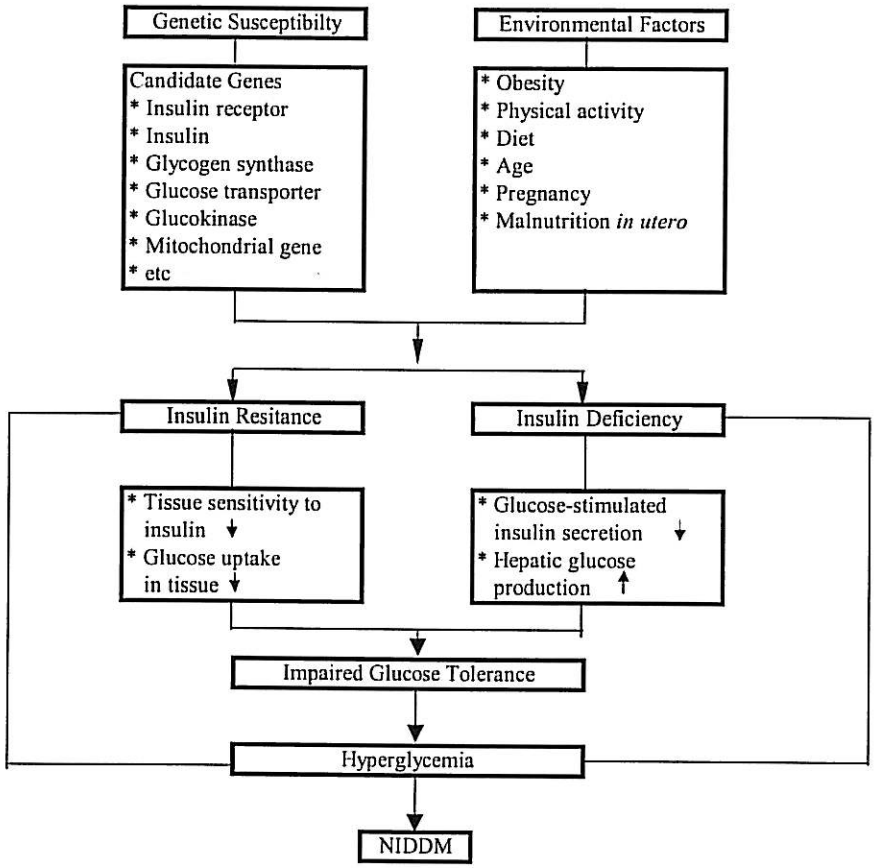

Figure 1.1 Schematic diagram of progressive pathogenesis of NIDDM (Jun *et al.*, 1999).

Figure 1.2 shows that glucose affects insulin release by acting in multiple ionic and metabolic mechanisms. β cells are sensitive to the concentration of glucose. If β cells are exposed to elevated glucose levels for more than 15 min, they become primed so that their response to glucose becomes greater than their initial response. Glucose inhibits ATP-sensitive K^+ channels (possibly by its stimulation of ATP production), which causes membrane depolarization. Depolarization activates Ca^{2+} channels resulting in Ca^{2+} entry and an increase in cytosolic Ca^{2+}. Extracellular Ca^{2+} production and energy production are required for stimulation of insulin secretion. During glucose stimulation of healthy β cells, normal insulin secretion takes place. However, in defective β cells, impaired insulin secretion may cause delayed insulin secretion. The insulin receptor substrate-1 (IRS-1) molecule is

thought to transmit the intracellular signal from the insulin receptor. The binding of insulin to the insulin receptor leads to activation of insulin receptor kinase through autophosphorylation of the insulin receptor (β-subunit). Insulin receptor kinase is essential for insulin action. In type II diabetes, the insulin receptor kinase activity appears to be lower in target tissue due to a decreased number of insulin receptors and a reduction in intrinsic insulin receptor kinase activity per-receptor.

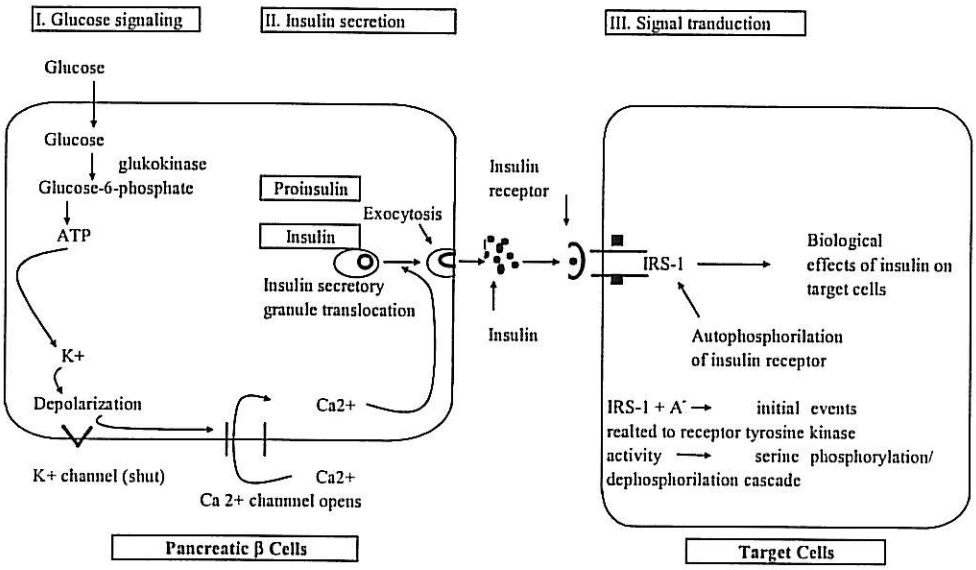

Figure 1.2 Schematic diagram of metabolic functions in β cells, insulin secretion in β cells, and insulin action in target tissues (Jun *et al.*, 1999).

CHAPTER 2

LITERATURE REVIEW

2.1 Insulin Receptor

The insulin receptor is present in virtually all vertebrate tissues although the concentration varies from as few as 40 receptors on circulating erythrocytes to more than 200,000 receptors on adipocytes and hepatocytes. The receptor gene is located on the short arm of human chromosome 19 is more than 150 kilobases in length and contains 22 exons which encode a 4.2 kb cDNA (Seino *et al.*, 1990).

The insulin receptor structurally is a unique member of the ligand-activated receptor tyrosine kinase family in that it is covalent heterotetramer, with its two α-subunits and two β-subunits linked by disulfide bonds (Fig. 2.1). The α–subunits mediate the binding of insulin to the receptor, while the β–subunits are responsible for communicating the ligand binding event to the signaling machinery via their intrinsic tyrosine kinase activity (White and Kahn, 1994). Both subunits are derived from a single proreceptor by proteolytic processing at a cleavage site consisting of 4 basic amino acids. There is one site of alternative splicing surrounding exon 11 which results in two receptor isoforms differing by 12 amino acids near the COOH-terminus of α-subunit. The mature heterotetramer ($\alpha_2\beta_2$) contains complex N-linked carbohydrate side chains capped by terminal sialic acids residues and migrates with a molecular mass of 300-400-kDa by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). The α-subunits are located entirely outside the cell and contain the insuling binding site(s) whereas the intracellular portion of β-

subunits contains the insulin-regulated tyrosine protein kinase (White, 1997; White and Kahn, 1994).

The insulin receptor is a tyrosine-specific protein kinase, and one of the earliest detectable responses to insulin binding is activation of this kinase and autophosphorylation of its β-subunit. Tyrosine autophosphorylation activates the phosphotransferase in the β-subunit and increases its reactivity toward tyrosine phosphorylation of other substrates (White *et al.*, 1985). Insulin stimulated phosphorylation of its receptor at 95-kDa β-subunit. Phosphorylation was specifically stimulated by insulin in a dose-dependent fashion after 1 and 15 minutes of hormone treatment (Kasuga *et al.*, 1982).

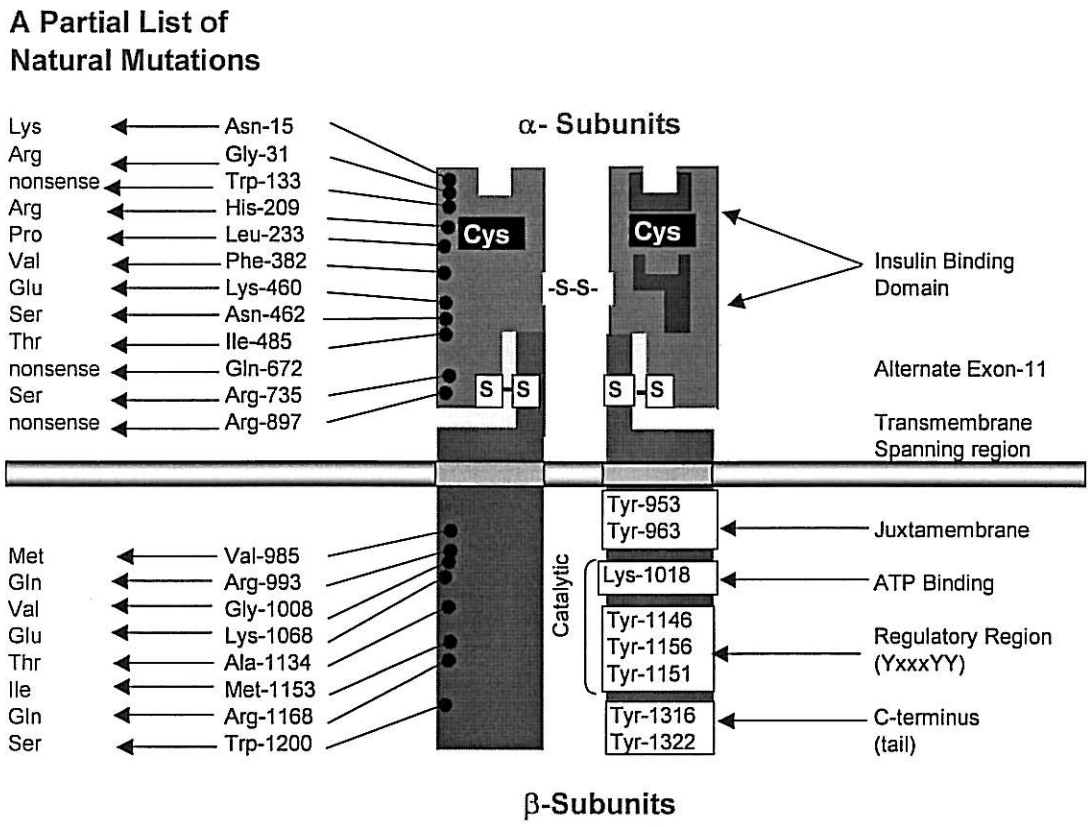

Figure 2.1 The model of insulin receptor (White, 1997; White and Kahn, 1994).

Insulin binds to the extracellular α–subunit of the IR, and induces a conformational change in the kinase domain of the trans membrane β-subunit resulting in activation of IR tyrosine kinase, an essential step for the downstream insulin signalling events. In type 2 diabetes, part of the insulin resistance is due to inability of insulin to activate the receptor kinase activity (see box). The Merck compound L783,281 is claimed to bypass this defect by specifically activating IR tyrosine kinase. Also depicted in the figure are other cellular sites wherein vanadium compounds and synthetic phosphoinositol glycans act (Fig. 2.2). This figure was taken from Balasubramanyam (2001).

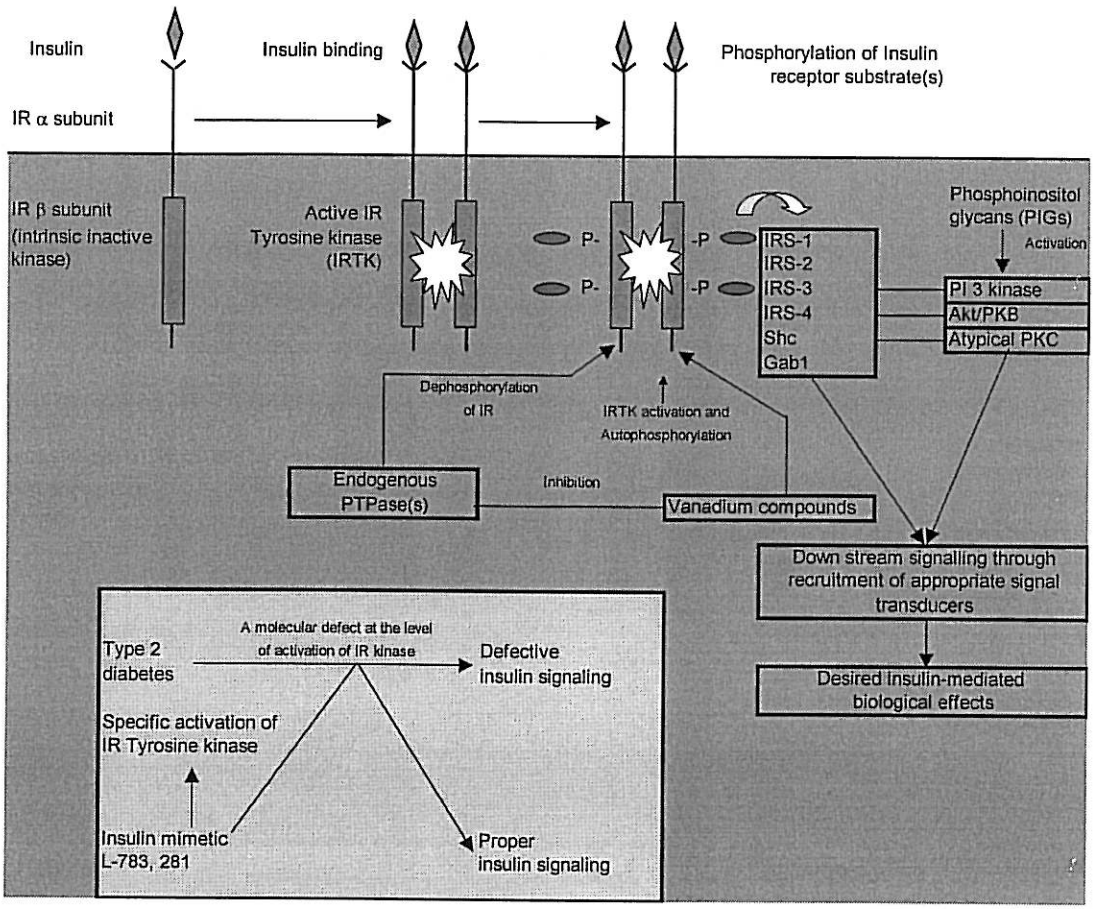

Figure 2.2 Type of insulin binding (Balasubramanyam, 2001).

2.2 Insulin Signaling

In mammals, insulin is the principal hormone controlling blood glucose and acts by stimulating glucose influx and metabolism in muscle and adipocytes and inhibiting gluconeogenesis by the liver. In addition, insulin modifies the expression or activity of a variety of enzymes and transport systems in nearly all cells. Insulin action is mediated through the insulin receptor, a transmembrane glycoprotein with intrinsic protein tyrosine kinase activity. The level of tyrosine kinase activity reflects the serum concentration of insulin and appears to mediate the insulin response through tyrosine phosphorylation of the receptor itself and substrates like insulin receptor substrate-1 (IRS-l). Non-insulin-dependent diabetes mellitus is due in large part to insulin resistance, a state where the target cells no longer respond to ordinary levels of circulating insulin. To understand the mechanisms of control of normal metabolism, as well as the pathogenesis of non-insulin-dependent diabetes mellitus, it is critical to understand the signaling pathways used by the insulin receptor (White, 1994).

Insulin mediates a wide spectrum of biological responses including stimulation of glucose uptake, glycogen, lipid and protein synthesis, antilipolysis, activation of transcription of specific genes, and modulation of cellular growth and differentiation. Since the discovery of insulin 77 years ago, great progress has been made in understanding the diverse signaling pathways that emanate from the insulin receptor itself to generate the pleiotropic actions of the hormone. Many of the molecular actions of insulin involve protein phosphorylation and dephosphorylation. Although the insulin receptor is a tyrosine kinase that undergoes ligand-dependent activation, many of the resulting downstream changes in phosphorylation/ dephosphorylation occur on serine and threonine as shown in Figure 2.3 (Taha and Klip, 1999).

2.3 Phosphorylation of Insulin Receptor

The pleiotropic actions of insulin are initiated by the binding of the hormone to its specific cell-surface receptors on target cells. The insulin-receptor interaction stimulates autophosphorylation of the receptor by its intrinsic tyrosine kinase, thereby activating phosphorylation of protein substrates (Hresko *et al.*, 1988). Phosphorylation of proteins has been postulated to be an important regulatory mechanism in the action of insulin and other hormones. Immunoprecipitation of the labeled receptor demonstrated that there are two major subunits of insulin receptor phosphorylatd with molecular weight of 135- and 95-kDa that corresponding to the receptor of alpha and beta subunit of the insulin receptor (Kasuga *et al.*, 1982 and Zick *et al.*, 1982). After autophosphorylation, the activated receptor phosphorylates endogenous substrates, such as insulin receptor substrate (IRS)-1 and -2. Phosphorylated tyrosine residues on these substrates then bind to a variety of other substrates and insulin action ensues (Virkamaki *et al.*, 1999). It is well established that insulin signaling, including activation of IR tyrosine kinase activity, is impaired in most patients with type 2 diabetes (Goldfine, 1999). This resistance to insulin then leads to hyperglycemia and other metabolic abnormalities of the disease (Taylor, 1999).

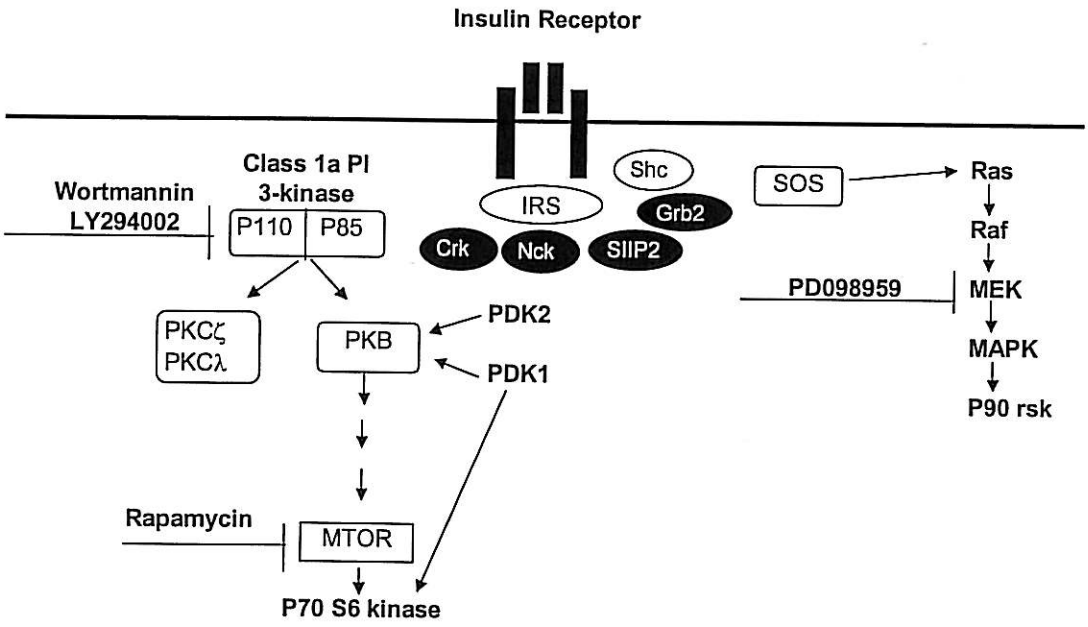

Figure 2.3 The insulin signaling pathway (Taha and Klip, 1999). The insulin receptor is a tyrosine kinase that undergoes autophosphorylation which increases the kinase activity of the receptor. The activated receptor phosphorylates IRS-proteins and Shc resulting in the activation of the Grb2/Sos and Ras/Raf/MEK/MAPK pathway. Phosphorylated IRS-proteins activate PI 3-kinase and its downstream targets, namely, PKB, mTOR, p70 S6 kinase and atypical PKCs (PKCζ and PKCλ). The sites inhibited by wortmannin, LY29,4002, rapamycin, and PD098059 are indicated.

CHAPTER 3

MATERIALS AND METHODS

3.1 Materials

3.1.1 Chemicals

All cell culture grade chemicals were purchased from Sigma-Aldrich and Gibco BRL and analytical grade chemical were purchased from Merck-Schuchardt (Mallinckrodt). Molecular weight markers for proteins (broad range) were purchased from Promega (Madison, USA). Anti phosphotyrosine (4G10) (HRP conjugate), anti-insulin receptor β-subunit, phosphotyrosine control (EGF-stimulated A431 cell lysate), secondary antibody (goat anti-rabbit IgG, HRP conjugate) were purchased from Upstate Biotechnology (Lake Placid, USA).

3.1.2 Cell Culture

3T3-L1 was purchased from European Collection of Cell Cultures (ECACC, UK).

3.2 Experimental Procedures

3.2.1 Cells Maintenance

TB/C3, CHO-K1 and 3T3-L1 preadipocytes were grown in RPMI , Ham F-12 and DMEM medium, respectively supplemented with 10% foetal bovine serum and 1% penicillin (10.000 U/mL) and 1% streptomycin (10.000 μg/mL). The cells were subcultured every 2 days after reaching confluent.

3.2.2 Adipocytes Differentiation (Adipogenesis)

The 3T3-L1 cells were cultured and maintained as previously described by Student *et al.* (1980) and Cheng (2001). Cell culture were propagated at 37°C in a humidified atmosphere of 5% CO_2 in DMEM containing 10% foetal bovine serum, 1 % penicillin (10.000 U/mL) and 1 % streptomycin (10.000 μg/mL). Cells were seeded and differentiated at 2-8 x 10^4 in 24-well and 96-well plates containing 1 and 0.1 mL medium, respectively.

Cells were grown until reaching confluent and subcultured every two days by trypsinization. Cell growth and viability were monitored by counting using tryphan blue exclusion method. The maximum growth was occurred at day-four of subculture.

Induction of differentiation was done one to two days post confluence. 3.3A). Cells were maintained in differentiation medium DMEM, 10% foetal bovine serum, 1% penicillin and streptomycin, and inducer cocktail (0.25 mM dexamethasone, 0.5 mM 1-isobutyl-3-methylxanthine/IBMX, and 1 μg/mL insulin) for four days, medium was changed every two days. At day 4, the dexamethasone and IBMX were removed with insulin remaining on the cells for an additional two days. Differentiation was allowed to continue in DMEM supplemented with 10% fetal bovine serum. The sample was tested at day 9-14 post-induction. Prior to the cellular assays, cells were serum starved in DMEM for three hours, washed two

times with KRPH buffer (5 mM Na_2HPO_4, 20 mM HEPES, pH 7.4, 1 mM $MgSO_4$, 1 mM $CaCl_2$, 136 mM NaCl, 4.7 mM KCl) and equilibrated 15 minutes in KRPH buffer.

3.2.3 Immunoprecipitation

Immunoprecipitation is a highly specific and effective technique for analytical separations of target antigens from crude cell lysates. By combining immunoprecipitation with other techniques, such as SDS-PAGE and immunoblotting, it can be used to detect and quantify antigens, determine relative molecular weights, monitor protein turnover and post-translational modifications, and check for enzyme activity.

Adipocytes were cultured and differentiated in 25 cm^2 T-Flask. Differentiated cells were treated with 100 nM insulin, 100 μg/mL cinnamtannin B1, 150 μg/mL water extract, combination of 100 nM insulin and 100 μg/mL cinnamtannin B and combination of 100 nM insulin, 150 μg/mL water extract. The cells were pretreated with wortmannin (500 nM), cytochalasin B (40 mM) or sodium orthovanadate (5 mM) to examine the specificity of the signaling pathway.

After treatment, cells were washed three times with ice-cold PBS pH 7.4 and lysed with 1 mL an ice cold immunoprecipitation buffer (10 mM phosphate, pH 7.2, 0.14 mM NaCl, 1% Igepal CA-630, 0.5% deoxycholate, 1 mM phenylmethylsulfonylfluoride, 50 mM NaF, 1.25 mM Na_3MoO_4 , 1.25 mM $Na_3 VO_4$, 12.5 mM $Na_4P_2O_7$,10 μg/mL pepstatin A, 10 μg/mL aprotinin, and 10 μg/mL leupeptin). Cell lysates were clarified by centrifugation at 14.000 rpm. The supernantant were collected and stored at -80°C for further analysis. 4 μg precipitating antibody (anti-insulin receptor β-subunit) was added to 300 μL cell lysates and incubated with rocking overnight at 4°C. 50 μL of Protein A/G Plus Sepharose (50% slurry in RIPA buffer) was added and incubated for one hour with rocking at 4°C. Beads were washed three to four times with immunoprecipitation

buffer. 30 µL of sodium dodecyl sulfate (SDS)-sample buffer (1 ml glycerol, 0.5 mL β-mercapthoethanol, 3 mL 10% SDS, 1.25 mL Tris-HCl pH 6.8 and 1 mg bromophenol blue) was added, and the beads were denatured for five minutes at 100°C. Proteins were separated on a 7.5% SDS-polyacrylamide gel electrophoresis gel at 120 volt for 75 minutes (Bio-Rad).

3.2.4 Western Blotting

The separated protein in SDS PAGE was transferred to nitrocelullose (Hybond C extra, Amersham) membrane at 100 V for 90 minutes (Trans-Blot, Bio-Rad). Blots were blocked in TBST (25 mM Tris, pH 7.4, 155 mM NaCl, 0.1% Tween 20) containing 3% milk (TBST-milk) at room temperature for 30 minutes. Primary antibody was incubated on the blots at a 1:2000 dilution in TBST-milk overnight at 4°C. Blots were washed several times with TBST, and the secondary antibody was added (1:5000 dilution) for 90 minutes at room temperature. Blots were again washed several times. Immunoreactive proteins were detected with chemiluminescense using ECL Western Blotting Analyses System (Amersham, Little Chalfont, Buckinghamshire, UK).

3.2.5 Enzyme-Linked Immunosorbent Assay (ELISA)

The use of enzyme-linked immunosorbent assay, or ELISA, provides an economical, rapid and highly sensitive method for screening a large number of samples. ELISA was used to detect and quantitate peptides, proteins or antibodies. The assay is based upon an antigen-antibody interaction and subsequent enzymatic action on a substrate yielding a soluble colored product.

3T3-L1 preadipocytes were seeded in 100 µL medium at density of 2 x 10^3 cells per well in 96-well plate. 2 days post confluent the cells were induced into

adipocytes as described in induction of cell culture. 11 days after induction, the cells were treated with the samples (100 nM insulin, 100 μg/mL cinnamtannin B1 and 150 μg/mL water extract) for 30 minutes. After treatment, the cells were fixed by adding 100 μl methanol/acetic acid (50: 50) to each well for 10 minutes. The solution was removed by inverting the plate and wash three times with PBS. The plate was tapped by upside down to remove excess PBS. Non specific antibody was blocked with 200 μl blocking buffer (3% skim milk in PBS) per well for 1 hour at 37°C. The cells were gently washed with PBS and the plate was tapped upside down to remove excess PBS. Antiphosphotyrosine antibody (5 μL) was diluted in 5 mL freshly prepared blocking buffer and added 50 μl to each well excluded a blank. The plate was incubated for overnight at 4°C. The cells were washed three times with PBS-Tween 0.05%. HRP conjugate secondary antibody in blocking buffer (1:5000) was added to each well and incubated 1 hour at room temperature. The complex antigen antibody washed tree times with washing solution by tapping the plate upside down. 100 μl of mixed ABTS reagent (2,2'-azino-di-(3-ethylbenzthiazoline-6-sulfonate and peroxidase solution B) (KPL, Gaithersburg, USA) was added to each well and incubate 30 minutes at room temperature and reaction was stopped by adding 50 μL 4% SDS. The plate was read with ELISA plate reader (Bio-Rad) at 410 nm.

CHAPTER 4

RESULTS AND DISCUSSION

4.1 Insulin Receptor Phosphorylation Analysis

Protein-tyrosine phosphorylation plays a central role in regulating a variety of fundamental cellular processes. The current hypothesis of insulin action proposes that interaction of insulin with its receptor leads to phosphorylation its own receptor with subsequent activation of the receptor tyrosine kinase activity. Immunoprecipitation of insulin receptor demonstrated that two major subunits of insulin receptor phosphorylated with molecular weights of 135,000 and 95,000 that correspond exactly to the position of the α- and β-subunit of the insulin receptor, respectively (Kasuga *et al.*, 1982 and Zick *et al.*, 1983).

Insulin binding to the insulin receptor activates a series of signal transduction events that result in specific biological actions in sensitive tissues. The signaling cascade starts with autophosphorylation of the insulin receptor through its tyrosine kinase activity and subsequent phosphorylation of insulin receptor substrates (IRS). Phosphorylation of IRS leads to activation of the phosphatidylinositol 3-kinase (PI3K) and the mitogen-activated protein kinase (MAPK) pathways. Stimulation of glucose transport and disposal, glycogen synthesis, and inhibition of lipolysis are mediated by PI3-K activity, whereas MAPK promotes cell growth (Saltiel and Kahn, 2001).

A metabolite (LY78,3281) from a *Pseudomassaria* fungus stimulates insulin receptor tyrosine kinase activity with high specificity without inhibition of

phosphatases, presenting another means to enhance insulin action (Zhang *et al.*, 1999). Given the recent evidence that defective phosphorylation of IRS-1 and IRS-2, PI 3-kinase and PKB are all implicated in the development of insulin resistance (Krook *et al.*, 2000).

4.2 Effect of Cinnamtannin B1 on Phosphorylation of Insulin Receptor

This work investigated the role of cinnamtannin B1 tyrosine posphorylation of insulin receptor of 3T3-L1 cells. Cell lysates were subjected to immunoprecipitation with anti-insulin receptor β-subunit antibody and the immunocomplex samples were subjected to SDS-PAGE, transferred to nitrocelluose membranes, and immunoblotted with monoclonal anti-phosphotyrosine antibody (4G10), as described in experimental.

Figure 4.22 demonstrated the typical insulin stimulated tyrosine autophosphorylation of insulin receptor β-subunit. EGF-stimulated A431 cell lysate was positively exhibit phosphorylation at 170-kDa. Untreated cells was not exhibit phosphorylation of insulin receptor except for molecular weight of 55-kDa that expressed by all cell lysates. Treatment with 100 nM insulin expressed phosphorylation of insulin receptor β-subunit at 95-kDa. 100 μg/mL cinnamtannin B1 alone augmented phosphorylation of insulin receptor.

To investigate whether cinnamtannin B1 potentiate the phosphorylation action of insulin, the cells were treated with the combination of cinnamtannin B1 and insulin Treatment of the cells with combination of cinnamtannin B1 and insulin activated phosphorylation similar to that of insulin alone. It was suggested that insulin and cinnamtannin B1 may be reacted competitively to insulin on receptor β-subunit.

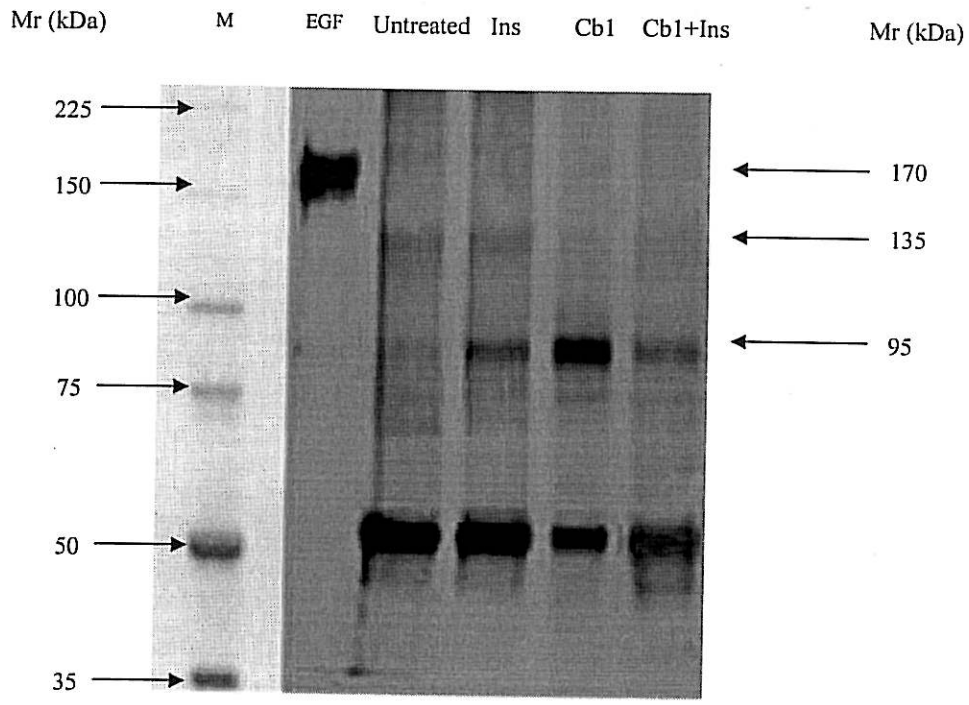

Figure 4.1 Effect of cinnamtannin B1 on phosphorylation of insulin receptor of 3T3-L1 adipocytes. 3T3-L1 adipocytes cells were left untreated, or treated with 100 nM insulin (Ins), 100 μg/mL cinnamtannin B1 (Cb1), M= marker. Proteins were separated by electrophoresis, blotted onto a membrane, and detected with an antibody to phosphotyrosine (4G10).

4.3 ELISA for Insulin Receptor Phosphorylation

Enzyme-linked immunosorbent assay (ELISA) on the plating cells was carried out to detect the phosphorylation of receptor β-subunit occurred in cell membrane based upon an antigen-antibody interaction and subsequent enzymatic action on a substrate yielding a soluble colored product with ABTS. The phosphorylation insulin receptor was detected by addition of antiphosphotyrosine antibody (HRP conjugated) as described in experimental section.

The phosphorylation was quantitatively assayed using ELISA, suggesting that cinnamtannin B1-induced signal transduction leading to phosphorylation is similar mechanism to that of induced by insulin. As shown in Figure 4.23, 100 μg/mL cinnamtannin B1 alone strongly activated phosphorylation of insulin receptor. Combination of cinnamtannin B1 and insulin yielded phosphorylation result more likely as insulin action alone.

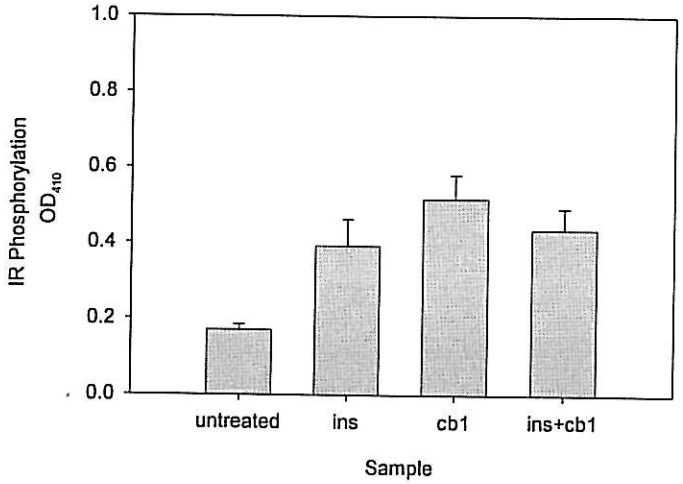

Figure 4.2 ELISA assay for the effect of cinnamtannin B1 on insulin receptor phosphorylation of 3T3-L1 adipocytes. Cinnamtannin B1 showed the highest activity in phosphorylation of insulin receptor. Results are means ± SE of three experiments.

4.4 Effect of Wortmannin, Cytochalasin B and Sodium Orthovanadate on Phosphorylation Activity of Cinnamtannin B1

In this study, the activity of cinnamtannin B1 on phosphorylation was evaluated by treating the cells with chemicals that are known for their activity such as sodium orthovanadate, wortmannin and cytochalasin B (Fig. 4.24). The cells were treated with sodium orthovanadate (5 mM), wortmannin (500 nM) or cytochalasin B (40 mM) prior to the addition of cinnamtannin B1.

Wortmannin has previously been shown to inhibit insulin dependent 2-deoxy-D- [1-^{3}H] glucose uptake in 3T3-L1 adipocytes by inhibiting PI-3-kinase (Arcaro and Wymann, 1993; Walker et al., 2000). Cytochalasin B is known to block glucose uptake by binding to the membrane (Jung and Rampal, 1977). The stimulation of phosphorylation was demonstrated using sodium orthovanadate, an inhibitor of protein phosphatase. Vanadate inhibits protein phosphotyrosine phosphatases and also activates insulin receptor β-subunit phosphorylation, the possibility was considered that vanadate-stimulated glucose uptake by 3T3-Ll adipocytes (Dubyak and Kleinzeller, 1980; Bernier *et al.*, 1988).

In order to examine whether the activity of cinnamtannin B1 on phosphorylation of insulin receptor was affected by these chemicals, the 3T3-L1 adipocyte was treated with 500 nM wortmannin, 40 mM cytochalasin B or 5 mM sodium orthovanadate 15 minutes prior addition of cinnamtannin B1. Differentiated cells were washed and glucose-starved as described in material and methods. Prior to addition of sample, the cells were pretreated with at final concentration of 500 nM. As shown in Figure 4.23, the activity of cinnamtannin B1 was fully inhibited by wortmannin. Pretreatment the cells with cytochalasin B inhibited all phosphorylation sites. It was interesting that cytochalasin B inhibited all insulin signaling caused the cell lost of communication. Pretreatment the cells with vanadate were not influence the phosphorylation activity of cinnamtannin B1.

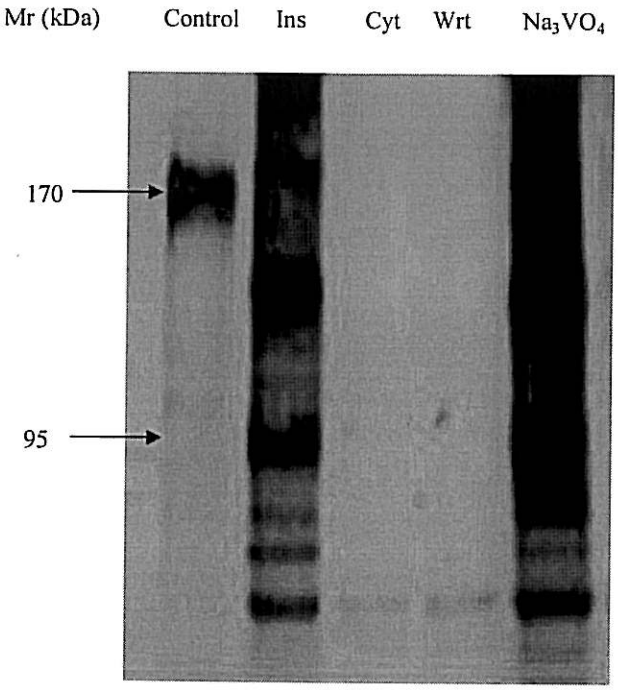

Figure 4.3 Effect of wortmannin, cytochalasin B and sodium orthovanadate on phosphorylation of insulin receptor. The 3T3-L1 adipocyte was treated with 500 nM wortmannin (Wrt), 40 mM cytochalasin B (Cyt) or 5 mM sodium orthovanadate (Na_3VO_4) 15 minutes prior the addition of cinnamtannin B1.

Wortmannin was isolated from *Penicillium wortmanni*. It is known that insulin stimulates PI 3-kinase and PI 3-kinase is involved in cell growth signaling pathways (Tomiyama, 1995). The effect of cinnamtannin B1 was clearly inhibited by wortmannin, a specific phosphatidylinositol 3-kinase (PI 3-kinase) inhibitor.

Cytochalasin B is known to enhance insulin release evoked by nutrient and non-nutrient secretagogues, including D-glucose, despite inhibiting D-glucose uptake and metabolism in human erythrocytes and pancreatic islets (Bloch, 1973; Jijakli *et al.*, 2002). Addition of cytochalasin B prior the treatment inhibited the phosphorylation activity of cinnamtannin B1. Similar activity in glucose uptake, cytochalasin B also inhibited the activity of insulin.

Vanadate ions, low molecular weight phosphate analogues, mimic most of the rapid actions of insulin in various cell types (Shechter, 1990). Vanadate has been shown to inhibit phosphatases that remove phosphate groups from phosphotyrosine in cell-free systems. Addition of vanadate to the culture medium of NRK-1 cells resulted in a maximal 40-fold increase in the level of phosphotyrosine in cell protein (Klarlund, 1985). Vanadate enhanced the state of activation of rat adipocyte glycogen synthase in a manner similar to that of insulin. No additional effect was observed when insulin and vanadate were added together. Vanadate also enhanced the degree of phosphorylation of the 95-kDa subunit of insulin receptor, selectively on tyrosine residues, in the solubilized rat adipocyte insulin receptor system. This demonstrates that insulin and vanadate have similar initial actions on receptor phosphorylation and also act similarly on an intracellular event, namely the activation of glycogen synthase (Tamura *et al.*, 1983).

CHAPTER 5

CONCLUSIONS

The effect of cinnamtannin B1 in phosphorylation was evaluated using western blot analysis. Both of cinnamtannin B1 and insulin stimulate phosphorylation of insulin receptor at 95-kDa corresponding to insulin receptor β-subunit. Cinnamtannin B1 (0.11 mM) exhibit a higher activity than insulin (100 nM) stimulation of insulin receptor. The combination of cinnamtannin B1 and insulin yielded no increasing in receptor phosphorylation comparing to the treatment with cinnamtannin B1 or insulin alone. The water extract was also stimulated insulin receptor phosphorylation. The quantitative activity of cinnamtannin B1 was confirmed by ELISA technique. ELISA detects the phosphorylation of insulin receptor in cells membrane based on antigen-antibody interaction. Cinnamtannin B1 showed high intensity of phosphorylation compared to insulin alone. The results support the data from western blotting analysis. Like insulin effect, the phosphorylation effect of cinnamtannin B1 was inhibited by wortmannin and cytochalasin B but increased by sodium orthovanadate.

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