The Effects of *Momordica charantia* and Cinnamon Extracts on Glucose Uptake and Adiponectin Secretion in 3T3-L1 Adipose Cells

Ben Roffey

School of Dietetics and Human Nutrition
McGill University
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Your file Votre référence
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Dedicated to my wife, Roxane, and my parents Ken and Sharon.

ABSTRACT

To examine the effects of *Momordica charantia* (MC) and cinnamon on glucose uptake and adiponectin secretion (AS) fat cells, 3T3-L1 adipocytes were treated with a water extract of cinnamon (CE) and three concentrations of MC water and ethanol extracts. The treatment combination of 0.2 mg/ml MC water extract and 0.5 nM insulin was associated with an increased glucose uptake into the cells (61%) and increased AS from the cells (75%). Without insulin, 0.2 mg/ml of CE increased glucose uptake (100%) and completely inhibited AS from the cells. Sub-optimal concentrations of insulin did not further enhance the CE activity and, in combination with 50 nM insulin, a dose-dependent decrease in glucose uptake was observed. The present results indicate that preferentially water-soluble component(s) in MC enhance the glucose uptake action of sub-optimal concentrations of insulin in 3T3-L1 adipocytes. This effect is accompanied by and may be a result of increased AS. CE increases glucose uptake in these adipocytes but inhibits AS.

SOMMAIRE

Pour examiner les effets du *Momordica charantia* (MC) et du cannelle sur l'assimilation de glucose, la sécrétion d'adiponectine (SA) et l'accumulation de céramide en cellules adipose, les adipocytes 3T3-L1 ont été traités avec un extrait d'eau de cannelle (EC) et trois concentrations des extraits d'eau et d'éthanol de fruit et de graines de MC. Les traitements ont compris les extraits en isolation et les extraits en combinaison avec deux concentrations d'insuline. La combinaison de traitement de 0.2 mg/ml d'extrait d'eau de MC et 0.5 nM d'insuline ont été associés à une assimilation accrue de glucose dans les cellules (61%) et ont augmenté la SA des cellules (75%). L'extrait d'éthanol de MC n'a pas été associé à un accroissement d'assimilation de glucose; cependant, on a observé une diminution dépendante de la dose de l'assimilation de glucose basal et de l'assimilation de glucose interposé par l'insuline avec l'extrait d'éthanol de MC en combinaison avec 50 nM d'insuline. Sans insuline, 0.2 mg/ml de EC a augmenté l'assimilation de glucose (100%) et a complètement empêché la SA des cellules. Les concentrations sub-optimales d'insuline n'ont pas plus augmenté l'activité de la EC et, en combinaison avec 50 nM d'insuline, on a observé une diminution dépendante de la dose de l'assimilation de glucose avec l'extrait. La variabilité élevée dans les concentrations intracellulaires de céramide a empêché toutes conclusions d'être dérivé à ce regard. Les résultats actuels indiquent que le ou les composant(s) préférentiellement hydrosoluble(s) du fruit et/ou de la graine de MC augmente(ent) l'action d'assimilation de glucose des concentrations sub-optimales d'insuline dans les adipocytes 3T3 - L1. Cet effet est accompagné par, et peut être un résultat de, l'accroissement de la SA des cellules. Le EC a augmenté l'assimilation de glucose dans ces adipocytes d'une façon concurrentielle avec l'insuline mais a empêché la SA.

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Ben Roffey (candidate) - Helping in the experimental design of the study, ordering the equipment, conducting all aspects of cell culture, performing all experimental assays, organizing the data, analyzing the data, and drawing conclusions from the analyzed data. The candidate wrote the manuscript under the guidance of the co-authors and made modifications to it in response to their comments.

Stanley Kubow (Supervisor, Associate Professor at McGill) –Advice and guidance on all aspects of the study including study design, assay techniques, interpretation and statistical analysis of data, solving equipment problems, and editing of the manuscript. Provided partial funding for the project.

Avtar Atwal (Co-Supervisor, Senior Research Scientist at Agriculture and Agri-Food Canada) – Introduced the idea for the topic of the study and offered advice on study design and interpretation of results. Editing of the manuscript. Provided funding for the study through Agriculture and Agri-Food Canada.

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ABBREVIATIONS

APS	Adaptor protein containing PH and SH2 domains
BMI	Body mass index
CAD	Coronary artery disease
CAP	Cbl activating protein
CAPK	Ceramide-activated protein kinase
CAPP	Ceramide-activated protein phosphatase
CE	Cinnamon extract
DAG	Diacylglycerol
DMEM	Dulbecco's modified Eagle's medium
DPM	Disintegrations per minute
FBS	Fetal bovine serum
GLUT4	Glucose transporter isoform 4
GIR	Glucose infusion rate
HDL	High-density lipoprotein
HEPES	N-2-Hydroxyethylpiperazine-N'-2-Ethanesulfonic Acid
HSL	Hormone sensitive lipase
IBMX	1-isobutyl-3-methylxanthine
IL-6	Interleukin-6
IR	Insulin receptor
IRS	Insulin receptor substrate
JNK	c-Jun N-terminal directed protein kinase
KRH	Krebs-Ringer-HEPES
LDL	Low density lipoprotein
LP	Lipid phosphate
LPL	Lipoprotein lipase
MAPK	Mitogen-activated protein kinases
MC	*Momordica charantia*
MCE	*Momordica charantia* ethanol extract
MCW	*Momordica charantia* water extract
MHCP	Methylhydroxychalcone polymer

MTT	(3,4-5 dimethyl thiazol-2-yl)-2,5-diphenyl tetrazolium
NEFA	Non-esterified free fatty acids
NFκB	Nuclear factor-κB
OPA	*O*-phthaldialdehyde
PDE	Phosphodiesterase
PDE3B	Cyclic nucleotide phosphodiesterase 3B
PDK1	Phosphoinositide-dependent protein kinase 1
PI3-kinase	Phosphoinositide 3'- kinase
PKA, PKB and PKC	Protein kinase A, B and C
PPAR-γ	Peroxisome proliferator-activated receptor gamma
PTP-1	Protein tyrosine phosphate-1
RXR	Retinoid X receptor
SAPK	Stress-activated protein kinase
SD	Standard deviation
STZ	Streptozotocin
TNFα	Tumour necrosis factor alpha
TZDs	Thiazolidinediones

TABLE OF CONTENTS

LIST OF FIGURES AND TABLES

Literature Review

Manuscript I

Manuscript 2

I. INTRODUCTION

In the year 2002, 151 million people worldwide were diagnosed with diabetes and this number is expected to rise to 221 million people by the year 2010 (Zimmet, 2002). More than 90% of these cases are type 2 diabetics suffering from severe insulin resistance. Research has begun to focus on adipose tissue as a possible central mediator of whole body insulin resistance. Evidence for this central role comes not only from the link between obesity and type 2 diabetes (Williams, 1999), but also from the role of adipose tissue in regulating serum lipid concentrations and, more recently, from the emerging role of adipose tissue as an endocrine organ (Mohamed-Ali *et al.,* 1998).

The specific and/or limited effects of current drug treatments for diabetes, combined with the dangerous side effects that most of them induce, has fueled the search for alternative medicines. *Momordica charantia* (MC) and cinnamon (*Cinnamomum sp*) have both shown potential, through scientific research and traditional use, to be used as natural anti-diabetics. MC (bitter melon, bitter gourd, karela), a plant native to China, India, Asia, and Africa, bears a fruit that is currently the most widely used traditional medicine to treat diabetes (Marles and Farnsworth, 1995). A few active components, polypeptide-p (Khanna *et al.,* 1981) and charatin (a mix of two steroid glycosides) (Lotlikar and Rajarama Rao, 1966), have been identified from the fruit and its seeds. These components, along with pure juice, methanol and ethanol extracts from the fruit, have shown hypoglycemic activity via *in vitro*, animal and human studies (Virdi *et al.,* 2003, Rathi *et al.,* 2002, Sitasawad *et al.,* 2000, Khanna *et al.,* 1981, Welihinda *et al.,* 1986). Although MC seems to improve the function of the pancreas (Sitasawad *et al.,* 2000, Ahmed *et al.,* 1998), liver (Rathi *et al.,* 2002), and skeletal muscle (Rathi *et al.,* 2002, Welihinda and Karunanayake, 1986) towards ameliorating insulin resistance, its mechanism has not been well defined and very little attention has been paid to the effects of MC on adipose tissue function and adipocytokine secretion. Between the two previous studies that have on focused on the effects of MC on adipose tissue, varying results were obtained in regards to glucose uptake and adipocytokine secretion was not measured (Ng *et al.,* 1986, Meir and Yaniv, 1982). As well, specific inhibitors of insulin-induced glucose uptake, such as wortmannin, have not been used with a combination of MC and

insulin to determine the similarity between MC and insulin induced glucose uptake in adipose tissue.

Adiponectin is a cytokine secreted solely by adipose tissue and has been shown to have both antidiabetic and antiatherogenic properties (Iwaki *et al.*, 2003). Plasma concentrations of adiponectin are lower in type 2 diabetics and obese individuals and have a strong negative correlation with insulin resistance (Hotta *et al.*, 2000, Abbasi *et al.*, 2004). In both obese and lipoatrophic mice, hypoadiponectinemia leads to insulin resistance while adiponectin supplementation alleviates the condition (Yamauchi *et al.*, 2001). Specifically, adiponectin activates insulin receptor substrate-1 (IRS-1)-mediated PI3-kinase and glucose uptake in muscle (Jazet *et al.*, 2003) and suppresses hepatic glucose production (Berg *et al.*, 2001). As of yet, no natural anti-diabetic treatments, including MC, have been studied in terms of their effect on adiponectin secretion.

Ceramide is the second messenger in the sphingomyelin signaling pathway and directly inhibits the insulin signaling pathway by decreasing insulin stimulated phosphorylation and activity of PKB/Akt proteins in the pathway (Adams *et al.*, 2004, Chavez *et al.*, 2003, Mei *et al.*, 2003, Powell *et al.*, 2004). Skeletal muscle ceramide levels are increased in obese insulin resistant humans (Adams *et al.*, 2004). In trying to elucidate the intercellular mechanisms by which an anti-diabetic functions, no natural treatment shown to positively influence glucose uptake, including MC, has been tested for its effect on cellular ceramide accumulation.

Cinnamon is the dried inner bark of various laurel trees in the cinnamomum family that is native to Sri Lanka and India but is cultivated extensively in the tropical regions of the world (Chevallier, 2000). Recently it has been discovered that water and ammonium hydroxide extracts of cinnamon have the ability to increase glucose uptake by 5 to 32-fold in a rat epididymal fat pad assay (Broadhurst *et al.*, 2000, Berrio *et al.*, 1992). Since then cinnamon has shown significant hypoglycemic activity and blood lipid parameter improvements in both animals and humans (Qin *et al.*, 2003, Qin *et al.*, 2004, Khan *et al.*, 2003). It is thought that the active component of cinnamon is a methylhydroxychalcone polymer (MHCP) that was isolated and found to potentiate the activity of insulin by two fold for glucose uptake and four fold for glycogen synthesis in 3T3-L1 adipocytes (Jarvill-Taylor *et al.*, 2001). Although cinnamon has been shown to

have potential use as an anti-diabetic from preliminary *in vitro* results to a human clinical trial, it has not been tested for its effects on adipocytokines or cellular ceramide accumulation.

Given both the intriguing results and limitations of the above outlined research, the experiments described in this thesis tested extracts of MC and cinnamon on 3T3-L1 adipocytes, an excellent model for insulin-sensitive glucose uptake, with the following objectives in mind:

1. To determine the ability of MC extracts to mediate 2-deoxy glucose uptake in 3T3-L1 adipose cells:
    a. To determine the presence of a dose response effect of MC extracts to enhance glucose uptake.
    b. To determine if MC extracts mediate glucose uptake independently of insulin or act to enhance the action of insulin.
    c. Using wortmannin to inhibit insulin signals mediating GLUT4 translocation and activation, determine if MC extracts stimulate glucose uptake by a similar pathway by which insulin stimulates glucose uptake.
2. To test for similarities between cinnamon aqueous extract and MC extracts on 2-deoxy glucose uptake in 3T3-L1 adipose cells in the same manner as the MC extracts.
3. To determine differences in activity between extracts:
    a. To test aqueous and ethanol extracts from a whole, unripe MC fruit for differences in effectiveness at mediating glucose uptake.
4. To determine the effect of active glucose uptake mediating MC and cinnamon extracts on adiponectin secretion from 3T3-L1 adipose cells.
5. To determine the effect of active glucose uptake mediating MC and cinnamon extracts on cellular ceramide accumulation in 3T3-L1 adipose cells.

II. LITERATURE REVIEW

2.1 Insulin Action in the Liver, Skeletal Muscle and Adipose Tissues

Insulin is a polypeptide synthesized and released by beta cells located in the islets of Langerhans that make up about 1% of the weight of the pancreas (Zeman, 1991). It is the only hormone capable of enhancing glucose uptake from the blood stream by regulating both glucose and lipid metabolism in favour of anabolic actions. The main tissues where insulin has receptor-mediated effects are the liver, skeletal muscle and adipose tissue. Consequently, in a normal functioning metabolic state, insulin has specific actions at these tissues.

The liver is the main site of glycogen synthesis and storage (Groff and Gropper, 2000). Through glycogenesis and the opposite pathway of glycogenolysis the liver is able to regulate glucose output into the blood stream to supply appropriate amounts of glucose to the central nervous system (Bergman, 2000). Insulin acts to suppress glucose output from the liver and increase glycogen synthesis by affecting the enzymes involved in these pathways (Zeman, 1991). It has been suggested that this effect of insulin may be indirect and is mediated by insulin's regulation of non-esterified fatty acids from the adipose tissue, which then in turn affect glucose metabolism in the liver (Bergman, 2000). This indirect effect of insulin may also occur through ATP-sensitive potassium channels located in the medial hypothalamus, a major integrator of hormonal signals that are activated by insulin to restrain hepatic gluconeogenesis (Pocal *et al.,* 2005). Insulin also enhances fatty acid synthesis in the liver through its enhancement of the production of acetyl-CoA and subsequent synthesis of fatty acids from acetyl-CoA (Zeman, 1991).

Skeletal muscle is responsible for 60-70% of glucose uptake from the blood stream through both an insulin dependent pathway and insulin independent uptake through muscle contraction (Smith, 2002). Glucose is taken into the muscle specifically through the GLUT4 protein and is converted into glycogen. Insulin acts through a receptor pathway similar to that in the adipose tissue to promote the translocation of GLUT4 to the cell surface and thus enhance glucose uptake (Koistinen and Zierath, 2002).

Insulin affects both glucose uptake and lipid metabolism in adipose tissue cells. It promotes the uptake of serum fatty acids into the adipocyte for storage as triglycerides by stimulating lipoprotein lipase (LPL) action on the cell surface to break down serum triglycerides into free fatty acids and glycerol for uptake into the adipocyte (Kopecky *et al.,* 2002). At the same time, insulin inactivates hormone sensitive lipase (HSL) within the cell preventing the enzyme from breaking down stored triglycerides into free fatty acids for release back into the blood stream (Zeman, 1991). Glucose that is transported into the adipose cell can be converted to glycogen but can also be synthesized into triacylglycerols by reesterification of glycerphosphate (a glucose glycolytic breakdown product) with fatty acids (Groff and Gropper, 2000).

Insulin mediates glucose uptake into adipocytes through a unique pathway(s) that has not been fully defined. The uptake of glucose requires the insulin-stimulated translocation of GLUT4 from its inner compartmentalization to the surface of the cell (Groff and Gropper, 2000). Insulin first binds to the extracellular insulin receptor (IR) alpha-subunits, which then activates the intra-cellular beta-subunits through a transmembrane signal (Mora and Pessin, 2002). The first substrates that are activated in the resulting intracellular signal transduction cascades are the insulin receptor substrate (IRS) proteins 1-4 (Litherland *et al.*, 2001). IRS-1 and IRS-2 are the most important of these receptor proteins during differentiation into adipocytes and in glucose transport regulation, although IRS-2 experiences the greater increase in expression and is more abundantly expressed in the mature adipocyte (Tamori *et al.*, 2002). In terms of glucose transport, the binding of the phosphorylated IRS proteins to the p85 subunit of the Class 1A phosphoinositide 3'-kinase (PI3-kinase) is the next most important step. This binding leads to subsequent association with the p110 catalytic domain of PI3-kinase near the plasma membrane (Litherland *et al.*, 2001). There is sufficient evidence to determine that the IRS interaction with PI3-kinase is a necessary steps in insulin signaling for glucose transport regulation. IRS activated PI3-kinase increases the synthesis of 3'-phosphoinositides which in turn activate phosphoinositide-dependent protein kinase 1 (PDK1) (Litherland *et al.,* 2001). At this point the signaling diverges to activate two down-stream kinases Akt (or PKB) and protein kinase C (PKC) (Mora and Pessin, 2002).

Downstream targets from these kinases to GLUT4 translocation have not been identified. Figure 1 shows the insulin signaling pathway as it occurs in an adipose cell.

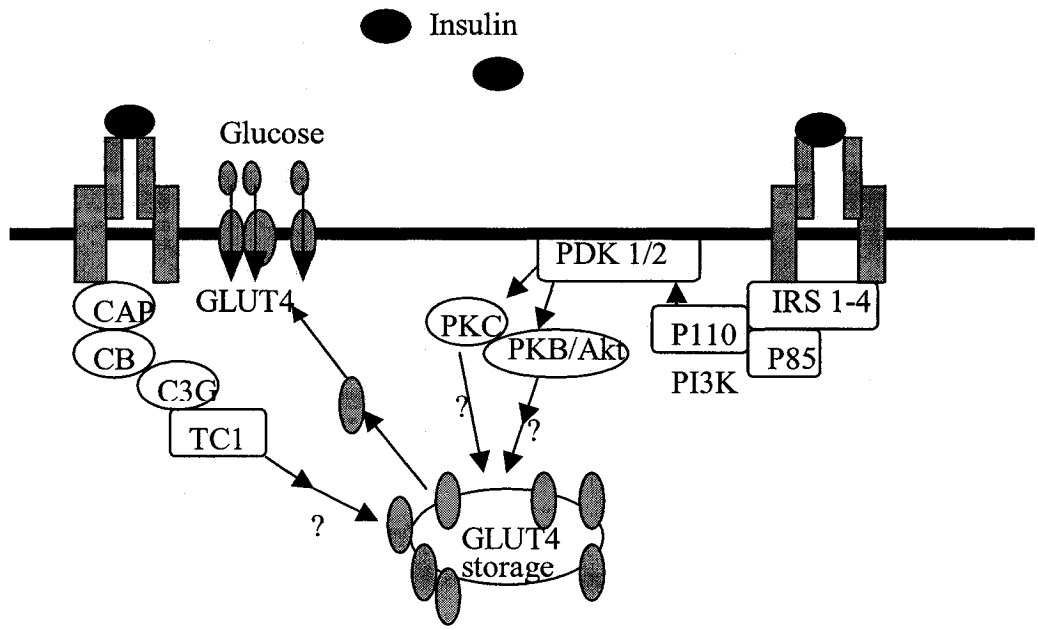


Fig. 1 Signaling events mediating insulin-stimulated glucose transport. Two parallel pathways are shown here. Adapted from Litherland, 2001.

There is much evidence to suggest the presence of at least one other PI3-kinase independent pathway that must act in concert to regulate GLUT4 translocation. The extracellular insulin receptors first couple to and phosphorylate Cbl through the adaptor protein containing PH and SH2 domains (APS) and Cbl activating protein (CAP) (Mora and Pessin, 2002). This becomes a recognition site for the CrkII-C3G complex that in turn activates TC10 leading to GLUT4 translocation (Mora and Pessin, 2002). Again, this pathway has not been defined beyond this point.

By inducing an intracellular response, insulin also activates a $p21^{ras}$ pathway that leads, through series of phosphorylation reactions, to the activation of mitogen-activated protein kinases (MAPK) (Wiese *et al.*, 1995). It is thought that a p38 MAPK-dependent pathway may activate cell surface GLUT4 and that this activation, along with the translocation of GLUT4, is necessary for glucose uptake (Bazuine *et al.*, 2005, Somwar *et al.*, 2001).

The insulin resistant state will negate all the actions of insulin mentioned above to a degree proportional to the degree of insulin resistance. Specifically in adipose tissue, insulin resistance will stimulate the release of free fatty acids into the blood stream as a result of increased lipolysis (Kopecky *et al.,* 2002). Insulin resistance will also decrease glucose uptake by the adipocyte. An effect marked by changes to the GLUT4 translocation pathway such as decreased IRS1 expression and impaired activities of PI3-kinase and PKB/Akt (Smith, 2002).

The specific pathways and sequences for the actions of insulin require much further investigation to pinpoint the root cause(s) and mechanisms of insulin resistance. Much of this research may focus on the adipose tissue as insulin appears not only to have a role in the lipid and glucose metabolism of this tissue, but may also partially regulate adipose cytokine expression and secretion that can affect whole body insulin resistance.

2.2 Wortmannin and Insulin Signaling

Wortmannin is a fungal metabolite that inhibits insulin stimulated glucose uptake with nanomolar efficiency. In 3T3-L1 adipocytes, wortmannin is capable of completely inhibiting insulin-stimulated glucose uptake at a concentration of 30 nM (Hausdorff *et al.,* 1999). This effect is partially mediated by the irreversible binding of wortmannin to the p110 isoform of PI3-kinase and subsequent decrease in GLUT4 translocation to the membrane (Hausdorff *et al.,* 1999). The insulin stimulated translocation of GLUT1 is even more sensitive to wortmannin and is completely inhibited at 1nM concentrations of wortmannin. For these reasons, wortmannin is often used as a negative control when experimenting with substances or extracts that stimulate glucose uptake in *in vitro* adipocytes (Jarvill-Taylor *et al.*, 2001, Radosevich *et al.*, 1998). This use allows the experimenters to speculate that the stimulatory effects of the substance or extract on glucose uptake rely on the activation of PI3-kinase. More recent evidence shows that wortmannin has a second higher affinity target that may or may not be a PI3-kinase. When 3T3-L1 adipocytes are left exposed to wortmannin during treatment with insulin and glucose, glucose uptake is inhibited by very low concentrations of wortmannin that do not affect GLUT4 translocation (Hausdorff *et al.*, 1999). This has led to the belief that full stimulation of glucose uptake by insulin also requires the activation of GLUT4 at the

cell surface, not just its translocation (Somwar *et al.*, 2001). When 3T3-L1 adipocytes are pretreated with wortmannin, which is then removed before the addition of insulin and glucose, the inhibition of glucose uptake again matches the inhibition of GLUT4 translocation (Hausdorff *et al.*, 1999). This has led to the conclusion that inhibition of the p110 PI3-kinase to decrease GLUT4 translocation is the lower affinity irreversible target of wortmannin while the higher affinity target is a reversible inhibition. While the identity of this second target remains undetermined, there is good evidence to show that this second target is involved in the activation of p38 MAPK and subsequent activation of GLUT4 (Somwar *et al.*, 2001). Specifically, the inhibition of p38 MAPK correlates strongly with the high sensitivity of glucose uptake to inhibition by wortmannin in 3T3-L1 adipocytes while the inhibition of PKC, Akt1, Akt2, and Akt3 correlated strongly with GLUT4 translocation (Somwar *et al.*, 2001). Despite these findings wortmannin, remains a commonly used and effective tool for determining the dependence of substances that stimulate glucose uptake on PI3-kinase activation and events required for insulin stimulated glucose uptake.

2.3 The Role of Adipose Tissue as an Endocrine Organ

It is currently not clear as to the degree to which the adipose tissue is a cause and/or result of insulin resistance and how this might occur mechanistically. The elevated levels of non-esterified free fatty acids (NEFA) that occur with obesity certainly play a role in peripheral tissue insulin resistance and altered glucose metabolism in the liver (Bjorntorp, 1997). Interestingly, severe depletion of adipose tissue can also result in severe insulin resistance and diabetes (Nadler and Attie, 2001). This suggests that a decrease in the number of normal functioning adipose cells in both the obese and lipoatrophic state is a major factor in the development of insulin resistance (Nadler and Attie, 2001).

White adipose tissue has traditionally been viewed to function mainly as a fuel depot storing excess energy in the form of triglycerides and fatty acids. However, over the last decade numerous discoveries have been made supporting the role of adipose tissue as an important endocrine organ capable of secreting a wide range of signaling molecules (adipocytokines). Some of these molecules include enzymes (LPL, adipsin),

cytokines [tumor necrosis factor-α (TNFα), interleukin 6 (IL-6)], and several hormones including leptin, adiponectin, acylation-stimulating protein and resistin (Mora and Pessin, 2002). Adipocytokines have a variety of functions including the regulation of insulin secretion, insulin sensitivity, glucose and lipid metabolism, energy balance, host defence, and reproduction (Mora and Pessin, 2002). The network of endocrine, paracrine and autocrine signals that originate from adipose tissue may perform metabolic functions enabling an organism to adapt to a range of metabolic challenges including starvation, stress, infection, and reproduction (Mohamed-Ali *et al.,* 1998). The pathways and mechanisms in the adipose signaling network and their effects and/or control on peripheral tissues, metabolic regulation and physiological homeostasis are still poorly understood and controversial. Despite knowledge gaps in this area it has become clear that adipose tissue plays a pivotal role in the pathogenesis of metabolic disease in part by influencing whole body insulin resistance through its action as an endocrine organ (Smith, 2002).

2.4 The Adipocytokines

Adipocytokines serve a variety of functions, although the regulation and specific actions of these molecules remains poorly understood. The signaling molecules produced by adipose tissue can both elicit and impair insulin resistance. Each adipocytokine has a unique role in regulating insulin sensitivity and lipid metabolism although their expression and function is complexly integrated into a variety of signals and controls influenced by metabolic state, adipose tissue mass, other adipocytokines and their receptors, the autonomic nervous system, and blood flow through the adipose tissue (Frayn *et al.,* 2003). Table 1 (below) shows a list of adipocytokines and their involvement with insulin resistance.

Leptin was one of the first adipocytokines to be discovered and is now shown to regulate food intake, body weight, energy expenditure and neuroendocrine function and plays a role in the maturation of reproductive function (Steppan and Lazar, 2002). Leptin acts centrally through the hypothalamus but also appears to have direct peripheral effects and has receptors on adipose tissue, skeletal muscle, liver, and pancreatic islet cells (Mohamed-Ali *et al.*, 1998). Although its role in controlling obesity has been well

defined (Friedman and Halaas, 1998), its effects on glucose metabolism and insulin sensitivity have been somewhat controversial. Leptin has been shown to stimulate fatty acid oxidation and accumulation in skeletal muscle (Minokoshi *et al.,* 2002), and the majority of studies suggest that leptin can improve glucose uptake in muscle through increased expression of IRS-1 and activation of PI3 kinase (Berti *et al.,* 1997, Kamohara *et al.,* 1997, Rossetti *et al.,* 1997). Yet an enhancement of insulin resistance by leptin has been suggested (Mohamed-Ali *et al.,* 1998, Kroder *et al.,* 1996). Leptin has been shown to inhibit insulin secretion by pancreatic β cells with no subsequent change in insulin sensitivity (Cases *et al.,* 2002). In the liver, leptin decreased glucose output and glycogenolysis (Rossetti *et al.,* 1997) but, in contrast, was observed to increase hepatic glucose output and decrease liver glycogen in a different study (Kamohara *et al.,* 1997). It is clear that leptin deficiency does not cause insulin resistance as leptin levels are raised in most obesity-associated type 2 diabetic mice and humans, but leptin resistance at its receptors may have an impact (Steppan and Lazar, 2002). Controversy in the above findings arises partly as a result of the complex and little understood pathways and the number of factors influencing leptin production. Not only influenced by temperature, food intake, exercise, and sleep, leptin production is stimulated by increased adipose cell size, TNFα, insulin and increased glucose uptake (Frayn *et al.,* 2003).

Table 1. Levels of adipocytokines during insulin resistance and their potential mechanism and effects on insulin sensitivity.

Adipo-cytokine	Levels in Obesity-Related Insulin Resistance	Effect on Insulin Sensitivity	Mechanism
Leptin	↑	↑ (contro-versial)	Activation of IRS-1 and PI3-kinase ↓ hepatic glucose output
Adiponectin/ ACRP30	↓	↑	↑ muscle glucose uptake (increased phosphorylation of IRS-1 and protein kinase B) ↓ hepatic glucose output
TNFα	↑	↓	Inhibit IRS-1 and PI3-kinase functioning ↓ GLUT4 expression ↓ PPAR –γ and CEBP/α
IL-6	↑	↓	Increased lipolysis (increased NEFA production)
Resistin	↑	↓ (contro-versial)	Unknown

TNFα and IL-6 (both pro-inflammatory cytokines) production by adipocytes is increased in insulin resistant rats and obese humans (Stephens *et al.,* 1997). Significant quantities of TNFα are produced by adipocytes but it is also produced by macrophages, lymphocytes and skeletal muscle. Its production can be influenced by insulin and other energy-balance signals and adipose tissue mRNA levels of TNFα are positively correlated with obesity and insulin resistance (Frayn *et al.,* 2003, Jazet *et al.,* 2003).

There is much evidence indicating its role in causing insulin resistance and decreased glucose uptake by inhibiting insulin receptor and IRS-1 phosphorylation (Hotamisligil, 2000). It has also been shown to downregulate GLUT4 and IRS-1 expression in 3T3-L1 adipocytes (Stephens *et al,* 1997). TNFα downregulates two adipocyte transcription factors: peroxisome proliferator activated receptor (PPAR)-γ and CEBP/α which influence production of adipocytokines involved in tissue glucose metabolism and genes involved in adipocyte lipid metabolism (Jazet *et al.,* 2003, Martin *et al.,* 1998). Mice lacking the TNFα gene remain highly insulin sensitive even in a state of obesity (Hotamisligil, 2000). IL-6 is produced by a variety of cells including adipocytes and although a strong correlation exists between IL-6 levels and insulin resistance its mechanism of action has not been well studied (Steppan and Lazar, 2002). It has been suggested that about 30% of total circulating IL-6 originates from adipose tissue (Mohamed-Ali *et al.*, 1997). IL-6 will increase hepatic triglyceride secretion and decrease adipose LPL activity, suggesting that it may decrease insulin sensitivity through increased NEFA production (Fruhbeck *et al.,* 2001). Although IL-6 administration has consistently been shown to cause insulin resistance and increased gluconeogenesis in the liver both *in vitro* (Senn *et al.*, 2002) and *in vivo* (Kim *et al.*, 2004, Stouthard *et al.*, 1995), its effects on glucose metabolism in humans has been contradictive. Infusion of IL-6 to human subjects has been shown to both increase plasma glucose levels in healthy subjects in a dose-dependent manner (Tsigos *et al.*, 1997), and, in contrast, to increase whole body glucose clearance in patients with metastatic renal cancer (Stouthard *et al.*, 1995). A third study looking at IL-6 infusion to healthy adults at rest could find no effect of either low or high doses of IL-6 on whole body glucose clearance (Steensberg *et al.*, 2003). Similar contradictions have been observed *in vitro* where chronic treatment and 24 hour exposure to IL-6 induced insulin resistance in 3T3-L1 adipocytes (Lagathu *et al.*, 2003, Rotter *et al.*, 2003) while 30 minute to 25 hour exposure of 3T3-L1 adipocytes to IL-6 in another study showed a consistent increase in glucose uptake (Stouthard *et al.*, 1996).

Resistin is the most recent adipocytokine to be identified. Specifically produced by white adipose tissue in rodents, this secretory molecule has raised levels in both genetic and diet induced models of diabetes and obesity (Steppan and Lazar, 2002).

Studies are suggesting a causative role for resistin in insulin resistance, although its regulation by food intake, receptor type and tissue location and downstream signaling pathways are unknown (Steppan and Lazar, 2002). Factors affecting resistin expression remain controversial (Jazet *et al.,* 2003). Resistin may also be rodent specific as resistin expression by mature human adipocytes is extremely low or absent (Frayn *et al.,* 2003).

These are but a few of the adipocytokines that are currently known and under research. There is clearly much more work to be done in elucidating mechanisms of action, receptors, and signaling pathways for all of these molecules before their role in glucose metabolism, insulin resistance, and progression of diabetes and related diseases can be determined. Figure 2 (below) shows the relationship of these adipocytokines and other factors leading to the development of type 2 diabetes. There is another adipocytokine that deserves closer examination beyond the above mentioned. Commonly referred to as adiponectin, this cytokine secreted solely by adipose tissue is gaining increasing interest due to its apparent strong relationship to diabetes and atherosclerosis.

2.5 Adiponectin: Metabolic and Physiological Effects

Adiponectin, also known as GBP-28, AdipoQ and Acrp 30, is an adipose tissue specific protein that displays evidence of both antidiabetic and antiatherogenic properties. Its expression is highly specific to adipose tissue as it is encoded by apM1 mRNA, the most abundant gene transcript in adipose tissue (Maeda *et al.,* 1996). Adiponectin is a 30-kDa, 244 amino acid protein belonging to the soluble defense collagen superfamily sharing structural homology with collagen X, VIII, complement factor C1q, and TNF-α (Shapiro and Scherer, 1998). Consisting of a signal sequence at the NH_2 end, a non-homologous sequence, a collagen domain, and a globular domain, the structure of adiponectin is very similar between mouse and human homologues differing only by 3 amino acids (Nakano *et al.*, 1996).

2.5.1 Adiponectin and insulin sensitivity

Human plasma concentrations of adiponectin range from 5 to 30 ug/ml, a concentration three times higher than most other hormones (Arita *et al.*, 1999). Plasma concentrations are lower in both obese and type 2 diabetic subjects compared to normal weight individuals (Hotta *et al.,* 2000, Abbasi *et al.*, 2004). A 10% reduction in BMI for

these same obese individuals corresponded with a 42% to 65% increase in plasma adiponectin (Hotta *et al.*, 2000). Despite this, the negative correlation between obesity and adiponectin concentration has been shown to be much more dependent on the degree of insulin resistance rather than BMI with adiponectin concentrations decreasing with increased insulin resistance (Abbasi *et al.*, 2004). Plasma insulin concentration and triglyceride levels are independently negatively correlated with adiponectin concentration while HDL cholesterol levels and age show positive correlations (Abbasi *et al.*, 2004). Low adiponectin levels have also been shown to be strong predictors of type 2 diabetes in Pima indians (Lindsay *et al.*, 2002). All of these studies indicate a strong relationship between adiponectin and insulin resistance but they do not clarify the cause and effect relationship on their own.

The results and observations from the above human trials are very well supported by animal studies in that adiponectin concentrations correlate in the same manner with obesity and insulin resistance (Yamauchi *et al.*, 2001, Hu *et al.*, 1996). Adiponectin knockout mice have shown decreased free fatty acid clearance from plasma and the development of insulin resistance on a high fat, high-sucrose diet (Maeda *et al.*, 2002). Lipotrophic mice, made so by severely reducing PPAR-γ activity over four weeks, showed clear diabetic symptoms as well as a complete absence of plasma adiponectin (Yamauchi *et al.*, 2001). More clearly emphasizing a direct role for adiponectin in regulating insulin sensitivity, insulin sensitivity was completely restored in these mice upon administration of both adiponectin and leptin, but only partially restored by either protein on its own. This potential use of adiponectin as an anti-diabetic supplement is supported further by studies showing that adiponectin injections abolish hyperglycemia and lower free fatty acid and triglyceride levels in *ob/ob*, streptozotocin induced and high fat/high sucrose diet diabetic mice (Berg *et al.*, 2001, Fruebis *et al.*, 2001). These effects are accompanied by weight loss in the high fat/high sucrose fed mice. There have been contrasting results as to whether the active, purified globular domain or the full length adiponectin is better at ameliorating insulin resistance (Berg *et al.*, 2001, Yamauchi *et al.*, 2001). It should be noted that endogenous adiponectin secreted by adipocytes is post-translationally modified into eight different isoforms (Wang *et al.*, 2002).

2.5.3 Regulators of Adiponectin Secretion and mRNA Levels

There are a number of agents involved in the regulation of insulin sensitivity that may mediate their effects, at least in part, by their effect on adiponectin secretion and gene expression. TNFα, one of the most potent hormones at instigating insulin resistance, severely but reversibly reduces adiponectin mRNA in 3T3-L1 adipocytes in a time and dose dependent manner, an effect that requires the activation of nuclear factor-κB (NF-κB) (Fasshauer *et al.,* 2002; Ruan *et al.,* 2002). IL-6 and dexamethasone down-regulate adiponectin mRNA in a similar reversible manner (Fasshauer *et al.*, 2003). All three of these agents also cause a decrease in adiponectin secretion to coincide with the lower mRNA levels. Glucocorticoids will inhibit adiponectin production (Halleux *et al.*, 2001). Catecholamines, specifically isoproterenol, reduce 3T3-L1 adipocyte adiponectin mRNA in a reversible, dose-dependent manner through beta-adrenergic stimulation via protein kinase-A (PKA) (Fasshauer *et al.*, 2001). Interestingly, while insulin has been shown to reduce adiponectin mRNA and gene expression in 3T3-L1 adipocytes (Fasshauer *et al.,* 2002) insulin will stimulate adiponectin secretion from the same cell line and in human visceral adipose tissue (Bogan and Lodish, 1999; Halleux *et al.*, 2001). This increased adiponectin secretion is due to the insulin stimulation of a distinct secretory compartment for adiponectin that is separate from that of GLUT4 but is also dependent on PI3-kinase activity (Bogan and Lodish, 1999). Different from insulin, chronic treatment with growth hormone increases adiponectin mRNA in 3T3-L1 cells without any reliance on PI3-kinase (Xu *et al.*, 2004). This effect is inhibited by the presence of insulin.

Thiazolidinediones (TZD), a class of drugs used to treat insulin resistance, are potent stimulators of adiponectin mRNA and secretion. Along with many other adipocyte genes, adiponectin is regulated through the transcriptional factor PPAR-γ (Rangwala and Lazar, 2004). TZDs are strong and specific activators of PPAR-γ and thus may exert their anti-diabetic effects at least partially through their secondary modulation of adiponectin expression (Ferre, 2004). Pioglitazone, troglitazone and rosiglitazone all significantly raise adiponectin serum concentrations in type 2 diabetics, parallel to improved insulin sensitivity and lipid parameters (Miyazaki *et al.*, 2004, Hiramatsu *et al.*, 2004, Tiikkainen *et al.*, 2004, Maeda *et al.*, 2001). The same results have been obtained when administering TZDs to both lean and obese mice and treatment of 3T3-L1 adipocytes

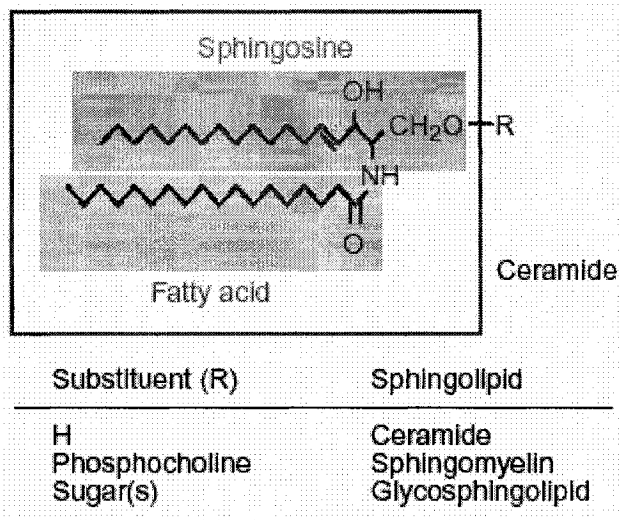
with TZDs mediating increases in adiponectin secretion and mRNA expression in a time and dose-dependent manner (Maeda *et al.*, 2001).

2.5.4 Adiponectin Mechanisms of Action Regulating Insulin Sensitivity

The site and mechanism of action for adiponectin in regulating glucose metabolism have not been clearly identified although adiponectin has shown effects on muscle and liver tissue. As well, the receptors for adiponectin have not yet been identified. Both full-length adiponectin and purified globular domains of the protein reduce plasma, muscle and liver triglyceride content in mice (Fruebis *et al.*, 2001, Yamauchi *et al.*, 2001). These effects appear to be the result of increased fatty acid oxidation in the muscle of the mice, and are associated with weight loss in the obese mice (Fruebis *et al.*, 2001, Yamauchi *et al.*, 2001). Globular fragments of adiponectin enhance insulin-stimulated tyrosine phosphorylation of insulin receptor, IRS-1 and AKT kinase in mouse skeletal muscle and IRS-1 in human skeletal muscle (Yamauchi *et al.*, 2001, Stefan *et al.*, 2002). In isolated rat primary hepatocytes, adiponectin enhances the ability of sub-physiological levels of insulin to suppress glucose-output (Berg *et al.*, 2001). This may be due to phosphorylation of acetyl-CoA and reduction of molecules involved in gluconeogenesis in the liver by adiponectin (Yamauchi *et al.*, 2002).

2.6 The Role of Ceramide in Insulin Resistance

All sphingolipids contain a long-chain sphingoid base (referred to as sphingosine in this form) with an amine group (Fig. 3). When this base unit forms an amide bond with a fatty acid molecule it forms the molecule ceramide. Addition of phosphocholine or carbohydrates to ceramide leads to sphingomyelin or glycosphingolipids, respectively.



(Malagerie-Cazenave *et al.*, 2002)

Fig. 2 General structure of ceramide and its components.

Sphingolipids were once regarded as structural inert components of cell membranes. Sphingomyelin, being the abundant and ubiquitous sphingolipid in mammalian cells, is mostly present in plasma membranes and in lipoproteins (Merrill and Jones, 1990). A discovery in 1986 that the sphingosine base could inhibit PKC- ζ led to further research indicating a variety of stimuli capable of activating sphingomyelinase which cleaves sphingomyelin to release phosphocholine and ceramide (Hannun *et al.*, 1986, Okazaki *et al.*, 1989). This cleavage is now seen as the initial step of an evolutionarily conserved sphingomyelin-ceramide pathway of which ceramide is the second messenger that regulates cellular differentiation, proliferation, and apoptosis by activating a variety of signaling cascades (Andrieu-Abadie *et al.*, 2001).

2.6.1 Ceramide Production and Signaling Cascades

The production of ceramide is stimulated by a number of external factors including TNFα, growth factors, antigens, heat, ionizing radiation, chemotherapeutic drugs, bacteria and viruses (Riboni *et al.*, 1997). These factors increase cellular ceramide concentration by acting on sphingomyelinase to break down the sphingomyelin molecule. Sphingomyelinase exists in several forms. The neutral magnesium-dependent sphingomyelinase located in the plasma membrane is most often activated by external stimuli but acidic or neutral magnesium-independent sphingomyelinases located in the

nucleus, cytoplasm and endosome/lysosome of the cell may also be (Andrieu-Abadie *et al.*, 2001). Ceramide can also be produced by *de novo* synthesis beginning with the condensation of serine and palmitoyl-CoA utilizing the enzyme serine-palmitoyltransferase (Stoffel, 1970). The key intermediate in this synthesis pathway is sphinganine which is converted to dihydroceramide by the acyl-CoA: sphingoid base N-acyltransferase that uses, as the acyl donor, fatty acyl-CoAs (Rother *et al.*, 1992). The dihydroceramide is then unsaturated to form ceramide.

Although ceramide can be converted back and forth between sphingosine and ceramide-1-phosphate, the most abundant form is ceramide and has been the focus of research in terms of signaling pathways. Ceramide has a number of direct targets including ceramide-activated protein phosphatase (CAPP), a ceramide-activated protein kinase (CAPK), and PKC-α and ζ (Riboni *et al.*, 1997). These in turn activate cascades such as the c-Jun N-terminal directed protein kinase (JNK)/stress-activated protein kinase (SAPK) cascade, mitogen activated protein kinase (MAPK) cascade and activate transcription factors such as NF-κB and activator protein-1 (AP1) (Riboni *et al.*, 1997). The activation of these pathways and transcription factors in turn regulate the expression of genes such as c-myc, c-jun, c-fos, Bcl-2 and Cox (Riboni *et al.*, 1997). It is the preferential activation of specific pathways that leads to varying expression of these genes that in turn modulate cell differentiation, proliferation, apoptosis, immune response and cytokine expression.

In addition to these better known effects of ceramide, there is mounting evidence that ceramide may play a role in inducing insulin resistance.

2.6.2 Evidence for the Potential Role of Ceramide in Insulin Resistance

Skeletal muscle ceramide content can be increased as much as two-fold in obese insulin resistant subjects compared to lean insulin-sensitive controls (Adams *et al.*, 2004). In these subjects, no other sphingolipid experienced the same increase. Muscle biopsies taken from the obese subjects revealed a concurrent decrease in the stimulation of AKT phosphorylation by insulin. These observations support a number of other studies indicating that ceramide may play a causative role in insulin resistance by directly affecting the insulin signaling pathway.

In both primary muscle cells biopsied from obese insulin resistant humans and cultured rat L6 myotubes and C2C12 myoblasts, increased ceramide levels are strongly correlated with inhibition of the PKB/Akt protein activity in the insulin signaling pathway (Adams *et al.*, 2004; Chavez *et al.*, 2003; Powell *et al.*, 2004). In the L6 myotubes, there were no changes in IRS-1 phosphorylation or association with the p85 subunit of PI3-kinase although there was a 5-fold increase in activation of the PKCζ (Powell *et al.*, 2004). Similarly, in the C2C12 cells, there was no change in IRS-1 associated PI3-kinase (Chavez *et al.*, 2003). In 3T3-L1 adipocytes it was first discovered that treatment of these cells with C6 ceramide results in significant decreases in GLUT4 gene expression (Long and Pekala, 1996). This specific PKB/Akt inhibition by ceramide is supported by the results of treating 3T3-L1 adipocytes with C_2 and C_6 ceramides showing a decrease in insulin-stimulated glucose uptake and decreased PKB/Akt phosphorylation and activity (Summers *et al.*, 1998, Mei *et al.*, 2003). In these cells C_2 ceramide has been shown to have no effect on any proteins upstream of PKB/Akt or MAPK in response to insulin (Summers *et al.*, 1998, Mei *et al.*, 2003) but with 12 hour C_2 ceramide treatment in the absence of insulin, basal glucose uptake was increased along with GLUT1 translocation to the membrane, IRS-1 associated PI3-kinase, MAPK and PKB/Akt activity (Mei *et al.*, 2003). However, while insulin stimulated PKB/Akt activity in the control cells, the 12 hour C_2 ceramide treatment negated any insulin-stimulated glucose uptake and PKB/Akt activity (Mei *et al.*, 2003).

Another novel target by which ceramide may affect insulin resistance is cyclic nucleotide phosphodiesterase (PDE) 3B which plays an important role in the antilipolitic action of insulin (Eriksson *et al.*, 1995). Treatment of 3T3-L1 adipocytes with C_2 ceramide resulted in a time-dependent decrease in PDE3B acitivity and expression along with an increase in lipolysis (Mei *et al.*, 2002). The ceramide treatment also inhibited the antilipolytic action of insulin.

These effects of ceramide support the strong correlation between ectopic fat accumulation and insulin resistance. As stated earlier, increased levels of ectopic fat leads to an increased rate of fatty acid turnover producing fatty acyl-CoA, DAG and ceramide as by-products (Defronzo, 1997). In obese insulin resistant subjects, muscle ceramide content was positively correlated with plasma free fatty acid concentration (Adams *et al.*,

2004). In C2C12 myotubes, treatment with saturated fatty acids increased concentrations of both ceramide and DAG (Chavez *et al.*, 2003a, 2003b). DAG instigates insulin resistance by inhibiting IRS-1 phosphorylation (Stratford *et al.*, 2001, Kellerer *et al.*, 1998). Due to the fact that their were no changes upstream of Akt/PKB in these C2C12 myotubes and that inhibition of *de novo* ceramide production prevented the insulin resistance caused by these fatty acids, it was determined that in this case ceramide, and not DAG, was responsible for causing the insulin resistance. In 3T3-L1 adipocytes this same treatment had no effect on PKB/Akt activity, ceramide or DAG concentrations (Chavez *et al.*, 2003b). This lack of effect may be attributable to the 3T3-L1 adipocytes ability to convert fatty acids into triacylglycerols rather than ceramide. Intralipid/heparin infusion to male subjects with normal glucose tolerance caused an increase in muscle total ceramide content that was negatively correlated with insulin sensitivity (Straczkowski *et al.*, 2004). It should be noted that plasma insulin concentrations may play a role in regulating cellular ceramide concentrations as total ceramide levels in the soleus, and red and white sections of the gastrocnemius of rats was significantly increased in STZ treated (insulin deficient) rats over control rats (Gorska *et al.*, 2004). In contrast to the findings of the above mentioned studies, fatty acid infusion in both normal glucose tolerant rats and humans has demonstrated no effect on ceramide concentration but an increase in insulin resistance due to raised DAG levels (Yu *et al.*, 2002, Itani *et al.*, 2002). This difference may be attributable to differences in fatty acid chain saturation as mono-unsaturated fatty acids do not show the effects of saturated fatty acids (Chavez *et al.*, 2003a).

2.7 Alternative Drug Therapies to Treat Type 2 Diabetes

The effects of physical activity and diet intervention, either direct or through weight loss and altered fat distribution, are beneficial as both treatment and preventative measures for type 2 diabetes, insulin resistance and detrimental serum lipid profiles but the maintenance of these lifestyle changes remains difficult for most patients (Neuhouser *et al.*, 2002). Many diabetics require pharmacological treatment as well, and the use of oral antihyperglycemic agents is recommended when lifestyle changes fail to result in the

achievement of target glucose levels, or if symptoms or severe hyperglycemia exist (Meltzer *et al.,* 1998).

Therapeutic oral compounds currently available include sulfonylureas, biguanides, disaccharidase inhibitors, and thiazolidinediones (Luo *et al.,* 1999). Insulin injection has limited treatment potential in the type 2 diabetes since it does not correct the fundamental problem of insulin resistance. Sulfonylureas stimulate pancreatic insulin secretion and in combination with insulin injections, sulfonyureas have shown slight improvements in glycemic control but pose a major risk for acute severe hypoglycemia (Marcus, 2000). Other side effects include weight gain, dermatological reactions, hepatitis, and hematologic effects (Marles and Farnsworth, 1995). Metformin is a plant derived biguanide that is capable of decreasing hepatic glucose production and increasing peripheral glucose uptake through its effect on the insulin sensitive transporter linked system (Marles and Farnsworth, 1995). Although not as effective as lifestyle intervention, metformin has shown potential to prevent the development of type 2 diabetes (Molitch *et al.,* 2003). The major drawback to metformin is its potential, albeit low, to cause lactic acidosis, especially in patients with a concomitant medical disorder (Marcus, 2000). Acarbose, a dissacharidase inhibitor, slows starch and sucrose absorption in the gut but causes flatulence, abdominal pain and diarrhea in the majority of the patients (Marcus, 2000, Metzler *et al.,* 1998).

Thiazolidinediones (TZDs) improve insulin resistance by acting as a ligand to the transcription factor PPAR-γ that consequently lowers serum NEFA levels by increasing adipose tissue uptake of free fatty acids (Henry, 2003, Kopecky, 2002). Due to their interaction with PPAR-γ, TZDs can also affect adipocytokine secretion (especially adiponectin) and the activity of enzymes and expression of proteins involved in the insulin signaling pathway in adipocytes (Rangwala and Lazar, 2004; Tiikkainen *et al.,* 2004). Unfortunately, due to these effects of TZDs, their use leads to weight gain and edema (Rangwala and Lazar, 2004). As well, obese individuals respond better to TZDs than do lean diabetics (Chilcott *et al.,* 2001). Other drugs, such as statins, fibrates, and niacin are being used alone or in combination to improve specific aspects of dyslipidemia in diabetics (Cottrell *et al.,* 2003). The specific and/or limited effects of these pharmacological treatments combined with their dangerous side effects, high price and

poor availability to rural populations limits their usefulness in combating the growing prevalence of diabetes in developing countries and has fueled the search for alternative medicines.

2.8 Current Research on Potential Anti-Diabetic Plants Using 3T3-L1 Adipose Cells

There are at least 25 single herbs and 9 vitamin/mineral supplements that are not currently in use but have undergone clinical trials to ascertain their effectiveness as a treatment for diabetes (Yeh *et al.,* 2003). Before being used in clinical trials, potential medicines undergo extensive *in vitro* and animal testing. In efforts to discover a potentially useful anti-diabetic drug from plant and food sources, hundreds of plants and extracts are being screened for potential activity. There are a large number of ways to examine potential antidiabetic activity both *in vitro* and *in vivo*, as demonstrated by variety of research performed on MC. However adipose tissue is beginning to be used more often for *in vitro* research as a screening procedure and for determining mechanism of action.

Although rat adipose tissue can be isolated and used *in vitro* (Meir and Yaniv, 1984), the 3T3-L1 adipocyte cell line has been used extensively as a model of *in vivo* adipose tissue cell function. 3T3-L1 cells are mouse fibroblasts that can be differentiated to become functional adipocytes (Frost and Lane, 1985). They can be studied in cell monolayers and, in contrast to isolated adipocytes, 3T3-L1 cells maintain cell viability and hormonal responsiveness for extended periods of time (Frost and Lane, 1985). They are one of the most sensitive tissue culture systems to elucidate insulin action as they respond to insulin with increased glucose uptake, and incorporation of glucose into lipid and glycogen (Wiese *et al.,* 1995). 3T3-L1 has an insulin-receptor pathway that appears to mimic the human adipocyte pathway and they release the same adipocytokines (adiponectin, TNFα, resistin, IL-6, leptin) as human adipocytes (Bierer *et al.,* 1998, Steppan and Lazar, 2002).

A number of studies have been done specifically looking at the glucose uptake and anti-diabetic potential of plant extracts using the 3T3-L1 cell line. Using radio-labeled 2-deoxyglucose, which is taken up by the insulin sensitive GLUT4 pathway in the

adipocytes, an extract from *Cryptolepis sanguinolenta*, that exhibited hypoglycemic action in STZ-treated diabetic model rats, was shown to enhance glucose uptake in 3T3-L1 cells (Beirer *et al.,* 1998). In similar glucose uptake tests, *in vivo* (mouse model) hypoglycemic extracts from *Lagerstroemia speciosa* L. and *Pycnanthus angolensis* were tested with 3T3-L1 cells and varied insulin levels to determine if the extracts acted on a separate glucose uptake pathway or if they acted by enhancing insulin sensitivity (Luo *et al.,*1999, Liu *et al.,* 2001). The 3T3-L1 model delineated separate mechanisms of action these two plant extracts in that the *L. speciosa* enhanced glucose uptake on its own but depressed the action of insulin, while *P. angolensis* had no effect on its own but potentiated the action of insulin on glucose uptake. GLUT4 expression in the cells was also measured to show a potential mechanism of action for the *L. speciosa* extract (Liu *et al.,* 2001). Another similarly conducted study on the glucose uptake stimulation by *Toona sinensis* leaf extract used calphostin C, an inhibitor of protein kinase C in the insulin-receptor/GLUT4 translocation pathway, to show that the extract enhanced glucose uptake through a pathway unique from that of insulin (Yang *et al.,* 2003). These studies and others are showing that 3T3-L1 adipose cells provide an excellent experimental model to quickly screen the effects of crude drugs on glucose uptake and at the same time allow measurements that can help determine mechanisms of action (Hong *et al.,* 2000).

2.9 The Role of *Momordica charantia* as an Alternative Therapy for Type 2 Diabetes

Momordica charantia (MC), also known as bitter melon, bitter gourd, karela, balsam pear and cundeamor, is the most widely used traditional medicine for treatment of diabetes. A member of the *Cucurbitaceae* family, MC grows well in warm climates and is commonly eaten, primarily in its unripe stage, as a vegetable in India, South America, Asia and Africa (Virdi *et al.,* 2003). Although used widely traditionally, MC has not been recommended for routine use due to weaknesses in the scientific data to prove efficacy and safety (Basch *et al.,* 2003).

The ability of MC to normalize blood glucose levels over a long period in insulin-resistant and mild to moderate hyperglycemic states has been shown in animal models (Virdi *et al.,* 2003, Rathi *et al.,* 2002, Sitasawad *et al.,* 2000) and has also demonstrated

its hypoglycemic ability in a few (although small and not well controlled) clinical trials with type 2 diabetic patients (Khanna *et al.,* 1981, Welihinda *et al.,* 1986, Leatherdale *et al.*, 1981).

Several potentially anti-diabetic active compounds have been isolated from MC although differing results based on extraction techniques indicate that these active compounds may act synergistically and/or in parallel to each other. In 1981, an 11 kDa polypeptide (dubbed polypeptide-p) was isolated from the fruit, seeds and pulp of MC and found to have a blood-sugar-lowering effect over a 12 hour period when injected into gerbils, langurs, and juvenile and maturity onset diabetic patients (Khanna *et al.,* 1981). Some of this effect may have been attributable to the zinc that became attached to the polypeptide during extraction, since zinc has been shown to have potential insulin-like effects *in vitro* (Ng *et al.,* 1986).

A saponin constituent (dubbed charantin) has been isolated as a mix of two steroid glycosides: β-sitosterol-D-glucoside and 5,25-stigmastadien-3-β-ol-D-glucoside (Lotlikar and Rajarama Rao, 1966). This constituent has shown hypoglycemic activity when administered to normal rabbits, rats, and cats (Lotlikar and Rajarama Rao, 1966). On the other hand, a saponin-free extract from MC juice has been shown to have a more potent hypoglycemic effect in type 2 diabetic model rats than did the saponin-containing fraction (Ali *et al.,* 1993). The majority of studies have used simple aqueous or alcohol extracts or pure fruit juice in order to determine the anti-diabetic effects and mechanisms of MC. With few exceptions (Singh *et al.*, 2004a), the majority of these studies show a better, long term hypoglycemic activity of MC aqueous extracts over ethanol or chloroform extracts (Ali *et al.*, 1993; Virdi *et al.*, 2003; Vikrant *et al.*, 2001; Rathi *et al.*, 2002).

Despite this research, the mechanism of action by which MC mediates glucose homeostasis has not been defined. In streptozotocin-induced diabetic rats, MC has been shown to have both a protective effect against apoptosis and oxidation in insulin secreting pancreatic β cells (Sitasawad *et al.,* 2000; Singh *et al.*, 2004b) and a restorative effect by markedly increasing the number of viable β cells (Ahmed *et al.,* 1998). These effects, however, are limited as explanations for the mechanisms for the hypoglycemic effect of MC since it has been shown that MC requires the presence of already viable β cells in

order to exert its hypoglycemic activity and may simply enhance insulin secretion by the pancreas (Karunanayake *et al.,* 1990; Ali *et al.,* 1993).

Other studies with diabetic rat models have shown that hypoglycemic activity of MC occurs without increased secretion of insulin and may be more a result of increased glucose utilization and storage by the liver (Sarkar *et al.,* 1996; Welihinda and Karunanayake, 1986). This observation is supported by results showing that an aqueous extract of MC partially restored liver the concentrations of key hepatic enzymes (hexokinase, phosphofructokinase and the substrate, glucose-6-phosphate) involved in glycogenesis (Rathi *et al.,* 2002). Along with the increased glycogen content in the liver, these studies found that glycogen content was also increased in the rat skeletal muscle indicating increased muscle tissue glucose uptake (Rathi *et al.,* 2002; Welihinda and Karunanayake, 1986). In KK-Ay mice, a species genetically bred to develop type 2 diabetes, oral administration of a water extract of MC fruit has been shown to cause an increase in glucose transporter isoform 4 (GLUT4) protein translocation to the plasma membrane in their skeletal muscle cells, coinciding with a decrease in blood glucose levels (Miura *et al.*, 2001). This same diabetic species of mice was used to show that a similar water extract of MC fruit was able to act synergistically with exercise to lower blood glucose to a greater degree than either treatment on its own (Miura *et al.*, 2004). An *in vitro* study using cultured L6 rat muscle cells showed that both water and chloroform extracts of MC were able to increase glucose uptake into these cells but only after 6 hours of simultaneous glucose and extract exposure to the cells (Singh *et al.*, 2004a). This increase in uptake occurred with extracts at a concentration of 5 ug/mL, while higher concentrations of the extract did not show this same increase and actually inhibited insulin-stimulated glucose uptake (Singh *et al.*, 2004a; Singh *et al.*, 2004b) . This MC-induced uptake was completely inhibited by simultaneous treatment with 100nM wortmannin.

Beyond just its hypoglycemic effect, MC was shown to increase serum high-density lipoprotein (HDL) concentrations and decrease hepatic cholesterol and triglyceride content when the powdered fruit was incorporated into the diet of rats fed a either a cholesterol-free or cholesterol-enriched diet (Jayasooriya *et al.*, 2000). Changes to lipid parameters were also seen when a methanol extract of the fruit was fed to diabetic rats,

resulting in significant decreases in serum triglyceride, low density lipoprotein (LDL) and a significant increase in HDL (Chaturvedi *et al.*, 2004). A water extract of MC fruit was able to prevent the diabetes-induced development of cataracts in rats fed the MC extract over a period of 4 months (Rathi *et al.*, 2002). Not only has MC extract displayed no evidence of nephrotoxicity and hepatoxicity in rats (Virdi *et al.*, 2003), a water extract of MC fruit has been shown to significantly prevent renal hypertrophy of the kidneys, polyuria (increased urine volume), and decrease urinary albumin levels compared to diabetic controls during a 40 day feeding trial on rats (Grover *et al.*, 2001). In a recent study, the high systolic blood pressure, increased plasma insulin levels, increased glucose uptake into the brush border vesicles of the jejunum, and decreased myelinated fibre area seen in STZ-induced diabetic rats were returned to the normal levels of the healthy controls after 10 weeks of MC fruit juice oral administration (Singh *et al.*, 2004b). The anti-diabetic effects of MC in this paragraph were all accompanied by improvements in blood glucose levels and/or glucose tolerance.

Although there has been significant work to show a hypoglycemic and anti-diabetic effect of MC in animals, the work has focused on effects on the pancreas, liver and skeletal muscle without any establishment of mechanistic pathway to show a direct effect of the MC on these tissues. There has been extremely little research looking at the effect of MC on adipose tissue functions, especially in the context of adipose tissue as a possible central regulator of insulin resistance and peripheral tissue glucose uptake. Given that many dietary ingredients that affect glucose metabolism may also influence lipid metabolism, there have only been a few studies testing the effects of MC in this regard. MC was found to return serum lipid parameters to near normal levels when MC fruit juice was fed daily to STZ-induced diabetic rats with increased plasma non-esterified cholesterol, triglyceride, and phospholipid levels and reduced serum HDL-cholesterol (Ahmed *et al.,* 2001). In normal rats fed cholesterol-free or enriched diets, however, MC whole fruit powder supplementation into the diet had no effect on these same lipid parameters with the exception of consistently increasing HDL-cholesterol levels (Jayasooriya *et al.,* 2000).

Concentrating more directly on adipose tissue lipid metabolism, Ng *et al.* (1986) studied the ^{3}H-glucose uptake, lipogenic and lipolytic activities of isolated rat and

hamster adipose tissue as affected by MC fractions obtained using the same extraction techniques (minus the use of zinc) performed by Khanna *et al.* (1981) to isolate polypeptide-p from both MC fruit and seeds. They found that the fruit "p-fraction" stimulated glucose uptake into the fat cells but inhibited lipolysis only in the hamster adipocytes. The seed "p-fraction" inhibited lipolysis and glucose uptake in both animal adipocytes. Further fractionation of the seed "p-fraction" by chromatography isolated two separate fractions, F1 and F2. F1, a saponin containing fraction, continued to inhibit lipolysis and glucose uptake while the F2 fraction stimulated glucose uptake. The anti-lipolytic activity of F2 was not tested due to lack of material. They suggested that the effects of the F1 fraction could be due to either the heat stable saponin or protein components it contained. The effects of the F2 fraction were suggested to be due to proteins smaller in size than the 11000 kDa component suggest by Khanna *et al.* (1981).

Only one other study has tested the ability of water and ethanol MC extracts to mediate glucose uptake in adipose tissue (Meir and Yaniv, 1984). The tests were performed on isolated rat epididymal adipose tissue, but the concentrations of MC extracts used were not stated and the oil flotation method they used for the glucose uptake assay showed a positive response to insulin only 65% of the time. Their results (data not provided in the paper) did not reveal an effect of MC on adipose tissue glucose uptake.

Still, the above studies have not acknowledged the possible insulin-resistance mediator role of adipose tissue through its endocrine functions in their study design, objectives or discussion. A more recent study has revealed a possible role for MC in the attenuation of weight gain and lowering of insulin resistance in normal rats fed a high fat diet (Chen *et al.,* 2003). They acknowledged the role of signaling molecules in mediating insulin resistance by including measurements of TNFα and leptin levels in the blood of the rats but their results did not show any effect of MC on these serum concentrations (Chen *et al.,* 2003).

In attempting to determine the effectiveness, the potential active components, and the mechanism of action for MC in alleviating hyperglycemia, there is a clear need for further research into the effects of MC on adipose tissue.

2.10 The Role of Cinnamon as an Alternative Therapy for Type 2 Diabetes

Cinnamon (*Cinnamomum sp.)* is the dried inner bark of various laurel trees in the cinnamomum familiy that is native to Skri Lanka and India but is cultivated extensively in the tropical regions of the world (Chevallier, 2000). Although cinnamon has been used medicinally for thousands of years, its potential use as an antidiabetic has not been explored until recently. Originally testing whole foods for their ability to potentiate the action of insulin in a rat epididymal fat cell assay based upon their chromium content, it was discovered that apple pie was able to increase glucose uptake. This effect was narrowed down to the cinnamon in the apple pie which potentiated insulin action up to four fold despite its low chromium content (Khan *et al.,* 1990). The capability of cinnamon to increase glucose uptake was confirmed in later studies using the rat epididymal fat pad assay and ammonium hydroxide extracts of cinnamon. These studies showed 5 to 32 fold increases in glucose uptake without the presence of insulin, an increase that was affected adversely by the bovine serum albumin concentration in the medium (Broadhurst *et al.*, 2000, Berrio *et al.*, 1992).

In trying to determine the mechanism of action for this effect by cinnamon, an ammonium hydroxide cinnamon fraction further purified using column chromatography was tested with this same assay in the presence of wortmannin. Wortmannin dose dependently inhibited glucose uptake normally induced by cinnamon alone (Imparl-Radosevich *et al.*, 1998). The authors concluded that cinnamon must act upstream of PI3-kinase and showed that this cinnamon extract also increased insulin receptor kinase autophosphorylation and decreased protein tyrosine phosphate (PTP)–1 activity, an enzyme that inhibits insulin signaling by dephosphorylating the insulin receptor at the cell surface (Imparl-Radosevich *et al.,* 1998).

To further determine the active components of cinnamon, the rat adipose cell assay was used with different species and purified extracts of cinnamon. They found no significant differences in activity between extracts from between 7 different species of cinnamon. Using HPLC and mass spectrometry, they further purified the cinnamon fraction and found that water-soluble polymeric compounds in the cinnamon had the ability to potentiate insulin action and to act as antioxidants as shown by their ability to inhibit the production of reactive oxygen species in platelets (Anderson *et al.*, 2004). It is

interesting to note that before HPLC separation the unpurified cinnamon fraction showed the greatest increase in glucose uptake on its own and that the presence of insulin with the cinnamon actually decreased this effect. Also, low concentrations of cinnamon actually decreased the effects of insulin on glucose uptake (Anderson *et al.,* 2004). A methylhydroxychalcone polymer (MHCP) isolated from the cinnamon fraction was further tested using 3T3-L1 adipocytes. They found that while MHCP only slightly increased glucose uptake and glycogen synthesis on its own, it potentiated the activity of insulin by two fold for glucose uptake and four fold for glycogen synthesis (Jarvill-Taylor *et al.*, 2001). This effect was inhibited by wortmannin but only when wortmannin was present during glucose uptake. When the wortmannin was only used in the pretreatment and removed during glucose uptake, the effect of MHCP alone was increased and the glucose uptake from the insulin and MHCP combined treatment was only partially inhibited (Jarvill-Taylor *et al.*, 2001).

The results of these *in vitro* assays have been confirmed *in vivo*. Rats fed a hot water extract of cinnamon daily for three weeks showed an almost 150% increase in glucose infusion rate (GIR) during euglycemic clamp testing, indicating a higher rate of glucose disposal in the body (Qin *et al.,* 2003). At the same time, IRS-β and IRS-1 tyrosine phosphorylation levels were increased and IRS-1/PI3-kinase association was increased in the skeletal muscle of the cinnamon fed rats (Qin *et al.*, 2003). Similarly, the hot water cinnamon extract had these same effects in rats fed a high-fructose diet that induced insulin resistance in the controls (Qin *et al.*, 2004). The most compelling evidence for the role of cinnamon in treating diabetes comes from a randomized placebo controlled clinical trial. Sixty type 2 diabetics (30 men and 30 women) were randomly assigned into groups and given 1, 3, or 6 g of cinnamon a day in the form of capsules for a period of 40 days. The placebo groups received equivalent amounts of wheat flour. After 40 days, all three levels of cinnamon led to similar decreases in fasting serum glucose from 18 to 29% (Khan *et al.*, 2003). Twenty days after cinnamon consumption ended, this effect only remained significant for the lowest level of cinnamon group. There were no significant changes in fasting serum glucose for the placebo groups. After 40 days, all three cinnamon groups also experienced reductions in total serum triglyceride levels (23 to 30%), serum cholesterol (13 to 26%) and LDL (10 to 24%) (Khan *et al.*,

2003). These changes remained significant 20 days post-cinnamon consumption. Again, there were no changes in any of these parameters for the placebo groups.

Although cinnamon has been well researched to show positive effects on glucose uptake via *in vitro* and *in vivo* experiments, and has demonstrated the ability to improve blood lipid parameters in humans, the mechanism of action by which cinnamon functions to lower insulin resistance and cinnamons effects on other aspects and complications of diabetes has not been well defined. Research should continue into the effects of cinnamon on intercellular and intracellular signaling molecules that are directly involved in mediating insulin resistance in order to determine a more complete picture of the mechanisms and outcomes of cinnamon intake by the diabetic human.

III. STUDY RATIONALE

The fruit juice and various extracts of MC have demonstrated hypoglycemic effects and improvements to lipid metabolism in both *in vivo* and *in vitro* situations. The theories on the mechanisms of action of MC and its bioactive components, however, remain varied and undefined.

Although the 3T3-L1 adipose tissue cell line has been established as an excellent model of insulin-sensitive glucose uptake (Wiese *et al,* 1995), this model has never been used as a testing medium for MC. MC extracts have been shown to improve glucose metabolism in both isolated and *in vivo* skeletal muscle, pancreas, and liver but without linking these effects to specific insulin dependent or independent pathways (Sarkar *et al.*, 1996, Rathi *et al.,* 2002, Meir and Yaniv, 1984). The 3T3-L1 cell line has been used with other fruit extracts and varying concentrations of insulin to demonstrate potential mechanisms for the enhancement of glucose uptake of such extracts (Luo *et al.,* 1999, Liu *et al.,* 2001). The two previous studies examining the effects of MC specifically on adipose tissue used different extraction techniques and found contrasting results for glucose uptake (Ng *et al.,* 1986, Meir and Yaniv, 1984). For the present thesis study, the 3T3-L1 cell line was used to determine if MC extracts, in combination with varying concentrations of insulin, can increase glucose uptake in an insulin sensitive adipose cell model and, if so, whether this action could be mediated by enhancing the action of insulin or by acting independently of insulin. As the active components of MC have not been fully determined, the use of both aqueous and ethanol extracts of fresh, unripe MC fruit and seeds will allow observations to be made on the polarity of the potential active components.

The activation of PI3-kinase has been shown to be a necessary step in the insulin-stimulated translocation of GLUT4 and subsequent glucose uptake in adipose cells. The fungal metabolite, wortmannin, has been shown to inhibit insulin-stimulated glucose uptake in both rat adipose tissue and 3T3-L1 cells in a dose-dependent manner by targeting the p110 isoform of PI3-kinase and another, as yet unidentified, target in the insulin pathway that is involved in the activation of cell surface GLUT4 (Okada *et al.,* 1994, Hausdorff *et al.,* 1999). Testing glucose uptake by the 3T3-L1 cells, as affected by

MC extracts, in the presence of wortmannin will demonstrate whether any MC stimulated glucose uptake uses the same GLUT4 translocation and activation pathway as insulin in adipose tissue. Although wortmannin has been shown to inhibit MC induced 2-deoxy-glucose uptake in cultured muscle cells, this effect has not been tested with combination treatments of MC and insulin nor in an adipose tissue cell line.

Cinnamon has been well shown to improve blood lipid profiles and glucose metabolism in both rats and type 2 diabetic humans (Qin *et al.*, 2003, Qin *et al.*, 2004, Khan *et al.*, 2003). These *in vivo* results are supported by a studies showing increased glucose uptake in isolated rat fat cells in the presence of cinnamon water extracts and the ability of the cinnamon isolate, MHCP, to enhance the action of insulin and act as an insulin mimetic when tested on 3T3-L1 adipose cells (Anderson *et al.,* 2004, Jarvill-Taylor *et al.*, 2001). Wortmannin has been shown to inhibit any increased glucose uptake caused by cinnamon (Imparl-Radosevich *et al.*, 1998, Jarvill-Taylor *et al.*, 2001). Testing for similiarities between the cinnamon water extract and the MC extracts will help identify the anti-diabetic potential of MC, as cinnamon has already shown positive results on glucose uptake both *in vitro* and in a human clinical trial.

The central role of adipose tissue in mediating peripheral insulin resistance, potentially through its function as an endocrine organ, is becoming well recognized (Mora and Pessin, 2002, Jazet *et al.,* 2003). Adiponectin is a cytokine secreted solely by adipose tissue and has been conclusively shown to be beneficial in ameliorating the effects of insulin resistance (Berg *et al.,* 2001, Steppan and Lazar, 2002). Only one study, using an *in vivo* rat model, has examined the effect of MC extracts on serum cytokine concentrations (TNFα and leptin) and the results did not demonstrate any effect of MC in this regard (Chen *et al.,* 2003). The effects of cinnamon on cytokine secretion have never been tested. Adiponectin is secreted by 3T3-L1 cells (Fasshauer *et al.,* 2003). Testing the effects of MC and cinnamon extracts on the 3T3-L1 secretion of this adipocytokine may show a potential, as yet unexplored, mechanism by which MC and cinnamon could affect indirectly insulin resistance.

Concentrations of the sphingolipid ceramide have been shown to positively correlate with insulin resistance and obesity in humans (Adams *et al.,* 2004). *In vitro* studies have shown ceramide to directly affect the insulin signaling pathway by inhibiting

the phosphorylation and activity of the PKB/Akt proteins (Summers *et al.*, 1998, Mei *et al.*, 2003). Neither cinnamon nor MC have been tested for their effects on cellular ceramide accumulation. Determining these effects may reveal another more direct mechanism by which MC and cinnamon could affect insulin resistance.

IV. MANUSCRIPT I

A Water Extract from *Momordica charantia* Fruit and Seeds Increases Glucose Uptake and Adiponectin Secretion in 3T3-L1 Adipose Cells in the Presence of a Sub-optimal Concentration of Insulin

Ben Roffey[1], Avtar Atwal[2], Tim Johns[1], Stan Kubow[1]

[1]School of Dietetics and Human Nutrition, McGill University, Macdonald Campus, 21,111 Lakeshore, Ste-Anne-de-Bellevue, QC, Canada H9X 3V9

[2]Bio-Ingredients Section, Food Research and Development Centre, Agriculture and Agri-Food Canada, Saint-Hyacinthe, Quebec, Canada J2S 8E3

4.1 Abtract

To examine the effects of *Momordica charantia* (MC) on glucose uptake, adiponectin secretion and accumulation of ceramide in fat cells, 3T3-L1 adipocytes were treated with three concentrations of water and ethanol extracts of MC fruit and seeds. Treatments consisted of the extracts alone and the extracts in combination with two concentrations of insulin. The treatment combination of 0.2 mg/ml MC water extract and 0.5 nM insulin was associated with an increased glucose uptake into the cells (61%) and increased adiponectin secretion from the cells (75%). The MC ethanol extract was not associated with an increase in glucose uptake; however, a dose-dependent decrease in basal glucose uptake and insulin-mediated glucose uptake was observed with the MC ethanol extract in combination with 50 nM insulin. The present results indicate that preferentially water-soluble component(s) in MC fruit and/or seed enhance the glucose uptake action of sub-optimal concentrations of insulin in 3T3-L1 adipocytes. This effect is accompanied by and may be a result of increased adiponectin secretion from the cells.

4.2 Introduction

In the year 2002, 151 million people worldwide were diagnosed with diabetes and this number is expected to rise to 221 million people by the year 2010 (Zimmet, 2002). More than 90% of these cases are type 2 diabetics suffering from severe insulin resistance. Research has begun to focus on adipose tissue as a possible central mediator of whole body insulin resistance. Evidence for this central role comes not only from the link between obesity and type 2 diabetes (Williams, 1999), but also from the role of adipose tissue in regulating serum lipid concentrations and, more recently, from the emerging role of adipose tissue as an endocrine organ (Mohamed-Ali *et al.,* 1998).

The specific and/or limited effects of current drug treatments for diabetes, combined with the dangerous side effects that most of them induce, has fueled the search for alternative medicines. *Momordica charantia* (MC; bitter melon, bitter gourd, karela), a plant native to the semi-tropical climate of China, India, Asia, and Africa, bears a fruit that is currently the most widely used traditional medicine to treat diabetes and is one of the most promising alternative medicines for the disease. A few active components, polypeptide-p (Khanna *et al.,* 1981) and charatin (a mix of two steroid glycosides) (Lotlikar and Rajarama Rao, 1966), have been identified from the fruit and its seeds. These components, along with pure juice, methanol and ethanol extracts from the fruit, have shown hypoglycemic activity via *in vitro*, animal and human studies (Virdi *et al,* 2003, Rathi *et al,* 2002, Sitasawad *et al.,* 2000, Khanna *et al.,* 1981, Welihinda *et al.,* 1986). Although MC seems to improve the function of the pancreas (Sitasawad *et al.,* 2000; Ahmed *et al.,* 1998), liver (Rathi *et al.,* 2002), and skeletal muscle (Rathi *et al.,* 2002; Welihinda and Karunanayake, 1986) towards ameliorating insulin resistance, its mechanism has not been well defined and very little attention has been paid to the effects of MC on adipose tissue function and adipocytokine secretion.

Although it has been shown that MC extracts are able to restore blood and hepatic lipid parameters to normal levels in diabetic rats (Jayasooriya *et al.,* 2000; Chaturvedi *et al.,* 2004), only two studies have tested MC extracts on isolated adipose cells. Both studies used isolated rat and hamster cells and found varying results for the effects of MC extracts on glucose and lipid metabolism in these cells (Ng *et al.,* 1987; Meir and Yaniv, 1982). Still, these studies have not explored the role that cytokine secretion from adipose

tissue may have in mediating whole-body insulin resistance. A more recent study has revealed a possible role for MC in the attenuation of weight gain and lowering of insulin resistance in normal rats fed a high fat diet (Chen *et al.,* 2003). They acknowledged the possible role of cytokines in mediating insulin resistance by including measurements of TNFα and leptin levels in the blood of the rats but their results did not show any effect of MC on these cytokine serum concentrations (Chen *et al.,* 2003).

Adiponectin is a cytokine secreted solely by adipose tissue and has been shown to have both antidiabetic and antiatherogenic properties (Iwaki *et al.,* 2003). Plasma concentrations of adiponectin are lower in type 2 diabetics and obese individuals and have a strong negative correlation with insulin resistance (Hotta *et al.,* 2000; Abbasi *et al.,* 2004). In both obese and lipoatrophic mice, hypoadiponectinemia leads to insulin resistance while adiponectin supplementation alleviated the condition (Yamauchi *et al.,* 2001). Specifically, adiponectin activated insulin receptor substrate-1 (IRS-1)-mediated PI3-kinase and glucose uptake in muscle (Jazet *et al.,* 2003) and suppresses hepatic glucose production (Berg *et al.,* 2001). Its antiatherogenic properties include a reduction of monocyte adhesion to endothelial cells (Ouchi *et al,* 1999) and inhibition of foam cell formation from macrophages (Ouchi *et al.,* 2001). In 3T3-L1 adipocytes, insulin stimulates the secretion of adiponectin through a phosphoinositide 3'-kinase (PI3-kinase) dependent pathway (Bogan and Lodish, 1999). Thiazolidinediones (TZDs), a class of approved anti-diabetic drugs, stimulate adiponectin secretion and mRNA expression by activating the transcriptional factor peroxisome proliferator-activated receptor (PPAR)-γ (Maeda *et al.,* 2001).

In the present study, water and ethanol extracts of MC fruit and seeds were used in combination with two insulin concentrations as treatments on 3T3-L1 adipocytes to study their effects on glucose uptake, adiponectin secretion and cellular ceramide accumulation. Ceramide is a sphingosine based lipid signaling molecule. It is considered to be the second messenger in the sphingomyelin signaling cascade and research is now linking ceramide to insulin resistance. Skeletal muscle ceramide levels are increased in obese insulin resistant humans (Adams *et al.,* 2004). Ceramide has been shown to directly inhibit the insulin signaling pathway by decreasing insulin stimulated phosphorylation and activity of PKB/Akt proteins in the pathway (Mei *et al,* 2003).

The results of this study show that a water extract from MC fruit and seeds potentiates the glucose uptake activity of sub-optimal concentrations of insulin and increases adiponectin secretion from 3T3-L1 adipose cells.

4.3 Materials and Methods

4.3.1 Materials

3T3-L1 fibroblast cells were purchased from American Type Culture Collection (Manassas, VA). 1-isobutyl-3-methylxanthine (IBMX), dexamethasone, 0.25% Trypsin-EDTA, 2-deoxy-D-[1,2-^{3}H]glucose, (3,4-5 dimethyl thiazol-2-yl)-2,5-diphenyl tetrazolium (MTT), wortmannin, dimethylsulfoxide (DMSO), C_6 ceramide, *O*-phthaldialdehyde (OPA) and potassium phosphate were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), penicillin-streptomycin, and human insulin were purchased from Life Technologies (Burlington, ON). General chemicals and lab equipment were purchased from either Sigma-Aldrich Chemical Co. (St, Louis, MO), Fisher Scientific Co. (Nepean, ON) or Ultident Scientific (St. Laurent, QC).

4.3.2 *Momordica charantia* Extract Preparation

Unripe MC fruit ("Moonlight" variety) was picked directly from the vine at a nearby farm in the province of Quebec. Within a few hours two whole fruit (422 g) (including seeds) were cut into 1 cm^3 pieces and freeze dried. The dried fruit was ground to homogeneous powder which was then divided into two approximately equal halves.

Momordica charantia Water Extract (MCW)

One half of the MC powder (18.03 g) was soaked in room temperature double distilled water (15:1) overnight with continuous stirring and protected from light. This was then filtered through Whatman #1 filter paper, the filtrate was collected and freeze dried. The freeze-dried sample (4.48 g) was crushed into powder and stored at -80°C until use.

Momordica charantia Ethanol Extract (MCE)

The other half of the MC powder (17.73 g) was extracted first with chloroform to remove lipid soluble components that would not be usable in cell culture assays. The powder was soaked in room temperature chloroform (9:1) overnight with continuous

stirring and protected from light. This was then filtered through Whatman #1 filter paper and the filtrate was discarded. The powder residue left after the filtration was collected to a clean Erlenmeyer flask, protected from light and left in a fume hood for 19 h to allow chloroform to completely evaporate from the sample. The dried residue was soaked in room temperature 95% ethanol (11:1) overnight with continuous stirring and protected from light. The solution was then filtered through Whatman #1 filter paper, the filtrate was collected and dried at 55°C using a rotovapor vacuum drier. To remove any remaining water the sample was freeze dried. The freeze-dried sample (1.51 g) was crushed into powder and stored at -80°C until use.

4.3.3 Cell Culture

3T3-L1 cells were maintained in high-glucose DMEM containing 10% FBS and 1% penicillin-streptomycin at 37°C in a humidified atmosphere of 5% CO_2. Cells were subcultured every 3 to 4 days at approximately 90% confluence. Cells plated onto 12 or 24-well plates were 1 to 5 passes from the original vial. Cells were grown in the plates to reach confluence in 3 days. At this point (day 0) cells were switched to differentiation medium (DMEM, 10% FBS, 0.25 μM dexamethasone, 0.25 mM IBMX, and 1 μg/ml insulin) for 3 days, with one medium change in between. On day 3, the dexamethasone and IBMX were removed leaving insulin on the cells for an additional 4 days, changing the medium every 2 days. Thereafter the cells were maintained in the original propagation DMEM, changing medium every 2-3 days, until use. Plates where cells were >90% differentiated were used for experiments between days 9 to 12 post-induction.

4.3.4 Cellular uptake of 2-deoxy-D-glucose

Glucose uptake activity was determined using 12-well plates. When >90% of cells in the plates were differentiated into adipocytes, cells were washed once with serum-free DMEM and incubated in 1 ml/well of the same for 2 h at 37°C. Cells were then washed once with Krebs-Ringer-HEPES (KRH) buffer (128 mM NaCl, 4.7 mM KCl, 1.25 mM $CaCl_2$, 1.25 mM $MgSO_4$, 10.0 mM Na_2HPO_4, 20 mM HEPES), and incubated in 1ml/well of the same for 30 min at 37°C. Treatment with insulin (0.5 nM or 50 nM) occurred in the presence and absence of MCW or MCE extracts dissolved in KRH buffer, which was allowed to proceed for 30 min. MCE extracts were first dissolved in DMSO to make a stock solution that was then further diluted into KRH buffer. The final

concentration of DMSO was 0.5% (v/v), which was included in basal and insulin controls. For measurement of glucose transport, 2-deoxyglucose was used together with the radiolabelled tracer, 2-deoxy-D-[1,2-^{3}H]-glucose, to give a concentration of 0.2 mM (0.5 mCi/mmol) yielding an activity of 0.1 μCi/ml. After 60 min at 37°C, glucose uptake was terminated by first placing the plates on a bed of ice while the medium was collected to vials and frozen at -20°C for later adiponectin analysis. The plates were washed with 3 ml/well ice-cold PBS and the cells were digested with 0.7 ml 1% Triton X-100 for 40 min at 37°C. Scintiverse BD (Sigma-Aldrich Chemical Co. St, Louis, MO), was added and tritium counts were obtained using a 1209 Rackbeta liquid scintillation analyzer (LKB-Wallac, Finland). Measurements were made in duplicate and corrected for specific activity.

4.3.5 MTT Viability Assay

Cell viability was assessed using 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay as described previously (Mosmann, 1983). The cells were seeded in 24-well plates. Adipocytes were washed once with serum-free DMEM and incubated in 0.5 ml/well of the same for 2 h at 37°C. Cells were then washed once with KRH buffer (128 mM NaCl, 4.7 mM KCl, 1.25 mM $CaCl_2$, 1.25 mM $MgSO_4$, 10.0 mM Na_2HPO_4, 20 mM HEPES), and incubated in 0.5 ml/well of the same for 30 min at 37°C. Treatment with MCW or MCE extracts dissolved in KRH buffer was allowed to proceed for 90 min at 37°C (in order to mimic the half h pretreatment and one h glucose uptake period used for glucose uptake experiments). During these steps DMEM control wells were aspirated and refilled with 0.5 ml/well fresh DMEM (10% FBS) each time the treated cells went through a medium change. At the end of the 90 min treatment time, all wells were aspirated and refilled with MTT solution (0.5 mg/ml MTT in glucose-free, phenol red free DMEM). The cells were incubated in this solution for 3 h at 37°C in an incubator. After 3 h the wells were aspirated and refilled with a 0.04N HCl in isopropanol solution. The wells were left to incubate for 5 min with gentle shaking until all the dark MTT colour had been converted to a yellow colour. An aliquot of 100 ul was collected from each well and transferred to a 96-well microplate. The absorbance for each well was read at 540 nm using a series 750 scanning spectrophotometer microplate reader

(Cambridge Technology Inc., Cambridge, MA, USA). Viability was expressed in terms of cell viability, as a ratio of the treatments to the KRH buffer control population.

4.3.6 Wortmannin Inhibition of Glucose Uptake

This assay was performed in exactly the same way as the glucose uptake assay but with the addition of 50 nM wortmannin at various stages. Preliminary tests with 50 nM wortmannin showed complete inhibition of insulin-dependent glucose uptake at this concentration. Treatment conditions chosen for this assay were KRH buffer control, 0.5 nM insulin alone, and the combination of 0.2 mg/ml MCW and 0.5 nM insulin. This treatment was shown to be the most effective treatment at increasing glucose uptake. The cells were exposed to the wortmannin during the KRH buffer pre-treatment (30 min), the treatment with 0.5 nM insulin and MCW (30 min) and during the 60 min glucose uptake period. Cells exposed to wortmannin were compared to controls.

4.3.7 Adiponectin Secretion

Adiponectin secretion by the treated 3T3-L1 adipocytes was measured using a Quantikine ELISA Mouse Adiponectin Immunoassay (R&D Systems, MN). Treatment conditions chosen for this assay were KRH buffer control, 0.5 nM insulin alone, and the combination of 0.2 mg/ml MCW and 0.5 nM insulin. This treatment was shown to be the most effective treatment at increasing glucose uptake. As stated earlier, the medium tested for adiponectin concentration was collected after the 60-min glucose uptake period during the glucose uptake assay.

4.3.8 Ceramide Analysis

Cells used for this assay were grown and differentiated in 60mm petri dishes. Two plates were used for each treatment in order to collect enough cells for ceramide quantitation. The aim was to collect $2x10^6$ cells. When >90% of cells in the plates were differentiated into adipocytes, cells were washed once with serum-free DMEM and incubated in 5 ml/plate of the same for 2 hours at 37°C. Cells were then washed once with KRH buffer and incubated in 5 ml/plate of the same for 30 minutes at 37°C. Treatment with insulin (0.5 nM) and/or MCW extracts dissolved in KRH buffer was allowed to proceed for 30 minutes. Glucose uptake was then initiated with the addition of 2-deoxy-D-[1,2-^{3}H]-glucose (0.2 mM, 0.5 mCi/mmol). After 60 minutes at 37°C, uptake was terminated by first placing the plates on a bed of ice while the medium was aspirated

using a pipette. The plates were washed twice with ice-cold PBS and then the cells were collected by scraping in 1.6 ml of PBS. The collected cells were then frozen in liquid nitrogen until the day of the experiment. For lipid extraction, the thawed cells were transferred to glass tubes and 6 ml of methanol:chloroform (2:1) was added. The methanol:chloroform contained 0.001% butylated hydroxytoluene (BHT) as an anti-oxidant. Each tube was vortexed and allowed to sit for 10 minutes. Then 2 ml of chloroform and 2 ml of alkaline water (0.8 mM NH_4PO_4) were added and vortexed. The tubes were centrifuged at 400 x g for 30 minutes. The bottom organic phase was collected, dried under N_2 and resuspended in 1 ml of chloroform. After collecting 200 μl for lipid phosphate determination, the remaining 800 μl was dried under N_2. The lipids were then hydrolyzed at 95°C for 1.5 hours in 0.5 ml of 1 N KOH in methanol to remove glycerolipids. The KOH fraction was dried under N_2 and the lipids were re-extracted using 0.5 ml methanol, 1 ml chloroform and 0.9 ml alkaline water. The bottom organic phase was again collected, dried under N_2 and resuspended in 50 μl of methanol. To derivatize the lipids, 50 μl of OPA solution (0.5 mg/ml OPA in 3% boric acid – pH 10.5, 0.5% 2-mercaptoethanol) was added and allowed to sit for 10 minutes after vigorous shaking. 100 μl of HPLC eluent (90% methanol, 10% 5mM KH_2PO_4) was added and left for 50 minutes. Using a Shimadzu SIL-6A autoinjector and the low volume injection setting, 15 μl of this solution was injected into an Alltech Nucleosil C18, 5 micron column (150x4.6mm) and eluted isocratically at a rate of 0.7 ml/min. Fluorescence was detected using a Shimadzu RF551 Spectroflurometric detector at excitation/emission wavelengths of 340/454 nm (Shimadzu, MD, USA). The ceramide peak was identified using a C6 ceramide standard to spike a sample and to create a standard curve that was linear from 50 pmoles to 4 nmoles.

Results are expressed as pmol ceramide/nmol lipid phosphate (LP). LP was determined by drying 50ul of the chloroform aliquot collected earlier in the extraction process under N_2. To hydrolyze the sample, 150 μl of 70% perchloric acid was added to the dried lipids and heated to 160°C for 1.5 hours. 800 μl of bidistilled water, 150 μl of 2.5% ammonium molybdate, and 150 μl of 10% ascorbic acid were added with vortexing in between each. The tubes were placed in boiling water for 5 minutes and absorbance of the cooled samples was read using a Beckman Du640 spectrophotometer at 820 nm

(Beckman Coulter Canada Inc., Mississauga, ON, Canada). A standard curve for lipid phosphate was created using Potassium Phosphate and was linear between 25 ng and 1 μg.

4.3.9 Statistical Analysis

All data are expressed as mean ± standard deviation (SD) and each value represents a minimum of three (n=3 to n=6) replicate experiments and all assay conditions were performed in triplicate. Data were analyzed using a one-way ANOVA and SAS version 8e (SAS Institute Inc, 1994). One-way ANOVA was employed to determine main treatment effects. When a significant ANOVA result was obtained, the method of least squares means test and Tukey's post-hoc test were used to test differences between treatment groups and control group. All residuals were tested for normality and were shown to be so. Level of significance was set at $p<0.05$.

4.4 Results

4.4.1 Glucose Uptake

In this assay, glucose uptake was allowed to proceed for one h before the cells were lysed and measured for intercellular glucose content. Preliminary results using a 10 min glucose uptake period were sufficient to show insulin mediated changes to uptake but the MC extracts had no effect. After the one h period of glucose uptake, significant insulin activation of 2-deoxy-glucose uptake was observed at both concentrations of insulin with the maximum stimulation detected at 50 nM insulin (Fig. 1 and 2). The uptake of 2-deoxy-D-glucose ranged between 2- to 6-fold for 0.5 nM (5322 ± 1394 dpm) and 50 nM insulin(17196 ± 3180 dpm), respectively, as compared to that of basal uptake (2758 ± 1094 dpm).

Adipocytes exposed to 0.2 mg/ml MCW with 0.5 nM insulin showed a significant increase (approximately 61%) in glucose uptake over the 0.5 nM insulin control ($p<0.05$) (Fig. 1). The two higher concentrations of MCW in this treatment group showed a similar trend but these were not statistically significant. Without the presence of insulin, MCW had no significant effect but demonstrated a trend to decrease glucose uptake compared to the control. Interestingly, MCW showed a similar trend in combination with 50nM insulin but showed no significant differences from the 50 nM control.

The presence of 0.5% DMSO was shown to have no effect on basal or insulin-stimulated glucose uptake. MCE extract at 0.2 mg/ml showed a trend of increased glucose uptake with the sub-optimal level of insulin but this trend did not reach significance (Fig. 2). However, dose response of MCE concentration to decrease glucose uptake on its own or with 50 nM insulin was intensified over the MCW results and showed a significant difference at the two highest concentrations of MCE (0.3 and 0.4 mg/ml) ($p<0.05$ and $p<0.01$, respectively).

4.4.2 MTT Assay

Both the MCW and MCE extracts did not affect cell viability differently from the KRH control. In this assay, the absorbance reading correlates with viable cell number and metabolic activity of the cells. In cells of equal activity, the test can be used to determine the percentage of viable cells. The KRH control cells experienced a decrease in absorbance measurement in comparison to the DMEM control (Fig. 3). There was no significant difference in absorbance between the treatments and the KRH control.

As with MCW, there was no significant difference in absorbance between the MCE treatments and the KRH control (Fig. 4). In this case, the KRH control cells again experienced a decrease in absorbance measurement in comparison to the DMEM control.

4.4.3 Effect of Wortmannin on MCW Stimulated Glucose Uptake

The effect of wortmannin on 2-deoxyglucose uptake in the absence and presence of 0.5 nM insulin and/or 0.2 mg/ml MCW is shown in Fig 5. In the absence of pre- and concurrent incubation with wortmannin, insulin caused a 1.3-fold stimulation of 2-deoxy-glucose uptake. The treatment combination of MCW and 0.5 nM insulin caused a 3-fold stimulation of 2-deoxy-glucose uptake in the absence of pre- and concurrent incubation with wortmannin. By treating the adipocytes with 50 nM wortmannin, increased uptake of glucose by treatment with a combination of MCW and 0.5 nM insulin, was completely inhibited to the same degree as the wortmannin inhibition of 0.5 nM insulin only treatment. The inhibition lowered glucose uptake to below basal levels.

4.4.4 Adiponectin Secretion

These adiponectin concentrations were measured in the medium collected after the one hour period of glucose uptake experiments. Adiponectin secretion by 3T3-L1 adipocytes was increased by the addition of 0.2 mg/ml MCW to the 0.5 nM insulin

treatment (Fig. 6). This increase was significant compared to basal adiponectin secretion ($p<0.01$) and to cells treated with 0.5 nM insulin alone ($p<0.05$). This increase was not significant over the 0.2 mg/ml MCW alone treatment which showed a trend to increase adiponectin secretion. This increased secretion of adiponectin due to the 0.2 mg/ml MCW with 0.5 nM insulin corresponds to the increased glucose uptake incurred by this same treatment on the same cells.

4.4.5 Cellular Ceramide Accumulation

No significant differences in intercellular ceramide accumulation were seen between the treatments with MCW and the control. Although, there appears to be a trend towards decreasing ceramide content in the cells compared to the control by treating them with the combination of 0.2 mg/ml MCW and 0.5 nM insulin, this decrease did not reach significance due mainly to high variability in the results ($p<0.751$) (Fig. 7). Only two repetitions were calculable for the 0.2 mg/ml MCW alone treatment which hindered the results. As well, the HPLC unit was not able to calculate the area of one of the ceramide peaks used to calculate the ceramide concentration for the MCW and 0.5 nM insulin treatment. These peaks with no calculated area were below the detection limit of the HPLC machine and were set as zero when determining ceramide concentration.

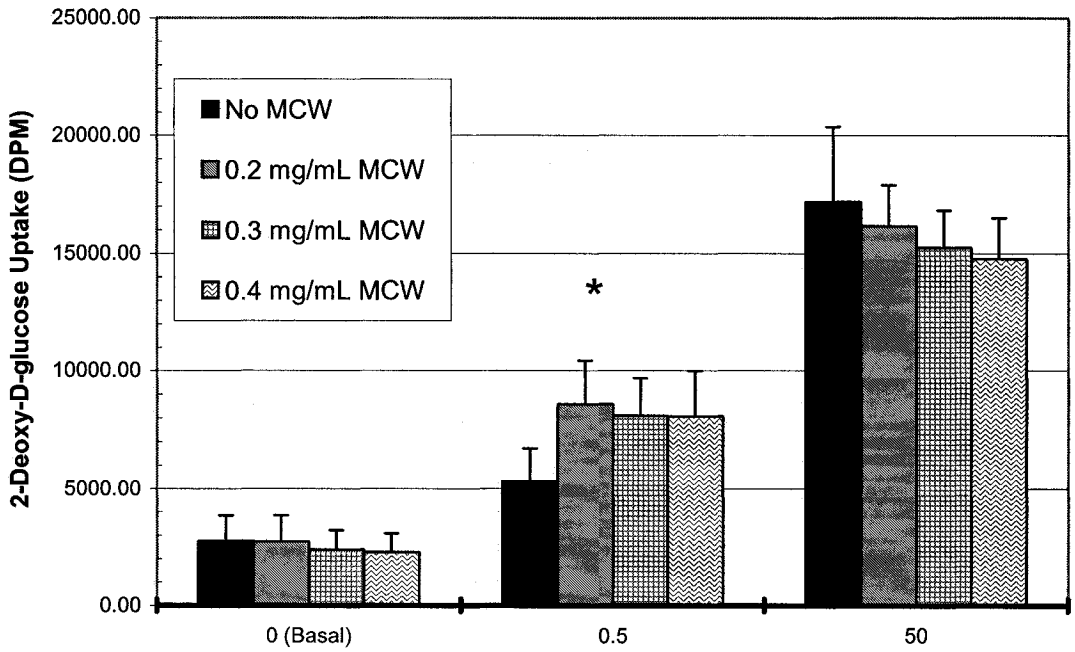


Fig. 1 MC water extract (MCW) on glucose uptake in 3T3-L1 adipocytes with or without the presence of 0.5 nM or 50 nM insulin. DPM denotes the disintegrations per minute. Data are mean uptakes ±SD from 3 or more independent experiments performed in triplicate, expressed as disintegrations per min (DPM). Significant difference between untreated cells (solid black bar) and the MCW treated cells was tested within groups based on insulin concentration. * $p<0.05$ (Using Tukey's post-hoc)

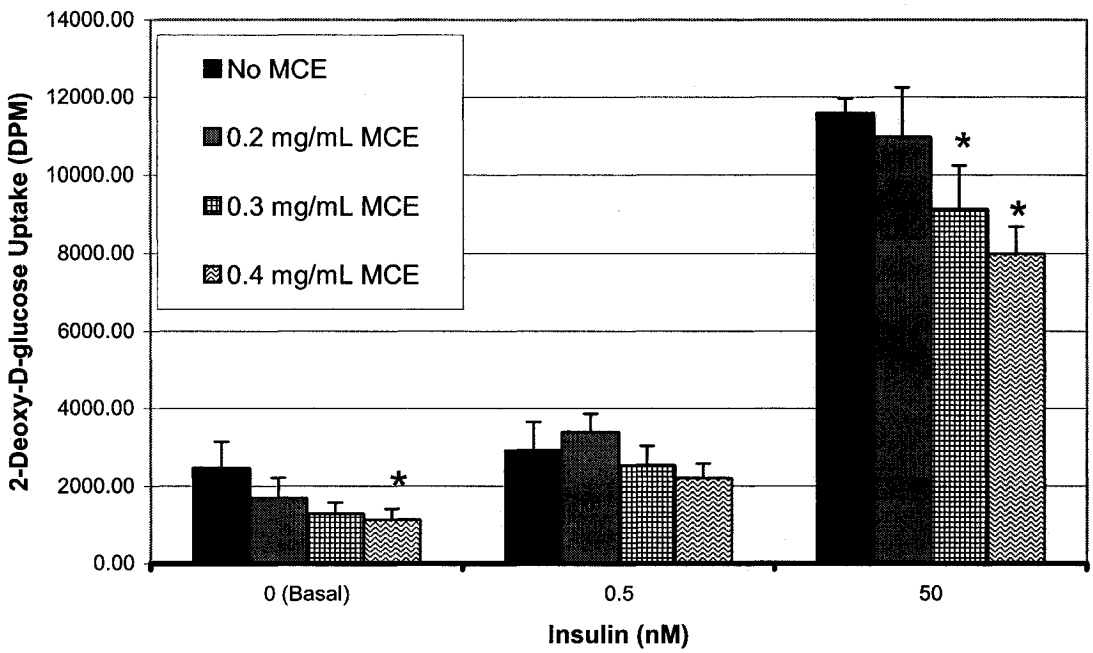


Fig. 2 MC ethanol extract (MCE) on glucose uptake in 3T3-L1 adipocytes with or without the presence of 0.5 nM or 50 nM insulin. DPM denotes the disintegrations per minute. Data are mean uptakes ±SD from 3 or more independent experiments performed in triplicate, expressed as disintegrations per min (DPM). Significant difference between untreated cells (solid black bar) and the MCW treated cells was tested within groups based on insulin concentration. * $p<0.05$ (Using Tukey's post-hoc)

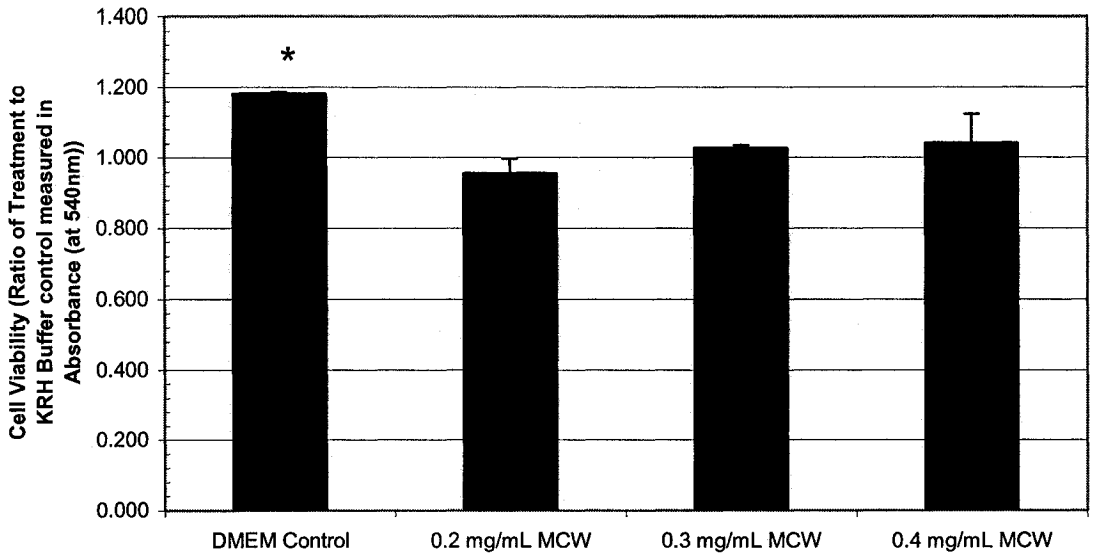


Fig. 3 The effect of MCW extract concentration on cell viability using the MTT assay. Cells were exposed to the MCW treatment for 90 min in order to mimic the half hour pretreatment and one hour glucose uptake used in the glucose uptake experiments. Data are mean ± SD of three replicate experiments and expressed as population growth, absorbance measured for the treatment divided by the absorbance measured for the KRH buffer control population (Absorbance = 0.144±0.01 at 540 nm) . Significant difference was tested between the treatments shown and the KRH buffer control (ratio = 1). * $p<0.05$ (using Tukey's post-hoc).

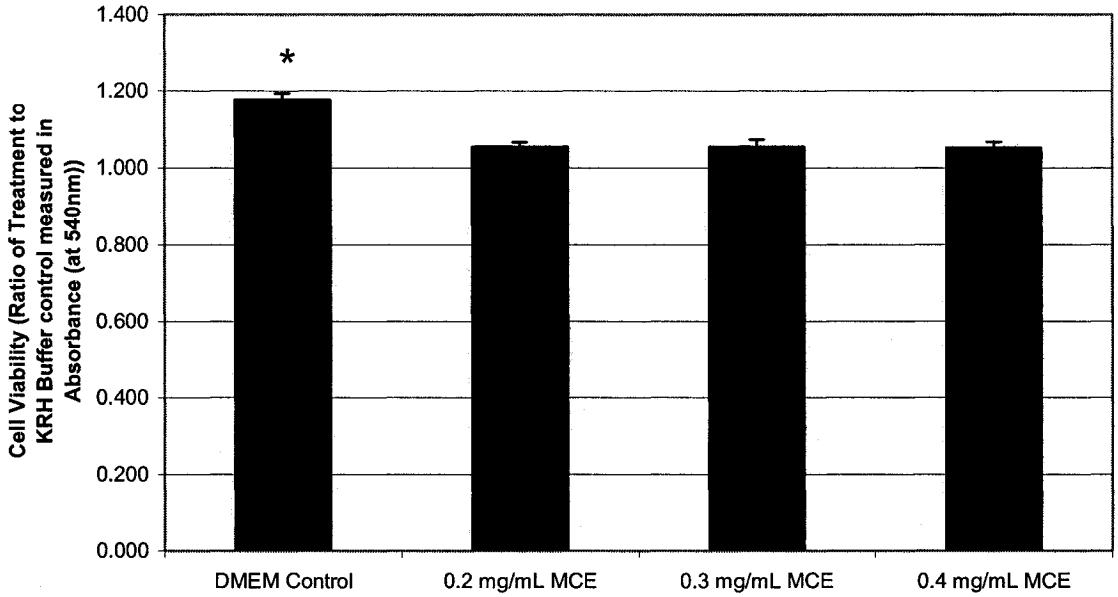


Fig. 4 The effect of MCE extract concentration on cell viability using the MTT assay. Cells were exposed to the MCE treatment for 90 min in order to mimic the half hour pretreatment and one hour glucose uptake used in the glucose uptake experiments. Data are mean ± SD of three replicate experiments and expressed as population growth absorbance measured for the treatment divided by the absorbance measured for the KRH buffer control population (Absorbance = 0.144±0.01 at 540 nm) . Significant difference was tested between the treatments shown and the KRH buffer control (ratio = 1). * $p<0.05$ (using Tukey's post-hoc).

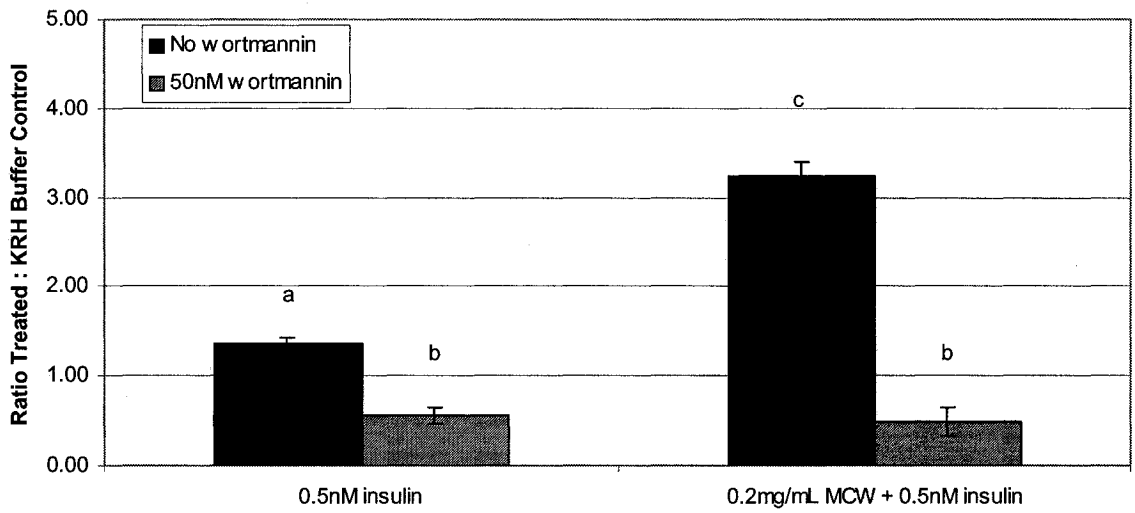


Fig. 5 The effect of 50 nM wortmannin on 3T3-L1 adipocyte glucose uptake when treated with 0.5 nM insulin alone and with 0.2 mg/ml MCW. Data are expressed as the DPM obtained in the treatment divided by the DPM from the KRH buffer control (basal glucose uptake). Data were derived from the triplicates of three independent experiments, and expressed as means ± SD. Significant difference is at $p<0.05$ (using Tukey's post-hoc).

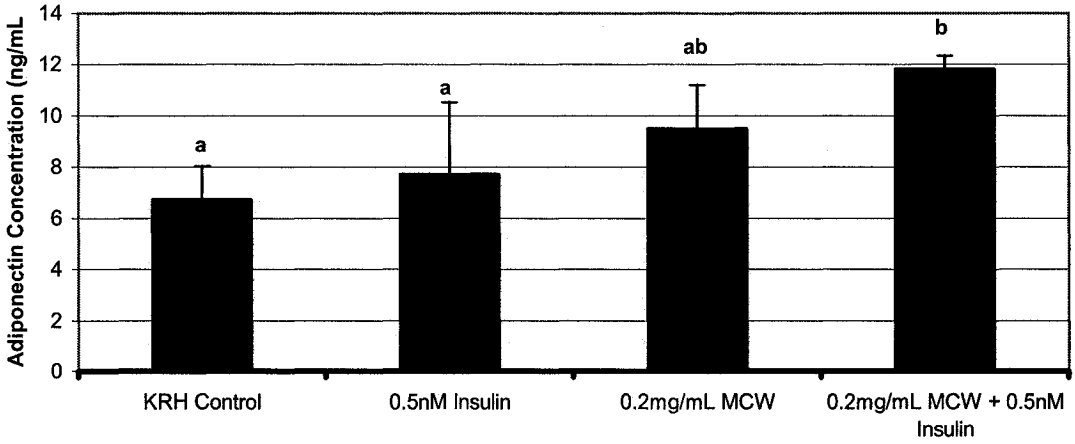


Fig. 6 Effect of MCW and 0.5 nM insulin on adiponectin secretion into the medium. The medium was collected from the 12-well plates at the end of the glucose uptake experiments after the 60 min exposure to ^{3}H-deoxy-glucose with or without 0.2 mg/ml MCW alone and 0.5 nM insulin alone or with 0.2 mg/ml MCW. The bars represent the mean ± standard deviation of at least three replicate experiments (n=3-6). Significant difference is at $p<0.05$ (using Tukey's post-hoc).

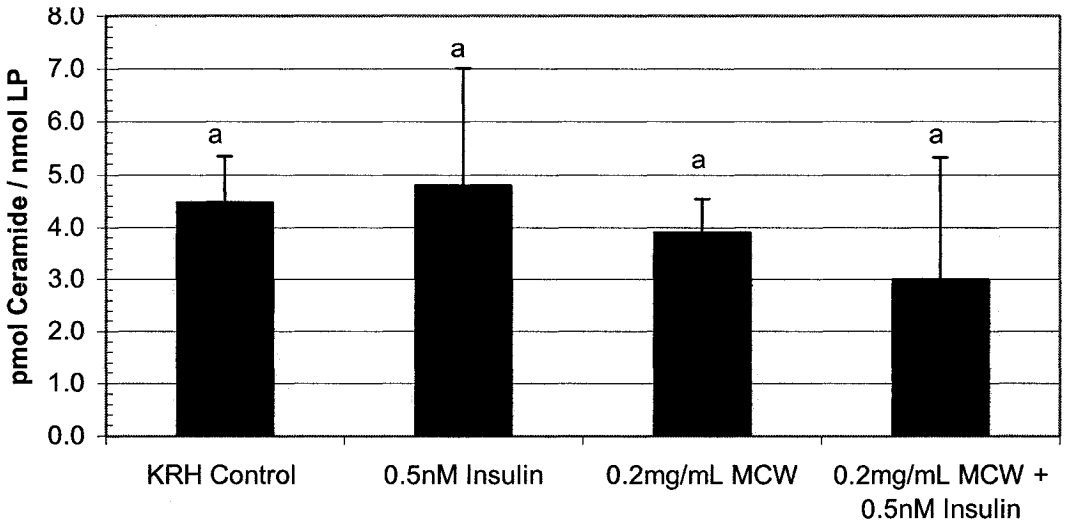


Fig. 7 Effect of MCW and 0.5 nM insulin on ceramide concentration within the 3T3-L1 adipocytes. The cells were pretreated in 60mm plates for 30 min with or without 0.2 mg/ml MCW alone and 0.5 nM insulin alone or with 0.2 mg/ml MCW. ^{3}H-Deoxy-glucose was added along with the treatments for 60 min after which the cells were collected and the lipids were extracted as described. The ceramide content was normalized per lipid phosphate content of the cells. Data are expressed as mean ± SD of two to three repetitions from different experiments. Significant difference was set at $p<0.05$ (using Tukey's post-hoc).

4.5. Discussion

MC has been widely used as a traditional medicine for the treatment of type 2 diabetes. Despite this, the precise mechanisms whereby MC may act to improve insulin sensitivity and glucose disposal in vivo are unclear. In the studies reported above, we demonstrate that 30 min of treatment with the water soluble MC extract increases the glucose uptake activity of sub-optimal levels of insulin and increases adiponectin secretion of 3T3-L1 adipocytes.

Along with its pathological alterations to lipid metabolism and liver glycogen storage, insulin resistance disrupts proper clearance of glucose from the blood stream by both skeletal muscle and adipose tissue. As the pancreas begins to fail, insulin secretion decreases to a point where the plasma insulin concentrations are not sufficient to overcome the insulin resistance and type 2 diabetes is the result (DeFronzo, 1997). A water extract from the MC fruit was able to increase glucose uptake in 3T3-L1 adipocytes by approximately 61% in the presence of 0.5 nM insulin, a suboptimal concentration of insulin, over treatment of the cells with 0.5 nM insulin alone (Fig. 1). This same extract did not increase glucose uptake under basal conditions or in the presence of 50 nM insulin. There has only been one other study in which MC has been tested on such an insulin sensitive tissue in a controlled immortalized cell culture method. The study by Singh *et al.* (2004) using L6 skeletal muscle myotubes found that 5 μg/ml fruit juice and a chloroform extract of the juice of MC (with the seeds removed) showed only very slight, but significant, increases in glucose uptake after 3 h exposure to glucose with the treatment and only showed a substantial significant increase after 6 h. In view of these latter findings, the lack of positive effect on basal glucose uptake with the MC extract in the present study is not surprising as the 3T3-L1 cells were treated only for one h. The glucose uptake results from the present study also indicate, however, that the MCW extract may be more effective in the potentiation of low levels of insulin rather than acting as an insulin mimetic agent. In support of this observation, other studies have shown that the MC juice was able to lower blood glucose levels in insulin resistant hyperglycemic rats but had no effect in streptozotocin (STZ)-induced diabetic rats lacking any viable insulin-secreting beta cells (Karunanayake *et al.,* 1990, Ali *et al,* 1993). Accordingly, it would appear that the hypoglycemic components(s) of MC either

improve the insulin-secreting capacity of viable beta-cells or potentiate the action of insulin. Similar results have been found for two isolates of *Pycnanthus angolensis* that increased the glucose uptake activity of 0.5 nM insulin by approximately 27 and 48% respectively but had no effect on their own (Luo *et al.,* 1999). These isolates were able to dose-dependently reduce glucose and insulin plasma levels in *ob/ob* and *db/db* mice that were both hyperglycemic and hyperinsulinemic but had no effect in STZ-induced insulin-deficient mice (Luo *et al.,* 1999).

The synergistic effect of MCW with insulin does not occur at the higher concentration of 50 nM insulin. When insulin induces glucose uptake in an adipose cell, it first binds to the insulin receptor (IR) protein on the surface of the cell, which sequentially activates isoforms of insulin receptor substrate (IRS), phosphotidinosytol-3-kinase (PI3-kinase), protein kinase B (PKB/Akt), and protein kinase C (PKC). In this manner, there is an induction of the translocation of the insulin-regulated glucose transporter, GLUT4 and to a much lesser degree GLUT1, to the cell surface (Litherland *et al.,* 2001). GLUT4 is predominantly responsible for insulin-stimulated glucose uptake (Litherland *et al.,* 2001). There is also evidence that glucose uptake may require activated cell surface GLUT4 proteins and that insulin may induce this activation through a p38 mitogen-activated protein kinase (MAPK) pathway (Bazuine *et al.,* 2005, Somwar *et al.,* 2001, Hausdorff *et al.,* 1999). Based on the difference of effect that MCW has on glucose uptake in the presence of 0.5 nM or 50 nM insulin seen with this study, it is possible that a component(s) in the MCW extract may decrease the number of active IR proteins available for insulin binding while the overall effect of the MCW extract is to increase the efficiency of the insulin pathway by acting downstream of the IR thereby allowing the low level of insulin that is able to bind to the receptors to increase GLUT4 translocation and activation to a greater degree.

In contrast to the MCW extract, the MCE extract did not potentiate significantly the glucose uptake activity of 0.5 nM insulin; however, a significant dose-dependently decrease in glucose uptake by the adipocytes was observed from the MCE extract under basal conditions or in the presence of 50 nM insulin (Fig. 2). It is conceivable that the MC component(s) potentiating the effect of 0.5 nM insulin are polar and preferentially water-soluble whereas the MC component(s) reducing glucose uptake in the absence and

presence of 50 nM insulin is preferentially ethanol-soluble. Numerous studies comparing the antihyperglycemic potential of water and alcohol extracts of MC on various rat models of diabetes have shown consistently that the water extract has more potent and long lasting plasma glucose lowering properties (Ali *et al.,* 1993, Virdi *et al.,* 2003, Vikrant *et al.,* 2001, Rathi *et al.,* 2002). The decrease in basal glucose uptake induced by MCE, however, was surprising. In the basal state, the glucose transport GLUT1 localizes significantly to the plasma membrane as well as the cell interior whereas GLUT4 remains predominantly in an intracellular compartment (Hausdorff *et al.,* 1999). For this reason, GLUT1 is largely responsible for basal uptake (Mueckler, 1994). It is thus possible that a preferentially ethanol-soluble component(s) of MC may decrease the number or activity of GLUT1 transporters at the cell surface.

Based on the results of the MTT assay in this study (Figs. 3 and 4), neither MCW nor MCE are toxic to the 3T3-L1 adipocytes and the changes to glucose uptake induced by these extracts cannot be explained by changes in the number of viable cells. This assay is based on the reduction of MTT into purple formazan pigment by the succinate-tetrazolium reductase system in the respiratory chain of the mitochondria (Gomez *et al.,* 1997). This system is active only in metabolically active cells and changes in MTT reductase activity are detectable even before membrane lysis, making the MTT assay a refined marker of cellular viability (Gomez *et al.,* 1997, Tollefson *et al.,* 1996).

Concurrent treatment of the adipocytes with 50 nM wortmannin inhibited the increased insulin stimulated glucose uptake caused by the treatment of the cells with 0.2 mg/ml MCW in the presence of 0.5 nM insulin. This inhibition decreased glucose uptake to below basal glucose uptake levels, similar to the inhibition of glucose uptake associated with 0.5 nM insulin alone. Wortmannin is a potent and irreversible inhibitor of the p110 isoform of PI3-kinase, thus preventing insulin-stimulated GLUT4 and GLUT1 translocation and inhibiting glucose uptake with nanomolar efficiency (Hausdorff *et al.,* 1999). For this reason, wortmannin is often used in cell culture to test the reliance of glucose uptake potentiating substances or extracts on this protein (Jarvill-Taylor *et al.,* 2001, Imparl-Radosevich *et al.,* 1998). More recent evidence shows that wortmannin has a second higher affinity, reversible target that may or may not be a PI3-kinase but is involved in the p38 MAPK pathway activation of cell surface GLUT4 (Somwar *et*

al.,2001). The results of this study thereby indicate that the water extract of MC, similar to insulin, relies on at least one of these wortmannin sensitive targets to increase glucose uptake. Further research is needed to positively identify the high affinity target of wortmannin and to determine whether MC relies on one or both of these wortmannin targets.

The combination of 0.2 mg/ml MCW and 0.5 nM insulin increased adiponectin secretion from the 3T3-L1 adipocytes (Fig. 6). In this study, treatment of the cells with 0.5 nM insulin did not increase adiponectin secretion; however, a study by Bogan and Lodish (1999) demonstrated that the treatment of 3T3-L1 adipocytes with 160 nM increased adiponectin secretion by as much as 100% over the control through a PI3-kinase dependent pathway. They concluded that insulin regulated the release of adiponectin from a distinct secretory compartment within the cell. Hence, it is likely that the insulin concentration of 0.5 nM tested in the present study was too low to cause significant changes to adiponectin secretion. On the other hand, 0.2 mg/ml of MCW on its own showed a tendency to increase adiponectin secretion. Moreover, the significant increase in adiponectin secretion induced by the combined MCW/insulin treatment may be a result of MCW acting in a similar way to insulin to release adiponectin from its secretory compartment. Thus, MCW may exert a synergistic effect with insulin to trigger adiponectin secretion. Alternatively, or in addition, MCW may be acting as a PPAR-γ ligand. PPAR-γ is a ligand activated transcription factor that plays an active role in insulin sensitivity and glucose metabolism of the adipocyte (Rangwala and Lazar, 2004), partly mediated by the regulation of adipocytokines. PPAR-γ is also involved in the maintenance of proteins involved in glucose metabolism such as IR, IRS, and GLUT4 whose expression is regulated by PPAR-γ (Tamori *et al.*, 2002). TZDs are a class of anti-diabetic drugs that act specifically as ligands to PPAR-γ and by doing so reduce the expression of the insulin desensitizing cytokines resistin (Haugen *et al.*, 2001), TNFα and IL-6 (Sigrist *et al.*, 2000) while inducing the expression of adiponectin (Maeda *et al.*, 2001, Iwaki *et al.*, 2003). Treatment of 3T3-L1 cells with different concentrations of TZD increased adiponectin secretion into the media by approximately 67 to 89% over the control after a 24 h exposure (Maeda *et al.*, 2001). In the present study, the adiponectin release was increased approximately 75% over vehicle control levels after only a one h

exposure to the MCW/insulin treatment during the glucose uptake period. Hence, it is possible that the MCW extract, like TZDs, may act as a ligand to PPAR-γ thus altering adiponectin secretion to favour insulin sensitivity. If this ligand activity is occurring with the MCW treatment, then the activation of PPAR-γ would also favourably regulate the expression and secretion of other adipocytokines and proteins involved in insulin signaling thereby increasing the efficiency of the insulin signaling pathway. Given that TZDs come with the side effects of weight gain and edema, research is beginning to focus on PPAR-γ ligands that selectively target genes regulated by PPAR-γ to improve glucose homestasis without the side effects (Rocchi *et al.,* 2001). Further research is warranted to study the mechanism of action of the MCW-mediated enhancement of adiponectin secretion and how the secretion and expression of other adipocytokines are affected in relation to the stimulation of glucose uptake by MCW.

The treatment of 3T3-L1 adipocytes with MCW extract alone and in combination with 0.5 nM insulin did not yield any significant results with respect to intercellular ceramide accumulation (Fig. 7). Although the MCW and 0.5 nM insulin treatment appears to slightly reduce ceramide concentration, the statistical probability of there being a true difference between the treatments and the control was too high to suggest even a trend. Further repetitions are required to determine if significant differences exist.

In summary, the treatment of 3T3-L1 adipocytes with a combination of 0.2 mg/ml water extract from fresh, unripe MC fruit and seeds and 0.5 nM insulin was able to increase significantly glucose uptake by the cells. The MCW + insulin combination that increased glucose uptake concurrently increased adiponectin secretion from the cells. This effect of MC on adiponectin secretion is a novel finding that may suggest an unexplored direction regarding the potential of MC, and other anti-diabetic plant extracts, to regulate the expression and secretion of cytokines that affect insulin sensitivity. Further research is required to identify the insulin sensitizing and desensitizing components in MC fruit and seeds and how they affect adipocytokine secretion in order to determine the mechanism of action and, ultimately, enhance the anti-diabetic potential of this traditionally used treatment for the disease.

V. Link Statement

As shown, the MC water extract potentiated the ability of 0.5 nM insulin to increase glucose uptake in 3T3-L1 adipocytes but had no effect in this regard on its own. Along with the increased glucose uptake into the 3T3-L1 adipocytes, the MC water extract and 0.5 nM insulin treatment also increased adiponectin secretion from these cells. The results suggested that component(s) of MC acted to increase the sensitivity of the adipocytes to insulin and concurrently increased adiponectin secretion from the cells.

In terms of its anti-diabetic potential, cinnamon has undergone multiple *in vitro* experiments exploring its ability to affect glucose uptake in fat cells (Broadhurst *et al.*, 2000, Berrio *et al.*, 1992, Imparl-Radosevich *et al.*, 1998, Jarvill-Taylor *et al.*, 2001), has had separate fractions and isolates tested to determine the active components (Imparl-Radosevich *et al.*, 1998, Anderson *et al.*, 2004, Jarvill-Taylor *et al.*, 2001), has demonstrated anti-diabetic activity in rodents (Qin *et al.*, 2003, Qin *et al.*, 2004), and has shown strong anti-diabetic effects in a randomized, double-blinded, placebo-controlled clinical trial with type 2 diabetic subjects (Khan *et al.*, 2003). Due to the extensive testing of cinnamon on fat cells, including the 3T3-L1 adipocyte cell line (Jarvill-Taylor *et al.*, 2001), and its progression of experiments culminating in strong results from a well-designed clinical trial, we believed that testing cinnamon would lend weight to the MC study by providing insight as to the potential effects of MC in humans based on our 3T3-L1 adipocyte experiment results using MC.

In addition, we wanted to test whether MC and cinnamon extracts have similar mechanisms of action for increasing glucose uptake in 3T3-L1 adipocytes and whether similar effects on adiponectin secretion from these cells could be observed with cinnamon extracts as noted with MC extracts.

VI. MANUSCRIPT II

A Water Extract from Cinnamon Increases Glucose Uptake but Inhibits Adiponectin Secretion in 3T3-L1 Adipose Cells

Ben Roffey[1], Stan Kubow[1]

[1]School of Dietetics and Human Nutrition, McGill University, Macdonald Campus, 21,111 Lakeshore, Ste-Anne-de-Bellevue, QC, Canada H9X 3V9

6.1 Abstract

To examine the effects of cinnamon (*Cinnamomum sp.)* on glucose uptake, adiponectin secretion and accumulation of ceramide in fat cells, 3T3-L1 adipocytes were treated with three concentrations of a water extract of cinnamon (CE). Treatments consisted of the extracts alone and the extracts in combination with two concentrations of insulin. Without insulin, 0.2 mg/ml of CE increased glucose uptake (100%) and completely inhibited adiponectin secretion from the cells. Sub-optimal concentrations of insulin did not further enhance the CE activity and, in combination with 50 nM insulin, a dose-dependent decrease in glucose uptake was observed with the extract.

6.2 Introduction

In the year 2002, 151 million people worldwide were diagnosed with diabetes and this number is expected to rise to 221 million people by the year 2010 (Zimmet, 2002). More than 90% of these cases are type 2 diabetics suffering from severe insulin resistance. Research has begun to focus on adipose tissue as a possible central mediator of whole body insulin resistance. Evidence for this central role comes not only from the link between obesity and type 2 diabetes (Williams, 1999), but also from the role of adipose tissue in regulating serum lipid concentrations (Zeman, 1991) and, more recently, from the emerging role of adipose tissue as an endocrine organ (Mohamed-Ali *et al.,* 1998).

The specific and/or limited effects of current drug treatments for diabetes, combined with the dangerous side effects that most of them induce, has fueled the search for alternative medicines. Cinnamon (*Cinnamomum sp.*) is the dried inner bark of various laurel trees in the cinnamomum familiy that is native to Skri Lanka and India but is cultivated extensively in the tropical regions of the world (Chevallier, 2000). Although cinnamon has been used medicinally for thousands of years, its potential use as an antidiabetic has not been explored until recently. Originally testing whole foods for their ability to potentiate the action of insulin based upon their chromium content, it was discovered that water and ammonium hyroxide extracts of cinnamon had the ability to increase glucose uptake by 5 to 32-fold in a rat epididymal fat pad assay (Broadhurst *et al.*, 2000, Berrio *et al.*, 1992). This cinnamon extract also increased insulin receptor (IR) kinase autophosphorylation and decreased protein tyrosine phosphate (PTP)–1 activity, an enzyme that inhibits insulin signaling by dephosphorylating the insulin receptor at the cell surface (Imparl-Radosevich *et al.,* 1998). It is thought that the active component of cinnamon is a methylhydroxychalcone polymer (MHCP) that was isolated and found to potentiate the activity of insulin by 2 fold for glucose uptake and 4 fold for glycogen synthesis in 3T3-L1 adipocytes but only had a slight effect without the presence of insulin (Jarvill-Taylor *et al.*, 2001).

Confirming the *in vitro* results, rats fed a hot water extract of cinnamon daily for three weeks showed an almost 150% increase in glucose infusion rate during euglycemic clamp testing (Qin *et al.,* 2003). In a randomized placebo controlled clinical trial, sixty type 2 diabetics (30 men and 30 women) were randomly assigned into groups and given 1, 3, or 6 g of cinnamon a day in the form of capsules for a period of 40 days. By the end of the trial, all three levels of cinnamon had led to similar decreases in fasting serum glucose from 18 to 29% with a similar degree of improvement in blood lipid parameters (Khan *et al.*, 2003).

In terms of the mechanism of action for these effects of cinnamon, these studies have not acknowledged the role that hormone and cytokine secretion from adipose tissue may have in mediating whole-body insulin resistance. Adiponectin is an adipocytokine secreted solely by adipose tissue and has been shown to have both antidiabetic and antiatherogenic properties (Iwaki *et al.*, 2003). Plasma concentrations of adiponectin are

lower in type 2 diabetics and obese individuals and have a strong negative correlation with insulin resistance (Hotta *et al.,* 2000, Abbasi *et al.,* 2004). In both obese and lipoatrophic mice, hypoadiponectinemia leads to insulin resistance while adiponectin supplementation alleviates the condition (Yamauchi *et al.,* 2001). Specifically, adiponectin activates insulin receptor substrate-1 (IRS-1)-mediated PI3-kinase and glucose uptake in muscle (Jazet *et al.,* 2003) and suppresses hepatic glucose production (Berg *et al.*, 2001). Its antiatherogenic properties include a reduction of monocyte adhesion to endothelial cells (Ouchi *et al.,* 1999) and inhibition of foam cell formation from macrophages (Ouchi *et al.*, 2001). In 3T3-L1 adipocytes, insulin stimulates the secretion of adiponectin through a phosphoinositide 3'-kinase (PI3-kinase) dependent pathway (Bogan and Lodish, 1999) and thiazolidinediones stimulate adiponectin secretion and mRNA expression by activating the transcriptional factor peroxisome proliferator-activated receptor (PPAR)-γ (Maeda *et al*, 2001).

In the present study, a water extract from cinnamon has been used in combination with various insulin concentrations as treatments on 3T3-L1 adipocytes to study their effects on glucose uptake, adiponectin secretion and intercellular ceramide accumulation. Ceramide is a sphingosine based lipid signaling molecule. It is considered to be the second messenger in the sphingomyelin signaling cascade and research is now linking ceramide to insulin resistance. Skeletal muscle ceramide levels are increased in obese insulin resistant humans (Adams *et al.,* 2004). Ceramide has been shown to directly inhibit the insulin signaling pathway by decreasing insulin stimulated phosphorylation and activity of PKB/Akt proteins in the pathway (Mei *et al*, 2003).

The results of this study show that a water extract from cinnamon mimics insulin by increasing glucose uptake by two-fold in 3T3-L1 adipocytes but, unlike insulin, concurrently inhibits the secretion of adiponectin from these same cells.

6.3 Materials and Methods

6.3.1 Materials

Refer to Manuscript I Materials section (4.3.1) for a full list of materials used in the experiment described here in Manuscript II.

6.3.2 Cinnamon Extract Preparation

Cinnamon sticks were purchased from a local grocery store and ground into a powder using a plant tissue grinder. The powder (98.5 g) was soaked in room temperature bidistilled water (5:1) overnight for 14 hours with continuous stirring. The resulting extract was centrifuged at 400g for 30 minutes to remove particulates and fiber. The supernatant was then filtered through Whatman #1 paper, the filtrate collected and freeze dried. The freeze-dried extract (2.9 g) was crushed into powder and stored at –80°C until use.

6.3.3 Cell Culture

Refer to Manuscript I Materials section (4.3.3) for a description of the techniques used to grow and maintain the 3T3-L1 cell culture

6.3.4 Glucose-Uptake

Glucose uptake activity was determined in the same manner as Manuscript I (section 4.3.4) with the exception that cells were treated with cinnamon extract (CE) rather than MC extracts.

6.3.5 MTT Viability Assay

The MTT cell viability assay was performed in the same manner as Manuscript I (section 4.3.5) with the exception that cells were treated with CE dissolved in KRH rather than MC extracts.

6.3.6 Wortmannin Inhibition of Glucose Uptake

The wortmannin assay was performed in the same manner as Manuscript I (section 4.3.6) with the exception that treatment conditions chosen for this assay were simply the KRH buffer control, 50 nM insulin alone, and 0.2 mg/ml CE alone.

6.3.7 Adiponectin Secretion

Adiponectin secretion was determined in the same manner as Manuscript I (section 4.3.7) with the exception that treatment conditions chosen for this assay were simply the KRH buffer control and the 0.2 mg/ml CE.

6.3.8 Ceramide Analysis

Ceramide analysis occurred in the same manner as Manuscript I (section 4.3.8) with the exception that treatment conditions chosen for this assay were 50 nM insulin or 0.2 mg/ml CE.

6.3.9 Statistical Analysis

All data were analyzed using a one-way ANOVA and SAS version 8e. Significant differences between treated and control (within groups based on insulin concentration for glucose uptake results) was determined using least-square means and Tukey's post-hoc. Level of significance was set at $p<0.05$. All data are expressed as mean ± standard deviation and each value represents a minimum of three (n=3-4) repetitions from different experiments. All residuals were tested for normality and were shown to be so except for the ceramide results that were not normal and were therefore transformed using the square root to become normal.

6.4 Results

6.4.1 Glucose Uptake

In this assay glucose uptake was allowed to proceed for one h before the cells were lysed and measured for intercellular glucose concentration. Preliminary results allowing a 10 min glucose uptake period were sufficient to show insulin mediated changes to uptake but the cinnamon extract had no effect. After the one h period of glucose uptake, significant insulin activation of 2-deoxy-glucose uptake was observed at both concentrations of insulin with the maximum stimulation detected at 50 nM insulin (Figs. 1 and 2). The uptake of 2-deoxy-glucose ranged between 2- to 5-fold for 0.5 nM (4339 ± 1760 dpm) and 50 nM insulin(13945 ± 2213 dpm), respectively, as compared to that of basal uptake (2645 ± 1248 dpm).

Adipocytes exposed to 0.2 mg/ml CE without the presence of insulin showed a significant increase (approximately 2-fold) in glucose uptake over the KRH buffer control ($p<0.01$) (Fig. 1). Any glucose uptake induced by the two higher concentrations of CE in this treatment group was not significant and returned to near basal uptake at the highest concentration. In combination with 0.5 nM insulin, CE did not show any significant changes to glucose uptake from the 0.5 nM insulin control but the uptake did show a trend to decrease with increasing CE concentration (Fig. 1). In combination with 50 nM insulin, the two highest concentrations (0.3 and 0.4 mg/ml) of CE significantly decreased glucose uptake ($p<0.01$) compared to the uptake with 50 nM insulin alone (Fig.1).

6.4.2 MTT Assay

The CE treatment significantly increased cell viability over the KRH control. This assay is based on the reduction of MTT into purple formazan pigment by the succinate-tetrazolium reductase system in the respiratory chain of the mitochondria (Gomez *et al.*, 1997). This system is active only in metabolically active cells and changes in MTT reductase activity are detectable even before membrane lysis, making the MTT assay a refined marker of cellular viability (Gomez *et al.,* 1997, Tollefson *et al.*, 1996). The absorbance reading correlates with viable cell number and metabolic activity of the cells.

The KRH control cells experienced a significant decrease in absorbance measurement in comparison to the DMEM control wells (Fig. 2). Treatment with the two lower concentrations of CE (0.2 and 0.3 mg/ml) increased absorbance to the same level as the DMEM control and were significantly different from the KRH control. The highest concentration of CE also increased absorbance to the same degree as the DMEM control but did not show significant difference from the KRH control.

6.4.3 Effect of Wortmannin on CE Stimulated Glucose Uptake

The effect of wortmannin on 2-deoxyglucose uptake presence of 50 nM insulin or 0.2 mg/ml CE is shown in Fig. 3. In the absence of pre- and concurrent incubation with wortmannin, 50 nM insulin caused a 7.5-fold stimulation of 2-deoxy-D-glucose uptake. The treatment combination of 0.2 mg/ml CE caused an almost 3-fold stimulation of 2-deoxy-glucose uptake in the absence of pre- and concurrent incubation with wortmannin. By treating the adipocytes with 50 nM wortmannin, increased uptake of glucose by treatment with 0.2 mg/ml CE and 50 nM insulin was completely inhibited back to basal uptake levels and slightly below basal levels respectively.

6.4.4 Adiponectin Secretion

These adiponectin concentrations were measured in the medium collected after the one h period of glucose uptake during the glucose uptake experiments. Since the 0.2 mg/ml CE treatment significantly increased glucose uptake in the adipocytes, adiponectin concentration was measured in this treatment medium. Compared to the KRH control medium which had an adiponectin concentration of 7.9 ng/ml, adiponectin secretion by 3T3-L1 adipocytes was significantly and dramatically decreased by treatment of the cells

with 0.2 mg/ml CE to the point that adiponectin was no longer detectable in the medium (Fig. 4).

6.4.5 Cellular Ceramide Accumulation

No significant differences in intercellular ceramide accumulation were seen between the treatment with 0.2 mg/ml CE and the control (Fig. 5). The HPLC unit was not able to calculate the area of 2 out of the 3 ceramide peaks used to calculate the ceramide concentration for the 0.2 mg/ml CE treatment. These peaks with no calculated area were below the detection limit of the HPLC machine and were set as zero when determining ceramide concentration. Although ceramide concentrations were appeared to be slightly lower with the CE treatment, the statistical probability of there being a true difference between the treatment and the control was too high to suggest even a trend. Further repetitions are required to determine if significant differences exist.

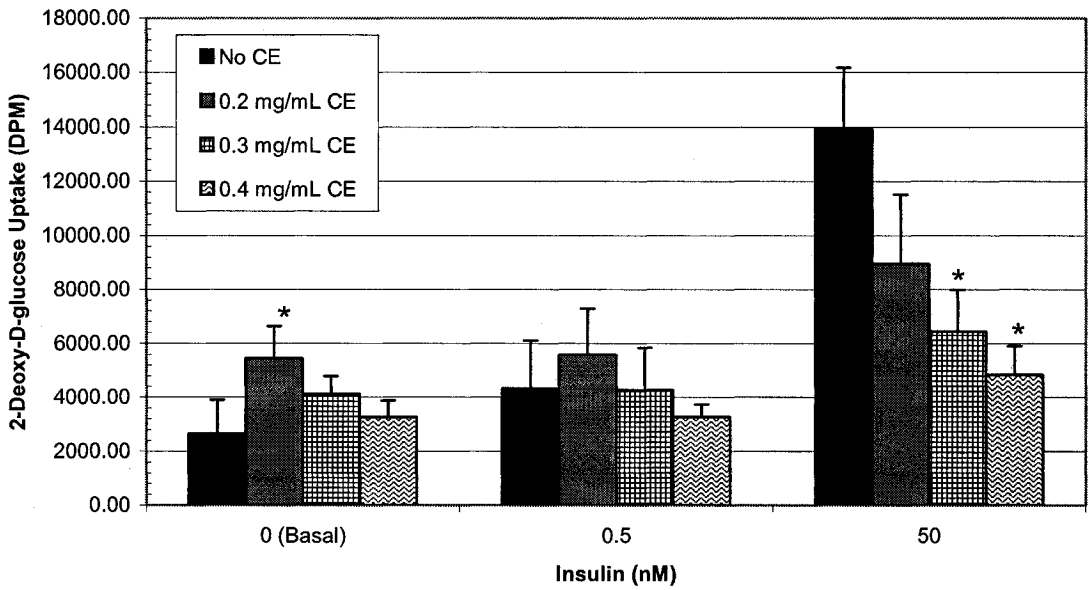


Fig. 1 Cinnamon water extract (CE) effect on glucose uptake in 3T3-L1 adipocytes with or without the presence of 0.5 nM or 50 nM insulin. DPM denotes the disintegrations per min. Data are mean uptakes ±SD from 3 or more independent experiments performed in triplicate, expressed as disintegrations per min (DPM). Significant difference between untreated cells (solid black bar) and the CE treated cells was tested within groups based on insulin concentration. * $p<0.05$ (Using Tukey's post-hoc)

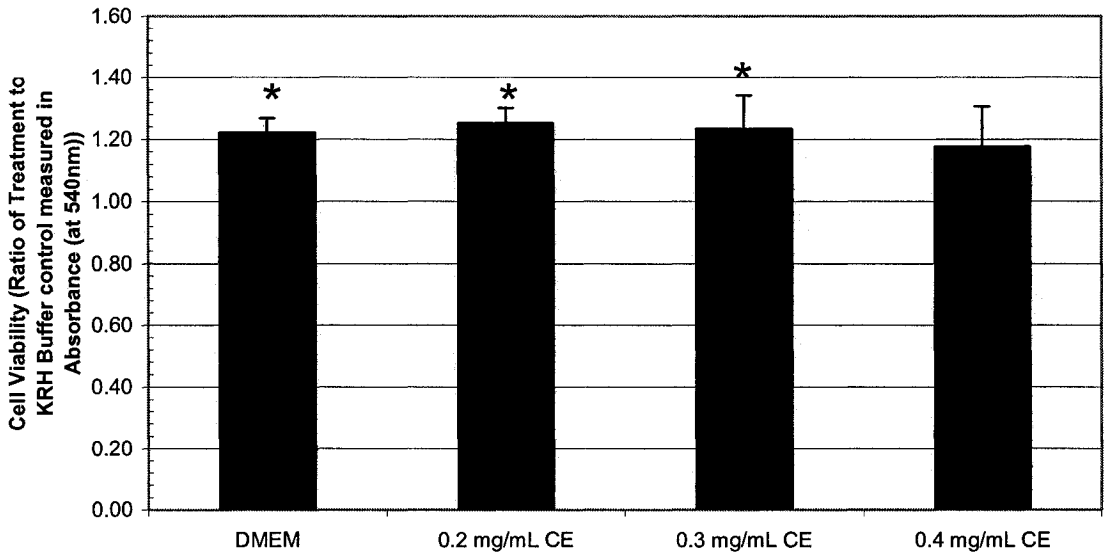


Fig. 2 The effect of CE extract concentration on cell viability using the MTT assay. Cells were exposed to the CE treatment for 90 min in order to mimic the half hour pretreatment and one hour glucose uptake used in the glucose uptake experiments. Data are mean ± SD of three replicate experiments and expressed as population growth, absorbance measured for the treatment divided by the absorbance measured for the KRH buffer control population (Absorbance = 0.151±0.01 at 540nm) . Significant difference was tested between the treatments shown and the KRH buffer control (ratio = 1). * $p<0.05$ (using Tukey's post-hoc).

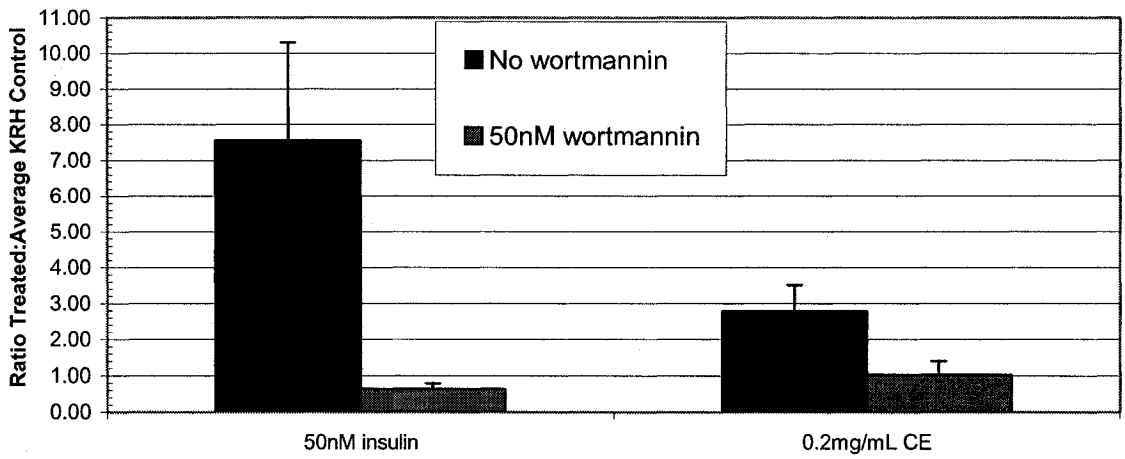


Fig. 3 The effect of 50 nM wortmannin on 3T3-L1 adipocyte glucose uptake when treated with 50 nM insulin alone and with 0.2 mg/ml cinnamon extract alone. Cells were exposed to wortmannin during from the beginning of the 30 min KRH buffer pre-treatment until the end of the 60 min glucose uptake period. Data are expressed as the DPM obtained in the treatment divided by the DPM from the KRH buffer control (basal glucose uptake). Data were derived from the triplicates of three independent experiments, and expressed as means ± SD.

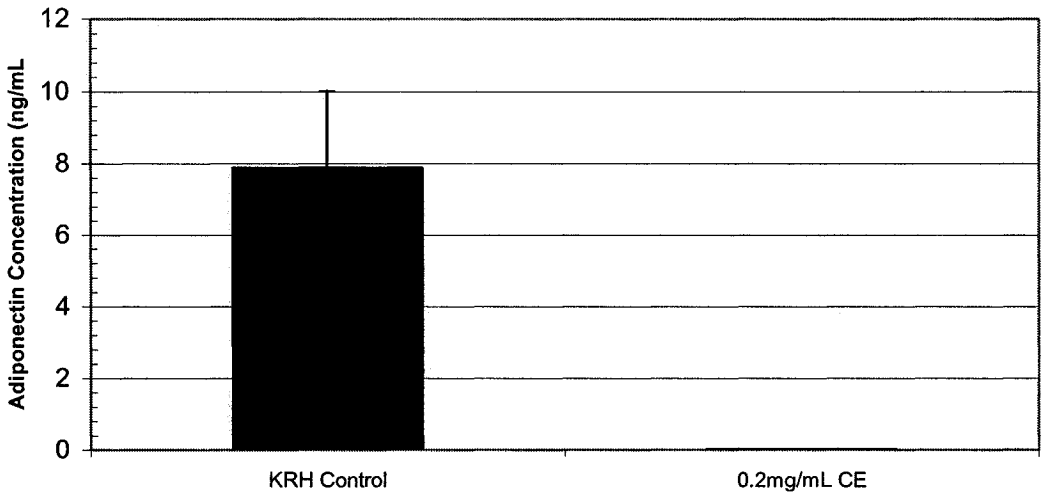


Fig. 4 Effect of cinnamon extract (CE) on adiponectin secretion into the medium. The medium was collected from the 12-well plates at the end of the glucose uptake experiments after the 60 min exposure to ^{3}H-Deoxy-D-glucose with or without 0.2 mg/ml CE. The bars represent the mean ± standard deviation of at least three repetitions (n=3-4) from different experiments.

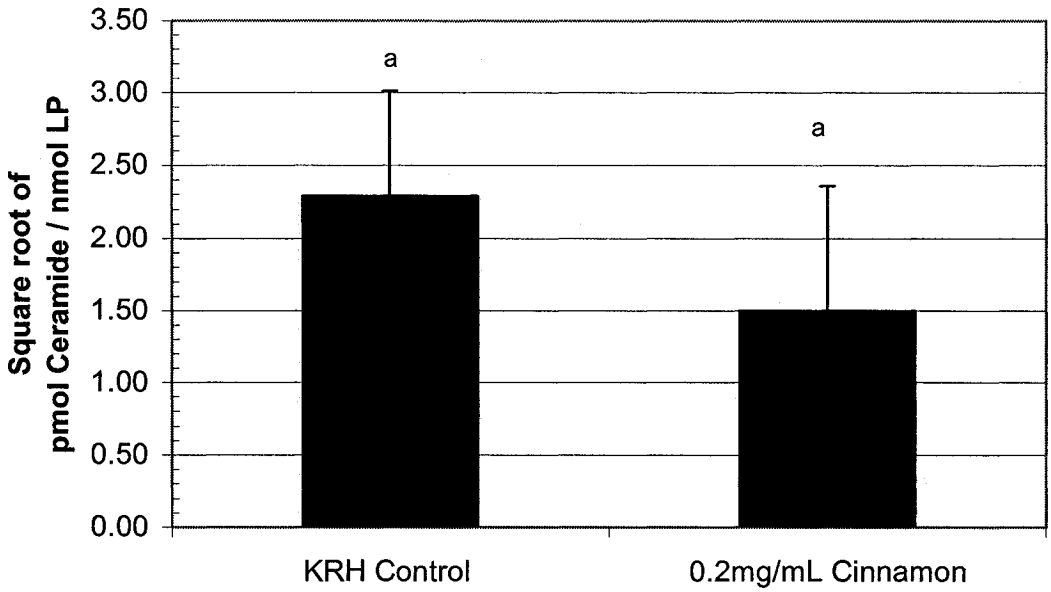


Fig. 5 Effect of 0.2 mg/ml cinnamon water extract on ceramide concentration within the 3T3-L1 adipocytes. The cells were pretreated in 60mm plates for 30 min with or without 0.2 mg/m MCW alone and 0.5 nM insulin alone or with 0.2 mg/ml MCW. ^{3}H-Deoxy-D-glucose was added along with the treatments for 60 min after which the cells were collected and the lipids were extracted as described. The ceramide content was normalized per lipid phosphate content of the cells. Residuals of the data were not normal so the square root of the data was taken to make them so. Data are expressed as mean ± standard deviation of two to three repetitions from different experiments. Significant difference was set at $p<0.05$ (using Tukey's post-hoc).

6.5 Discussion

The present study shows the ability of a water extract from cinnamon to increase glucose uptake in 3T3-L1 adipocytes without the presence of insulin. At a concentration of 0.2 mg/ml the cinnamon water extract (CE) used in this study increased glucose uptake by 3T3-L1 adipocytes by two-fold over basal uptake (Fig. 1). At concentrations above 0.2 mg/ml, however, glucose uptake stimulation observed with CE was eliminated as glucose uptake remained at the basal level at the 0.4 mg/ml dose of CE. Despite the positive outcome of glucose uptake stimulation with the CE dose of 0.2 mg/ml, the present findings indicate that when CE was combined with 50 nM insulin, CE decreased the ability of insulin to increase glucose uptake in a dose-dependent fashion (Fig. 1). Similar results have recently been shown in a rat epididymal fat pad assay where low concentrations of cinnamon water extract reduced insulin stimulated glucose uptake

(Anderson *et al.,* 2004). It is not uncommon for plant extracts to increase glucose uptake on their own but negatively impact the effects of insulin and this does not necessarily predict their effects during a clinical trial (Liu *et al.*, 2001, Hong *et al.*, 2000). In that regard, a water extract of *Lagerstroemia speciosa* that showed similar results to the present findings on glucose uptake in 3T3-L1 adipocytes was shown to decrease blood glucose levels in Type II diabetics by 30% over 2 weeks (Judy *et al.*, 2003). The results of the MTT assay indicate that the changes to glucose uptake instigated by CE cannot be fully explained by differences in the number of viable cells among the treatments. The concentrations of CE used in the experiment appear to increase the number of viable cells or at least the metabolic activity of the cells over the KRH control (Fig. 2). The increase in cell viability, however, was minimal and so could not support the two-fold increase in glucose uptake caused by treatment of the cells with 0.2 mg/ml CE and also cannot explain the dose-dependent decrease in glucose uptake observed with CE in the presence of insulin. In contrast to the present findings, when the MHCP isolate from cinnamon was tested on 3T3-L1 cells, it exhibited an insulin-sensitizing effect (Jarvill-Taylor *et al.*, 2001). Hence, in concert with the present study results, this indicates that various components of CE are affecting glucose uptake in a different manner and that some CE components present in the water extract may be repressing the activity of insulin-sensitizing components such as MHCP.

In determining the mechanism by which cinnamon works, its water extracts have been shown to increase IR phosphorylation and decrease PTP-1 activity in *in vitro* adipose cells (Imparl-Radosevich *et al.,* 1998). Cinnamon water extracts also increase IRS-1 phosphorylation and IRS-1/PI3-kinase association in the skeletal muscle of rats (Qin *et al.*, 2003), indicating that cinnamon acts upstream of PI3-kinase near the surface of the cell. The results of the present study indicate that CE may be competing with insulin at some point along the insulin signaling pathway because at each concentration of CE, glucose uptake occurred to the same amount between the CE on its own and the same concentration of CE with 0.5 nM insulin (Fig. 1). With 50 nM insulin, CE dose-dependently decreased glucose uptake but never to a degree of uptake below that seen with CE on its own (Fig. 1). These results indicate that CE, especially at higher concentrations, is predominantly controlling glucose uptake by the 3T3-L1 adipocytes.

The CE does not appear to act directly on the cell surface as, unlike insulin, one hour of concurrent CE and glucose exposure was required to show any changes in glucose uptake due to the treatment. As theorized previously with the MHCP component of cinnamon (Jarvill-Taylor *et al.*, 2001), the CE active component(s) may have taken this extra time to pass through the cell membrane and reach the point in the cell at which they can exert their effect.

In the present study, the ability of wortmannin to completely inhibit the increased glucose uptake caused by 0.2 mg/ml CE to basal uptake levels (Fig. 3) shows that, similar to insulin, CE relies on a wortmannin sensitive protein to increase glucose uptake. This effect of wortmannin on CE-induced glucose uptake also confirms that the increase in glucose uptake seen with CE is not simply a result of a difference in viable cell number or cell metabolic activity. Wortmannin is a potent and irreversible inhibitor of the p110 isoform of PI3-kinase, thus preventing insulin-stimulated GLUT4 and GLUT1 translocation and inhibiting glucose uptake with nanomolar efficiency (Hausdorff *et al.*, 1999). For this reason, wortmannin is often used in cell culture to test the reliance of glucose uptake potentiating substances or extracts on this protein (Jarvill-Taylor *et al.*, 2001, Radosevich *et al.*, 1998). More recent evidence shows that wortmannin has a second higher affinity, reversible target that may or may not be a PI3-kinase but is involved in the p38 MAPK pathway activation of cell surface GLUT4 (Somwar *et al.*, 2001). Further research is needed to positively identify the high affinity target of wortmannin and to determine whether the glucose uptake activity of CE relies on one or both of these wortmannin targets.

The most striking and novel finding of the present study is that although 0.2 mg/ml CE was able to increase glucose uptake, this treatment appeared to concurrently inhibit all detectable adiponectin secretion from the 3T3-L1 adipocytes (Fig. 4). Given that insulin has been shown to stimulate adiponectin secretion from 3T3-L1 adipocytes by increasing the release of adiponectin from a distinct, secretory compartment within the cell (Bogan and Lodish, 1999), this CE treatment cannot be considered a complete insulin-mimetic. Also, given the mounting research to show adiponectin as insulin-sensitizing and anti-atherogenic adipocytokine, these adiponectin secretion results do not support CE as an antidiabetic in this regard. CE may be inhibiting adiponectin secretion

by affecting the same pathway that insulin uses to regulate the compartmental release of adiponectin or by acting on PPAR-γ. PPAR-γ is a transcriptional factor that affects insulin-sensitivity and glucose metabolism in part by regulating the expression and secretion of adipocytokines, including adiponectin. Thiazolidinediones (TZDs) are a class of antidiabetic drug that act specifically as PPAR-γ ligands and have been shown to increase both glucose uptake and adiponectin secretion in 3T3-L1 adipocytes (Maeda *et al.*, 2001). Clearly, CE has the opposite effect of these well known anti-diabetic drugs in terms of adiponectin secretion but it remains to be determined which component(s) of CE are inhibiting the secretion and how this inhibition is occurring.

In summary, at a concentration of 0.2 mg/ml the water extract of cinnamon used in the present study was able to act as an insulin-mimetic in terms of its ability to increase glucose uptake by 2-fold in 3T3-L1 adipocytes. However, with this increased uptake, CE concurrently showed an opposite effect to insulin by completely inhibiting adiponectin secretion from the cells. Also, in combination with insulin, CE showed a dose-dependent inhibition of insulin-stimulated glucose uptake. Although these results indicate both pro and antidiabetic effects of CE, pure cinnamon powder has been shown to improve blood glucose and lipid parameters in Type II diabetics (Khan *et al*, 2003) and it may be that components in CE become altered or remain unabsorbed during digestion. Given the potency of adiponectin to mediate whole-body insulin resistance and atherogenesis, however, the results of the present study indicate that further research is needed regarding the effects of cinnamon on the secretion of adiponectin and other hormones and cytokines that regulate insulin sensitivity. Such studies will help determine the overall and long-term effects of cinnamon consumption on diabetes and its complications.

VII. SUMMARY AND CONCLUDING REMARKS

The major findings of this thesis are that water extracts from both MC and cinnamon can increase glucose uptake and affect adiponectin secretion from 3T3-L1 adipocytes but in different ways from each other.

At a concentration of 0.2 mg/ml, the MCW extract in combination with 0.5 nM insulin was able to increase glucose uptake into the cells to a greater degree than 0.5 nM insulin alone. On its own or in combination with 50 nM insulin, this concentration of MCW had no effect on glucose uptake. In combination with the sub-optimal concentration (0.5 nM) of insulin, the two higher concentrations of MCW had similar effects to the 0.2 mg/ml concentration but without a statistically significant increase in glucose uptake. The ethanol extract of MC did not increase glucose uptake with any combination of insulin but dose-dependently decreased glucose uptake compared to respective controls on its own and in combination with 50 nM insulin. These results suggest that a preferentially water soluble component(s) of MC is able to increase the glucose uptake activity of sub-optimal concentrations of insulin while a preferentially ethanol soluble component(s) decreases the activity of both insulin-sensitive and basal glucose uptake.

Similar to MCW, the water extract from cinnamon was able to increase glucose uptake at a concentration of 0.2 mg/ml. However, this increase was only seen with CE on its own, without the presence of insulin. In the presence of 0.5 nM insulin, CE did not increase glucose uptake over the 0.5 nM insulin control. Instead, at each concentration of CE, glucose uptake occurred to the same amount between the CE on its own and the same concentration of CE with 0.5 nM insulin. In combination with 50 nM insulin, CE significantly and dose-dependently decreased glucose uptake. These differences between the MC and cinnamon extracts effects on glucose uptake suggest that MC contains a water-soluble component(s) that increases the sensitivity of 3T3-L1 adipocytes to sub-optimal concentrations of insulin while cinnamon contains a component(s) that both mimic and compete with insulin to affect glucose uptake.

In this thesis, the MTT assay was used successfully to demonstrate the lack of toxicity of the MC and cinnamon extracts on the 3T3-L1 cells and to show that any

changes to glucose uptake due to the extracts was not simply a result of changes in the number of viable cells.

Although different in their relationship with insulin to increase glucose uptake, wortmannin was able to inhibit the effects of both MCW and CE and maintain glucose uptake to below basal levels in the presence of these extracts. These results indicate that the activity of both extracts rely on the activation of PI3-kinase and/or another, as yet unidentified, wortmannin sensitive target to increase glucose uptake. As wortmannin prevents the translocation and activation of GLUT4, it seems that both MCW and CE may rely on these same effects to increase glucose uptake.

The most surprising and novel finding of this thesis was the difference between the MCW with 0.5 nM insulin treatment and the CE alone treatment on the secretion of adiponectin from the 3T3-L1 adipocytes. While 0.2 mg/ml MCW on its own showed a trend to increase adiponectin secretion, in combination with 0.5 nM insulin this increase was greater and became statistically significant. This effect of MC on adiponectin secretion is a major new finding that may point in an as yet unexplored direction examining the potential of MC, and other anti-diabetic plant extracts, to regulate the expression and secretion of hormones and cytokines that affect insulin sensitivity. Unexpectedly, 0.2 mg/ml CE completely inhibited all adiponectin secretion from the adipocytes. Given the ability of adiponectin to increase whole body insulin sensitivity, this result does not support the anti-diabetic activity of cinnamon shown in rats and humans or the present studies findings on its ability to increase glucose uptake.

Unfortunately, the effect of MC and cinnamon on intercellular ceramide accumulation in 3T3-L1 adipocytes could not be determined in this thesis due to a low number of repetitions and high variability in the results. No significant differences were found between the active glucose uptake extracts and their controls on the concentration of ceramide accumulated in the cells. Further repetitions of this experiment are required to reveal any significant differences that may occur.

The 3T3-L1 adipocytes used in the present study cannot fully mimic the functioning of adipocytes within the diabetic human as the *in vitro* cells are not exposed to the range of signaling molecules secreted by other cells in the body, the food breakdown products carried in the blood and other factors that can alter the functioning

and growth of adipose cells in the human body. Varying the length of exposure and removing the wortmannin from the media during the glucose uptake period, would have shown which, if not both, wortmannin-sensitive target that the MC and cinnamon extracts relied on to increase glucose uptake by the cells. This study could have been strengthened by testing the effect of MC and cinnamon extracts on the secretion of other adipocytokines known to affect insulin sensitivity such as TNFα, IL-6 and resistin. Testing the effects of MC and cinnamon extracts on PPAR-γ expression could have offered a clearer explanation as to how the extracts altered adiponectin secretion. As well, further repetitions of the ceramide experiment may have revealed a mechanism by which the tested extracts were able to alter glucose uptake in the adipocytes.

Future studies in terms of the anti-diabetic potential of both MC and cinnamon extracts should focus on two issues, the active component(s) of the extracts and the mechanism of action by which the extracts affect diabetes and its complications in the clinical situation. Identifying the active component(s) through fractionation, isolation and molecular identification techniques (HPLC, gas liquid chromatography, mass spectrometry, etc.) and *in vitro* experiments similar to the present study, may allow the potency and effectiveness of the extracts to be increased by increasing the concentration of the active component(s) in the extract. The extracts should then be further tested *in vivo* to show their effects not only on glucose and lipid blood parameters but also on cytokine and hormone levels in the blood. These results combined with measurements of PPAR-γ expression and intercellular ceramide accumulation in adipose and skeletal cells isolated from the subjects should reveal how the extracts affect the *in vivo* diabetic situation and its complications along with the mechanism(s) of action by which these effects occur.

The results of this thesis are the first to show the ability of a water extract from MC fruit and seeds to increase glucose uptake in the presence of sub-optimal concentrations of insulin. Research into the specific component(s) of MC causing the increased glucose uptake shown in this thesis would be useful towards increasing the anti-diabetic potential of MC extracts by isolating and concentrating the active component(s). This thesis is also the first to demonstrate an effect of natural, potential anti-diabetics on the secretion of adiponectin. This finding is important given the strong

role that adiponectin plays in regulating whole body insulin sensitivity and its protective effects against atherosclerosis and inflammation. Given the strong but opposite effects of MC and cinnamon extracts on this adipocytokine, further research is warranted to understand exactly how these extracts control adiponectin expression and secretion, how this effect on adiponectin is related to the extracts effects on insulin resistance, and if the extracts affect the expression and/or secretion of other signaling molecules in a similar manner.

VIII. NOVEL FINDINGS

The novel findings demonstrated in this thesis are summarized below:

1) A combination of 0.2 mg/ml fresh, unripe MC fruit (seeds included) water extract and 0.5 nM insulin increases 2-deoxy-glucose uptake into 3T3-L1 adipocytes and simultaneously increases adiponectin secretion from the same cells.
2) The component(s) of MC fruit that increase 2-deoxy-glucose uptake into the 3T3-L1 cells are preferentially water soluble.
3) The increase in 2-deoxy-glucose uptake into 3T3-L1 adipocytes caused by the combination of 0.2 mg/ml MC water extract and 0.5 nM insulin is completely inhibited by treatment of the adipocytes with 50 nM wortmannin.
4) A 0.2 mg/ml cinnamon water extract that increases 2-deoxy-glucose uptake into 3T3-L1 adipocytes without the presence of insulin, simultaneously inhibits adiponectin secretion from the adipocytes.

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APPENDICES