

Green Tea Polyphenols: a Natural Therapeutic Approach for Metabolic Syndrome and Diabetes Prevention

A thesis submitted for the Degree of Doctor of Philosophy

by

Jane Jung Yeon Kim



School of Medical and Molecular Biosciences

Faculty of Science

University of Technology Sydney, Australia

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Declaration

This thesis titled “Green Tea Polyphenols: a Natural Therapeutic Approach for Metabolic Syndrome and Diabetes Prevention” is of original work. The work presented in this thesis was carried out under the supervision of A/Prof Xianqin Qu and A/Prof Chris Zaslowski at University of Technology, Sydney. This thesis is of original work and has not been submitted by the candidate for the award of any other degree.

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Abbreviations

2DOG	2-deoxyglucose
ACC	acetyl-CoA carboxylase
AGI	Alpha-glucosidase inhibitors
AMPK	AMP-activated protein kinase
ANOVA	one-way analysis of variance
ALT	alanine transaminase
AST	aspartate transaminase
ATCC	American Type Culture Collection
ATPIII	Adult Treatment Panel III
BBR	berberine
BMI	Body mass index
BSA	bovine serum albumin
BW	body weight
cAMP	Cyclic adenosine monophosphate
CEBP α	CCAAT-enhancer-binding protein- α
CHM	Chinese herbal medicine
ChREBP	carbohydrate-responsive element-binding protein
CM	complete media
CREB	cAMP response element-binding protein
CRP	C-reactive protein

CVD	cardiovascular disease
DAG	diacylglycerol
DBP	diastolic blood pressure
DMEM	Dulbecco's Modified Eagle Medium
DMSO	dimethyl sulfoxide
EC	epicatechin
ECG	epicatechin gallate
EGC	epigallocatechin
EGCG	epigallocatechin gallate
EGIR	European Group for the Study of Insulin Resistance
fatty acyl-CoA	fatty acyl-coenzyme A
FBS	fetal bovine serum
FFA	free fatty acids
FAS	fatty acid synthase
G-6-Pase	glucose-6-phosphatase
GDH	glutamate dehydrogenase
GLUT1	glucose transporter 1
GLUT2	glucose transporter 2
GLUT3	glucose transporter 3
GLUT4	glucose transporter 4
GOD-PAP	glucose oxidase-peroxidase
GS	glycogen synthesis

GSK3 β	glycogen synthase kinase 3 β
GTP	green tea polyphenols
HOMA-IR	homeostasis model assessment of insulin resistance
HDL	high-density lipoprotein
HE	haematoxylin and eosin
HFD	high-fat diet
FPG	fasting plasma glucose
HSL	hormone-sensitive lipase
IDF	International Diabetes Federation
IFG	impaired fasting glucose
IFN	interferon
IGT	impaired glucose tolerance
IL	interleukin
IRS	insulin receptor substrate
ISDN	isosorbide dinitrate
LC-MS	liquid chromatography-mass spectrometry
LDL	low-density lipoprotein
LKB1	liver kinase B1
MAPK	mitogen-activated protein kinase
MCP-1	monocyte chemotactic protein-1
MetS	metabolic syndrome
MI	myocardial infarction

MLC	myosin light chain
MTT	3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide
NAD	nicotinamide adenine dinucleotide
NAFLD	non-alcoholic fatty liver disease
NASH	non-alcoholic steatohepatitis
NCEP	National Cholesterol Education Program
NEFA	non-esterified free fatty acids
NIDDM	non-insulin dependent diabetes
NO	nitric oxide
OGTT	oral glucose tolerance test
ORO	oil red O
PAI-1	plasminogen activator inhibitor-1
PCOS	polycystic ovarian syndrome
PDE3b	phosphodiesterase 3b
PDH	pyruvate dehydrogenase
PDK1	PI-dependent kinase 1
PEPCK	Phosphoenolpyruvate carboxykinase
PI3-K	phosphoinositide 3-kinase
PKA	protein kinase A
PKB/Akt	protein kinase B
PKC	protein kinase C
PPAR γ	peroxisome proliferator activated receptor- γ

PVD	peripheral vascular disease
PVDF	polyvinylidenedifluoride
RBP4	retinol binding protein-4
SBP	systolic blood pressure
SM	starving media
SREBP-1c	sterol regulatory element binding protein-1c
STZ	streptozotocin
T2D	type 2 diabetes
TAG	triacylglycerol
TG	triglycerides
TNF- α	tumour necrosis factor- α
TZD	thiazolidinedione
VLDL-C	very low-density lipoprotein-cholesterol
WHO	World Health Organization
WTH	Waist-to-Hip
ZF	Zucker fatty (fa/fa)

Abstract

Metabolic syndrome (MetS) is a collection of interrelated disorders that increase the risk of type 2 diabetes (T2D) and cardiovascular disease. Abnormalities associated with MetS include, but are not limited to, central obesity, insulin resistance, glucose intolerance, hyperglycaemia, hyperlipidaemia and hypertension. MetS has caused an encumbrance to public health globally. Due to the complex nature of MetS and the lack of availability of effective medications, there is an urgent need for the implementation of novel oral agents to manage MetS and prevent T2D and cardiovascular complications.

The beneficial effects of green tea polyphenols (GTP) for MetS have been recently reported. However, the direct effects and mechanisms of GTP on abnormalities of glucose and lipid metabolism are not fully understood. This thesis investigated effects and mechanisms of GTP in obesity, insulin resistant and metabolic dysfunctions through *in vitro* and *in vivo* studies. The effects of GTP on biochemical parameters and its actions in major organs involved in glucose homeostasis, fat metabolism and insulin sensitivity such as skeletal muscle, liver and adipose tissue were elucidated. The study was further extended to investigate the effect of GTP on a model of non-alcoholic fatty liver disease (NAFLD).

In 3T3-L1 adipocytes, GTP-EGCG (epigallocatechin gallate) improved glucose uptake and inhibited lipolysis significantly. It was revealed that GTP-EGCG significantly increased glucose uptake by up-regulating expressions of IRS-1, PKB/Akt and GLUT4. The probable mechanism for decreased lipolysis with GTP-EGCG may be through down-regulation of PKA, as observed in this thesis. GTP-EGCG also enhanced glycogen synthesis in a dose-dependent manner and significantly increased glycogen synthesis two-fold compared with insulin alone in HepG2 cells. Western blotting revealed that phosphorylation of GSK3 β and

GS was significantly increased in GTP-EGCG treated HepG2 cells compared to non-treated cells. GTP-EGCG also significantly inhibited lipogenesis and the likely mechanism behind this inhibition involved enhanced expression of phosphorylated AMPK α and ACC in HepG2 cells.

A marked state of insulin resistance was observed in high fat diet (HFD) fed obese Zucker fatty (ZF) rats compared to their lean littermates, which is associated with significant defects in the insulin-signalling/glucose transport system. GTP significantly improved fasting metabolic parameters, including glucose, insulin, TG, NEFA and cholesterol, and improved insulin sensitivity and glucose intolerance in HFD ZF rats. The likely molecular mechanisms involved are regulation of the elements of the insulin-signalling pathway such as PKB/Akt, GLUT4 translocation and regulation of PKC translocation in skeletal muscle of HFD ZF rats. Data from this thesis also strongly supports that GTP reduces fat accumulation in the liver and hepatic insulin resistance. This is evidenced by significant reduction in serum levels of ALT and AST hepatic TG and lipid content in HFD ZF rats administered GTP. Mechanisms are likely to up regulate GSK3 β and GS expressions thereby enhancing glycogen synthesis and down-regulating *de novo* lipogenesis through regulation of AMPK α , ACC and PEPCK expressions.

Overall, the results presented in this thesis provide insight into GTP as a potential therapeutic agent for MetS and related disorders such as T2D, obesity and NAFLD. GTP may be a valuable natural and cost-effective therapy for the treatment and prevention of metabolic disorders.

1
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CHAPTER 1: INTRODUCTION

1. Introduction

1.1. Metabolic Syndrome

Metabolic syndrome (MetS) is a collection of interrelated metabolic abnormalities which include centrally distributed obesity, decreased high-density lipoprotein (HDL) cholesterol, elevated triglycerides (TG), elevated blood pressure, and hyperglycaemia (Alberti & Zimmet, 2005). Recent studies have shown that individuals suffering from metabolic syndrome have an increased risk of both type 2 diabetes (T2D) and cardiovascular disease (CVD) (Lombard & Ascott-Evans, 2002; Grundy et al., 2005). MetS is therefore thought to be a driver of these modern day epidemics and has become a major public health challenge worldwide (Alberti et al., 2009). MetS is believed to affect at least one in four adults in the US (Alberti & Zimmet, 2005) and the prevalence of MetS is now fast attaining epidemic proportions worldwide including for children and adolescents (Poyrazoglu et al., 2014). A better understanding of the underlying biological mechanisms that cause metabolic syndrome to lead to atherosclerotic CVD, T2D and other metabolic disorders such as non-alcoholic fatty liver disease (NAFLD) could help to open up avenues for research into potential therapeutic interventions.

1.1.1. The Definition of Metabolic Syndrome

The identification of the concept of MetS can be first attributed to the work of the Swedish physician, Kylin, who first noted in 1923 a syndrome characterised by the co-occurrence of hypertension, hyperglycaemia and gout (Kylin, 1923). Several decades later in 1947, Vague (Vague et al., 1979) observed that obesity was associated with the metabolic abnormalities

1 often seen with T2D and with CVD. It was not until 1988 that the clinical definition of MetS
2 was further developed by Reaven (Reaven 1988), when he described MetS as involving a
3 group of metabolic abnormalities that included insulin resistance as the main
4 pathophysiological feature. He gave this syndrome the name Syndrome X.

5 Giving MetS a precise clinical definition has proven to be elusive. In the last decade, several
6 working definitions of MetS have been developed; the most widely recognised of which are
7 from the World Health Organization (WHO) (WHO, 1999), Adult Treatment Panel III
8 (ATPIII) (NCEP, 2001), the European Group for the Study of Insulin Resistance (EGIR)
9 (Balkau & Charles, 1999), and the International Diabetes Federation (IDF). Central obesity,
10 insulin resistance, dyslipidaemia (hypertriglyceridaemia and low levels of high-density
11 lipoprotein cholesterol), elevated blood pressure and impaired glucose tolerance are
12 commonly accepted as the major features of MetS (Table 1). However, at present, no
13 consensus exists for specific thresholds or establishing the diagnosis of each of these traits as
14 components of the syndrome (Kassi et al., 2011). As a case in point, unlike the other
15 classifications, the IDF classification places value on country/ethnic-origin of individuals in
16 setting the criteria for waist circumference in assessing central obesity (Table 1).

Table 1. The definition of metabolic syndrome (adapted from Bruce & Bryne, 2009)

	WHO (Impaired glucose tolerance or diabetes and/or insulin resistance and two other factors)	EGIR (Presence of fasting hyperinsulinaemia [the highest 25%] and two other factors)	ATP-III (Three or more of the following factors [TAGs and HDL counted separately])	IDF (Central obesity and two other factors)
Central obesity	WHR ≥ 0.9 (men), 0.85 (women) and/or BMI > 30 kg/m ²	Waist ≥ 94 cm (men), ≥ 80 cm (women)	Waist ≥ 102 cm (men), > 88 cm (women)	Waist > 94 cm (European men), > 90 cm (Asian men), > 80 cm (women)
Blood pressure (mmHg)	$\geq 140/90$	$\geq 140/90$ or treated for hypertension	$> 130/85$ or treated for hypertension	≥ 130 SBP or ≥ 85 DBP or treated for hypertension
Dyslipidaemia (mmol/l)	TAGs ≥ 1.7 , HDL < 0.9 (men), < 1.0 (women)	TAGs ≥ 2.0 or HDL-cholesterol < 1.0 or treated for dyslipidaemia	TAGs ≥ 1.7 , HDL-cholesterol < 1.0 (men), < 1.3 (women)	TAGs ≥ 1.7 , HDL-cholesterol < 1.04 (men), < 1.29 (women)
Dysglycaemia (mmol/l)	Fasting glucose ≥ 6.1 and/or 2 h post-challenge glucose ≥ 7.8 on diabetes	Fasting plasma glucose > 6.1 , but non-diabetic	Fasting plasma glucose ≥ 6.1	Fasting glucose ≥ 5.6 or previous diagnosis of impaired glucose tolerance or diabetes
Insulin resistance	Glucose uptake during hyperinsulinaemic-euglycaemic clamp in lowest quartile for population	Presence of fasting hyperinsulinaemia (ie, among the highest 25% of the non-diabetic population)	<i>Not applicable</i>	<i>Not applicable</i>
Other factors	Impaired glucose tolerance or diabetes and/or insulin resistance and two other factors	Presence of fasting hyperinsulinaemia (the highest 25%) and two other factors	Microalbuminuria (urinary albumin excretion rate > 20 μ g/min or albumin/creatinine ratio > 30 mg/g)	<i>None</i>

BMI, body mass index; DBP, diastolic blood pressure; HDL, high-density lipoprotein; SBP, systolic blood pressure; TAG, triacylglycerol; WHR, waist/hip ratio.

The pathogenesis of the MetS is poorly understood, and has been proved to be complex and multifactorial. While insulin resistance and abdominal obesity have received the most attention as the main underlying features of MetS, there are many other etiological factors that have been linked to the development and progression of MetS. For example, it has been suggested that chronic inflammation is linked with visceral obesity and insulin resistance, which involves the production of abnormal adipocytokines such as tumor necrosis factor- α (TNF- α) interleukin-1 (IL-1), IL-6, leptin, and adiponectin (Kaur, 2014). This is thought to contribute to the development of a pro-inflammatory state and ultimately a chronic,

subclinical vascular inflammation, which results in pro-thrombotic state, atherosclerosis and endothelial dysfunction (Yudkin, 2007; Prieto et al., 2014).

1.2.Components of Metabolic Syndrome

1.2.1. Insulin Resistance

Insulin resistance is characterised by a diminished response to the biological effects of insulin and is associated with obesity, abdominal distribution of fat, elevated TG levels, low HDL cholesterol, small LDL particle size, hypertension and significant rises in inflammatory cytokines (Resnick et al., 2003). Insulin resistance is established as one of the most clinically accepted causative factors of MetS (Gustafson et al., 2007) and a major risk factor T2D and CVD (Reusch, 2002). Insulin resistance is also closely associated with other risk factors, such as central obesity, hypertension, dyslipidaemia, and pro-inflammatory and pro-thrombotic states.

Normally, insulin binds to insulin receptors on target organ cells, resulting in a series of cellular events that promote intracellular glucose transport and metabolism. However, in insulin resistance, there is an inability of peripheral target tissues to react appropriately to normal concentrations of insulin (Jellinger, 2007). In such circumstances, to maintain euglycaemia, the pancreas compensates by secreting increased amounts of insulin (Petersen & Shulman, 2002). After a period of compensated insulin resistance, impaired glucose tolerance eventually develops despite elevated insulin concentrations as insulin resistance increases. Finally, pancreatic β -cell failure results in decreased insulin secretion.

Studies have shown that chronic increase of fat storage in adipose tissue leads to impairment of adipocytes to store TG and therefore activating inflammatory cytokines and pathways (e.g.

1 TNF- α , interferon (IFN)- γ , IL-1 β , and IL-6) and impairing adipogenesis and insulin
2 signalling (Gual et al., 2005; Gustafson & Smith, 2006; McArdle et al., 2013). For example,
3 mice with adipocyte-specific knockout of the insulin receptor have shown inability of insulin
4 to suppress lipolysis, impairment of insulin-stimulated glucose uptake and TG synthesis in
5 adipocytes (Rask-Madsen & Kahn, 2012). As a result, fat is stored in other cell types,
6 including the liver and skeletal muscle, causing ectopic lipids and increased concentrations of
7 FFA, and ultimately leading insulin resistance in adipose tissue, muscle and other
8 surrounding tissues (McArdle et al., 2013).

9 Also, skeletal muscle is the major target organ for glucose uptake in the body and responsible
10 for 80% of glucose disposal in humans (McArdle et al., 2013). A defect in muscle glycogen
11 synthesis has been observed to play significant role in insulin resistance, caused by
12 significant alterations in rate-limiting steps such as glycogen synthase, hexokinase II, and
13 GLUT4 (Shulman, 2000).

14 Accumulation of fat in the liver also commonly occurs with insulin resistance. In hepatic
15 insulin resistance, insulin is unable to suppress gluconeogenesis and glycogenolysis and
16 therefore results in increased glycogen synthesis in hepatocytes (Rask-Madsen & Kahn,
17 2012). An important and early sign of hepatic insulin resistance is the production of hepatic
18 LDL, via altered rate of apoB synthesis and *de novo* lipogenesis, or increased FFA from
19 adipose tissue into the liver (Meshkania & Adelib, 2009). Also, hepatic insulin resistance has
20 been shown to cause chronic inflammation, depicted by the production of abnormal
21 adipokines such as TNF- α , IL-1, IL-6, leptin and resistin (Meshkania & Adelib, 2009; Li et
22 al., 2013). A strong association between fatty liver and hepatic insulin resistance has been
23 observed, however exact linking mechanisms still remain controversial. Factors such as
24 increased calorie intake, lipid storage defects, muscle insulin resistance, genetics and role of

DAG may contribute to the association between fatty liver and hepatic insulin resistance. Studies in both humans and animal models of fatty liver have demonstrated that DAG, leads to activation of PKC ϵ , resulting in hepatic insulin resistance, whereas other studies have found that intracellular compartmentation of DAG is a critical factor in determining whether increased hepatic DAG content results in hepatic insulin resistance and will likely explain why some cases of fatty liver are not associated with hepatic insulin resistance (Samuel et al., 2004; Birkenfeld & Shulman, 2014)

1.2.2. Central Obesity

Obesity refers to a condition of gaining excess body weight from the over-accumulation of fats. Obesity is defined as having a body mass index (BMI) value of $\geq 30 \text{ kg/m}^2$, where BMI for an individual is calculated by as his or her body mass (kg) divided by the square of his or her height (m^2). Over-accumulation of fats in the body may be due to an increase in the number or size of adipocyte cells or a combination of both. According to the WHO, over one billion adults are overweight on a worldwide basis, with at least 300 million being obese. The prevalence of obesity is also increasing in adolescents and children (Moller & Kaufman, 2005; Bays et al., 2005).

In individuals with MetS, adipose tissues lose their storage function, which results in the accumulation of lipids in the liver and skeletal muscles and central abdomen (Schaffler et al., 2006). The significance of central obesity lies in the fact that insulin sensitivity is inversely correlated to the amount of central fats present (Coon et al., 1992; Moller & Kaufman, 2005). Studies have shown that central obesity exerts a strong influence on the development of insulin resistance, MetS and CVD (Despres & Lemieux, 2006; Gastaldelli et al., 2002; Serrano Rios, 1998) because central obesity is associated with a failure to suppress the free

fatty acid (FFA) concentrations through visceral fat tissue lipolysis. This results in an increased CVD risk profile, with impaired endothelial dysfunction, vascular smooth muscle cell proliferation, and alteration of circulating LDL-cholesterol and HDL-cholesterol (Wyne, 2003). This constantly increasing release of FFA also provides increased substrate for hepatic production of TG. Increased accumulation of TG in the liver leads to increased production of very low-density lipoprotein-cholesterols (VLDL-Cs), which leads to dyslipidaemia (Chapman & Sposito, 2008) and is likely to contribute to the development of NAFLD and insulin resistance (Angulo et al., 2007).

1.2.3. Glucose Intolerance

Closely related with insulin resistance, is glucose intolerance, which is also one of the accepted major components of MetS. Glucose intolerance is characterised by higher than normal plasma glucose levels over a measured period of time. It has been observed that a higher degree of glucose intolerance is met with a higher degree of insulin resistance (Reaven, 2006) and thus can be used as a surrogate estimate of insulin resistance as well as an independent predictor of T2D (Warram et al., 1990).

1.2.4. Dyslipidaemia

Dyslipidaemia has a strong influence on metabolic and cardiovascular risk factors. Dyslipidaemia associated with MetS is defined by the presence of abnormal lipid amounts (e.g. TG, cholesterol and fats) in the bloodstream. Dyslipidaemia may be manifested by the elevation of TG total cholesterol, low-density lipoprotein (LDL) cholesterol, and a significant reduction in high-density lipoprotein (HDL) cholesterol (Solano & Goldberg, 2006). The precise pathogenesis of dyslipidaemia is still unknown, yet studies strongly suggest that insulin resistance plays a major part in the development of dyslipidaemia. The main causes of

1 the dyslipidaemia may be due to increased FFA from insulin-resistant fat cells resulting in
2 increased FFA into the liver which promotes TG production, which ultimately stimulates the
3 secretion of apolipoprotein B (ApoB) and LDL cholesterol (Mooradian, 2009). Not only are
4 these changes significantly associated with insulin resistance, but also have been indicated to
5 increase the risk of CVD (Assmann & Schulte, 1992).

6 **1.2.5. Hypertension**

7 MetS is also strongly associated with hypertension. In T2D, insulin resistance and secondary
8 hyperinsulinemia are present and are both very commonly associated with hypertension,
9 atherosclerosis, and obesity (Mendizábal et al., 2013). Reaven explains that insulin-resistant
10 or hyperinsulinaemic subjects have a higher likelihood of developing hypertension. Also,
11 patients with hypertension and high triacylglycerol and low HDL-cholesterol are at greatest
12 risk of CVD (Reaven, 2006). One of the possible mechanisms that may trigger hypertension
13 associated with MetS is excessive visceral fat, which results in insulin resistance. The
14 subsequent hyperinsulinaemia promotes the increase in sodium reabsorption by the kidneys,
15 which is likely to result in higher blood pressure (Halpern et al., 2010). Furthermore, as
16 obesity increases sodium retention, obese subjects require increased arterial pressure to
17 maintain sodium balance leading to impairment of renal-pressure natriuresis (Mendizábal et
18 al., 2013).

19 **1.2.6. Pro-coagulant and Pro-inflammatory Factors**

20 Chronic inflammation may be stimulated by factors such as excessive diet, physical inactivity
21 and ageing (Woods et al., 2012). These factors may contribute to increased secretion of
22 cytokines in adipose tissue and eventually lead to MetS associated insulin resistance and
23 T2D. Alternatively, resistance to the anti-inflammatory actions of insulin may also result in

1 elevated levels of pro-inflammatory cytokines causing low-grade inflammation (Esposito &
2 Giugliano, 2004). Although the exact mechanisms of inflammation are still debated, current
3 research supports the following theories. For example, TNF- α has been shown to play a
4 primary role in stimulating the production of leptin, IL-6 and other inflammatory mediators.
5 TNF- α is increased during weight gain and stimulates adipogenesis and lipolysis, and is also
6 likely to increase blood pressure (Sonneberg et al., 2004). Some other cytokines that are
7 associated with the development of insulin resistance, obesity and CVD include elevated
8 leptin, C-reactive protein (CRP) and NF- κ B. In addition, obesity is associated with low levels
9 of apM1 and adiponectin (Lee & Pratley, 2005; Yang & Barouch, 2007).

10 **1.2.7. A Novel Component of the Metabolic Syndrome: Non-alcoholic** 11 **Fatty Liver Disease**

12 In recent years the liver has been shown to be highly susceptible to accumulate ectopic fats.
13 NAFLD is defined as a disorder with excess fat in the liver due to non-alcoholic causes. The
14 disorder ranges from simple fatty liver (steatosis) to more severe non-alcoholic steatohepatitis
15 (NASH). Ectopic fat accumulation in the liver has been shown to have a direct link to T2D
16 and diabetic complications such as CVD (Toledo & Kelly, 2006). Fatty liver disease is
17 commonly found in T2D patients, with an estimated prevalence ranging from 21% to 78%
18 and is also common to obese individuals (Toledo & Kelly, 2006).

19 To date, the cause of NAFLD is not fully understood, however, studies have been carried out
20 to identify and comprehend the pathogenesis and mechanisms involved. Research suggests
21 that increased FFA into the liver resulting from impaired insulin-suppressed lipolysis in
22 adipose tissue may be a strong contributor to hepatic steatosis. Also, accumulation of TG in
23 hepatocytes has been shown to cause an imbalance between hepatic TG synthesis and
24 utilisation (Yki-Jarvinen, 2002). Recent studies have also shown that severe NAFLD may be

strongly linked to the pathogenesis of CVD (Ballestri et al., 2014), possibly through increased oxidative stress, subsequent lipid peroxidation (Paschos & Paletas, 2009) and release of pro-atherogenic factors from the liver such as CRP, fibrinogen, plasminogen activator inhibitor-1 and other inflammatory cytokines (Targher et al., 2008). Hepatic activation of NF κ B via overexpression of I κ B kinase β has also shown to promote the onset of hepatic insulin resistance (Cai et al., 2005; Tilg & Moschen, 2008).

NAFLD is also strongly associated with obesity but in particular, body fat distribution appears to be significant in the onset and pathogenesis of NAFLD. Excessive intra-abdominal fat, namely central obesity, may be a key causative factor for NAFLD, due to its strong link with insulin resistance and increased circulating FFAs. Overall, studies support that insulin resistance and hyperinsulinaemia play a key role in the pathogenesis of both MetS and NAFLD (Paschos & Paletas, 2009).

1.3. Metabolic Syndrome and Type 2 Diabetes

MetS is regarded as a strong determinant of T2D. It has been observed that the risk factors of MetS, in particular, insulin resistance, obesity, central adiposity, hypertension and hypertriglyceridaemia have also been described as major risk factors for T2D (Hanson et al., 2002). According to recent studies, individuals with MetS are at significantly increased risk of developing T2D and progression to CVD (Kengne et al., 2012).

T2D, also known as non-insulin dependent diabetes mellitus (NIDDM), is the most common form of diabetes affecting 90 - 95% of people with diabetes worldwide and 85 - 90% in Australia. T2D is a complex group of metabolic disorders, where both genetic and lifestyle factors play a major part. Excessive weight gain, inactivity, high blood pressure and poor diet

are major risk factors for its onset. T2D causes dysfunctions in multiple organs or tissues leading to complications, the most common and life-threatening being CVD (Stumvoll et al., 2005).

T2D is characterised by hyperglycaemia as a result of impaired secretion or action of insulin (Rao, 2001). The fasting plasma glucose (FPG) levels within ≥ 6.1 and < 7 mmol/l (or blood glucose of ≥ 5.6 and < 6.1 mmol/l), which are slightly higher than the normal physiological level, are referred to as impaired fasting glucose (IFG) levels. The 2-hour post glucose load values of ≥ 7.8 and < 11.1 mmol/l for plasma (or ≥ 6.7 and < 10 mmol/l for blood) are referred to as impaired glucose tolerance, which indicates insulin resistance. Finally, plasma glucose levels ≥ 7 mmol/l (or ≥ 6.1 mmol/l for blood) or the 2-hour oral glucose tolerance measurements of ≥ 11.1 mmol/L for plasma (or ≥ 10 mmol/l for blood) can be a diagnostic criterion for diabetes (American Diabetes Association, 2007; Henareh et al., 2004; Sacks et al., 2002).

Causes of T2D include impairment of insulin action and insulin secretion, impaired glucose uptake, β -cell dysfunction, glucotoxicity, lipotoxicity, increased adipocytokines and inflammatory factors (Matsuzawa et al., 2004; Permana et al., 2006). Also, obesity associated with dyslipidaemia leads to ectopic deposition of lipids in muscle, liver, pancreas, kidneys and heart (Dulloo et al., 2004), which plays a pivotal role in the development of T2D and progression to diabetic complications.

1.4. Metabolic Syndrome and Cardiovascular Disease

CVD refers to diseases of the heart and the blood vessels. Although the causes of CVD are diverse, atherosclerosis and hypertension have been known to be the most common.

1 Interestingly in recent years, it has been reported that clinical components of MetS such as
2 dyslipidaemia, insulin resistance, hyperinsulinaemia, glucose intolerance, hypertension and
3 central obesity are also strong risk factors for CVD (Hanson et al., 2002), giving rise to the
4 term “cardiometabolic risk” (Després et al., 2008). In a study, the prevalence of CVD was
5 approximately 3-fold higher in subjects with MetS than those without MetS (Isomaa et al.,
6 2001). Also, evidence from a systemic review and meta-analysis carried out by Mottillo et al.,
7 showed that MetS was associated with a 2-fold increase in risk of CVD, CVD mortality, and
8 stroke, and a 1.5-fold increase in risk of all-cause mortality. MetS was also associated with an
9 approximate 2-fold increase in risk for myocardial infarction (Mottillo et al., 2010).

10 The pathophysiological mechanism by which MetS increases cardiovascular risk remains
11 unclear. A likely explanation is that insulin resistance, which is currently known as one of the
12 key underlying components of MetS, may progress to hyperinsulinaemia and
13 hyperglycaemia, and therefore provoke peripheral vasoconstriction and sodium retention
14 (Mottillo et al., 2010). LDL cholesterol may also increase, leading to elevated TG, low HDL
15 cholesterol, elevated apolipoprotein B, elevated small LDL cholesterol, and consequently,
16 atherosclerosis and CVD (Vincent, 2014). As a result of these lipid imbalances, individuals
17 with the MetS also exhibit a prothrombotic and proinflammatory state (Mottillo et al., 2010).

18 Central obesity, also a major risk factor and component of MetS, has shown to be also
19 strongly correlated with cardiovascular complications such as coronary heart disease (CHD),
20 hypertension, stroke and heart failure (Poirier et al., 2006; Casanueva et al., 2010). Numerous
21 studies have reported an association between BMI/waist-to-hip ratio and stroke and obese
22 subjects have a higher chance of developing CHD due to significant alterations in cardiac
23 structure and function (Poirier et al., 2004). There is also an increased risk of sudden cardiac
24 death in obesity (Poirier et al., 2006). This is likely to be caused by alterations of the normal

1 physiological balance of adipocytokines, insulin resistance, endothelial dysfunction and a
2 pro-atherogenic state (Ritchie & Connell, 2007; Emanuela et al., 2012).

3 Overall, a combination of clinical components of MetS such as insulin resistance,
4 hyperinsulinaemia, glucose intolerance and central obesity and conventional cardiovascular
5 risk factors such as hypertension, dyslipidaemia and smoking results in a significantly greater
6 risk for CVD (Ritchie & Connell, 2007). However, exact molecular mechanisms for this
7 association are still undetermined and debated, so currently there is a lack of therapeutic
8 agents for the treatment and prevention of MetS related CVD and cardiovascular
9 complications.

10 **1.5.Pathogenesis of Metabolic Syndrome**

11 **1.5.1. Impaired Insulin Signalling in Skeletal Muscle**

12 Skeletal muscle is the major site of glucose uptake in the postprandial state in humans.
13 Approximately 80% of glucose uptake occurs in skeletal muscle, under euglycemic
14 hyperinsulinemic conditions (DeFronzo & Tripathy, 2009). In the early stages of T2D,
15 impaired glucose disposal by skeletal muscle is the primary defect responsible for insulin
16 resistance. Insulin prompts a diverse range of biological responses, such as maintaining
17 glucose homeostasis and regulating carbohydrate, protein and lipid metabolism. Circulating
18 glucose levels are tightly regulated by mechanisms involving a sophisticated and coordinated
19 role of insulin in reducing hepatic glucose production and stimulating glucose transport into
20 muscle and fat (Saltiel & Kahn, 2001; Ouwens et al, 2005). Several studies have
21 demonstrated that activation and role of insulin causes the uptake of glucose, FFA and amino
22 acids into surrounding adipose tissue, muscle and the liver (Foster & Klip, 2000). This further
23 promotes the storage of nutrients in the form of glycogen, lipids and protein. T2D occurs

when the body becomes resistant to the effects of insulin seemingly due to defects in the insulin-signalling pathway (Li et al., 2000; Saltiel & Pessin, 2000).

Insulin binds to its receptor leading to the auto-phosphorylation of the β -subunits and the tyrosine phosphorylation of insulin receptor substrates (IRS) (Bjornholm & Zierath, 2005). Activation of the insulin-signalling pathway results in the translocation of the glucose transporter 4 (GLUT4) from cytoplasmic vesicles to the cell membrane (Bevan, 2001; Grovers et al., 2004). It has been identified that normal signalling through the insulin pathway is critical for the regulation of intracellular and blood glucose levels.

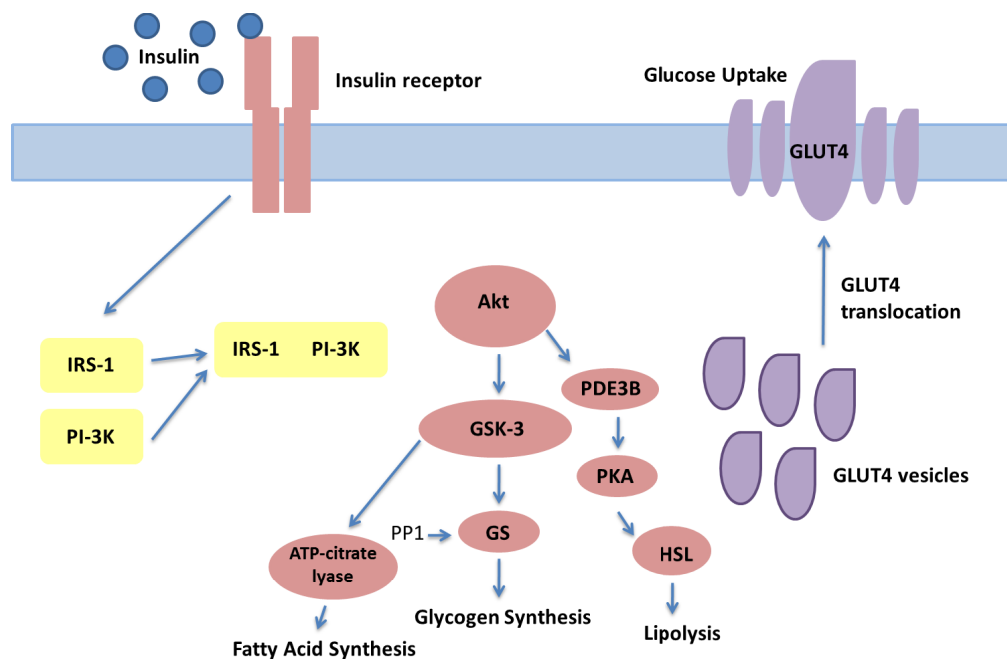


Fig 1.1. Insulin-signalling pathway Activation of the insulin signalling pathway results in translocation of the GLUT4 from cytoplasmic vesicles to the cell membrane resulting in increased glucose uptake. The activated Akt also regulates and phosphorylates components of PKA protein kinase C (PKC) isoforms and glycogen synthase kinase (GSK3), regulating glycogen synthesis and lipolysis in muscle and adipose tissue.

The cascading occurs as the insulin receptor, which is a heterotetrameric protein, contains two transmembrane β -subunits and two α -subunits, which are extracellular. Insulin action is

initiated with the binding of insulin to the α -subunit of its receptor, consequently stimulating the tyrosine kinase activity that is intrinsic to the β -subunit of the receptor. Insulin binding results in autophosphorylation of the tyrosine residues, and the kinase domain undertakes a conformational change, which acts as an origin for activation of the kinase and interaction of downstream molecule that participates in the insulin-signalling cascade. This autophosphorylation is followed by phosphorylation of the insulin-receptor substrates (IRS) (Saltiel & Pessin, 2000). Phosphorylation of IRS leads to activation of downstream signaling molecules, through three main pathways: the IRS/phosphatidylinositol 3- (PI3) kinase pathway; (RAS)/mitogen-activated protein kinase (MAPK) pathway; and the Cbl-associated protein (CAP)/Cbl pathway (Morino et al., 2005; Archuleta et al., 2009).

Tyrosine phosphorylation of IRS activates PI3-kinase pathway, leading to activation of 3-phosphoinositide-dependent protein kinase 1 (PDK1) and Akt kinase. The PI3-kinase-Akt pathway is responsible for many downstream effects of insulin (Asano et al., 2009). The activated Akt regulates and phosphorylates components of the GLUT4 complexes, protein kinase C (PKC) isoforms, glycogen synthase kinase (GSK3) and p70S6kinase. Thus activation of the PI3K-Akt pathway plays a critical role in glycogen synthesis by mediating the insulin induced phosphorylation and inhibition of GSK3 (Yu et al., 2002).

In skeletal muscle and adipose tissue, glucose uptake is facilitated by insulin through the stimulation of GLUT4 from intracellular sites to the plasma membrane. For example, in an animal experiment by Chen et al. (Chen et al., 2007), insulin resistant rats showed low expressions of GLUT4 in adipose and muscle tissues, which led to low glucose influx into the cells. Moreover, disruption of GLUT4, in muscle, leads to glucose intolerance and insulin resistance which indicate that glucose transport mediated by GLUT4 is essential in maintaining muscle glucose homeostasis (Abel et al., 2001; Zaid et al., 2009). Also disruption

of GLUT4 in adipose tissue of mice results in secondary insulin resistance and impaired glucose tolerance, indicating that alteration in expression of GLUT4 could lead to the insulin resistance and diabetes (Cline et al., 1999; Jacques et al., 2001).

1.5.2. Adipose Tissue Dysfunction and Adipocytokines

Adipose tissue has been historically understood as a storage place of fatty acids, however, recent studies suggest that adipose tissue play a major role in glucose and lipid metabolism, and further controls a large number of hormones and adipocytokines (Hajer et al., 2008). Many pro-inflammatory and pro-thrombotic adipocytokines are secreted from macrophages located within adipose tissues (Matsuzawa et al., 2004; Permana et al., 2006). Examples of pro-inflammatory and pro-thrombotic adipocytokines include TNF- α , IL-6, C- reactive protein (CRP), monocyte chemoattractant protein-1 (MCP-1), retinal binding protein-4 (RBP4), resistin, and plasminogen activator inhibitor-1 (PAI-1) (Donath, 2008). The majority of these adipocytokines have direct effects in causing insulin resistance (Tomas et al., 2002).

Chronic imbalance of calories consumed versus expended promotes storage of excess energy in adipocyte intracellular TG stores. This upturn in fat mass manifests as both increased intracellular lipids - greater adipocyte size (hypertrophy) and increased numbers of adipocytes (hyperplasia). Adipocyte hypertrophy, evident in both obese and T2D subjects, was originally considered the sole route whereby adipose tissue mass increased in adults. However, adipocyte hyperplasia (adipogenesis) is now known to contribute to the increased adipose tissue mass of obesity (Hajer et al., 2008).

In central obesity, increased adipose tissues cause an increase in secretions of TNF- α (Matsuzawa et al., 2004). There are many studies about both in vitro and in vivo experiments that show TNF- α as a significant causative factor of obesity-related insulin resistance

(Feinstein et al., 1993; Hotamisligil et al., 1993; Moller, 2000; Wellen & Hotamisligil, 2005).

TNF- α also causes adhesion of molecules in the endothelium, which contributes to atherosclerosis (Maeda et al., 2002). TNF- α also contributes to insulin resistance by inhibiting the expression of genes that are essential for insulin signalling and adipocyte differentiation - CEBP α (CCAAT-enhancer-binding protein- α), PPAR- γ , GLUT4, IRS1, adiponectin, and long-chain fatty acid acyl-CoA synthase), providing another molecular basis for insulin resistance (Hajer et al., 2008).

Similar to TNF- α , IL-6 is secreted mostly from central adipose tissues, and is also a causative factor of insulin resistance and MetS. IL-6 stimulates production of CRP from the liver, an important inflammatory marker that is closely linked to CVD (Pradhan et al., 2001). A significant increase in levels of CRP has been found to be associated with a high prevalence of patients with MetS and increased risk for T2D (Dehghan et al., 2007; Lizardi-Cervera et al., 2007). A high level of CRP causes low-grade inflammation that is often present in obese individuals (Pepys & Hirschfield, 2003; Canello & Clement, 2006).

Others factors include MCP-1, which is an inflammatory adipocytokine that has been found to be proportional to the degree of insulin resistance. In vitro studies showed that there was a relative reduction in glucose uptake in 3T3-L1 adipocytes incubated with MCP-1 compared to the control adipocytes. Similarly, RBP4 has been shown to possibly cause insulin resistance and glucose intolerance as evidence shows that RBP4 decreases the expression of GLUT4 and lowers glucose uptake in adipocytes (Yang et al., 2005). RBP4 has also shown to decrease glucose uptake by skeletal muscles while it increases glucose output by the liver, ultimately leading to hyperglycaemia (Yoshikazu, 2006).

Resistin causes insulin resistance as well as inflammatory responses, by producing other pro-inflammatory adipocytokines such as TNF- α and IL-6 (Chapman & Sposito, 2008). Rodents treated with resistin became insulin resistant with increased circulating glucose levels and hepatic glucose production; whereas the vehicle-treated animals showed low glucose levels (Moller & Kaufman, 2005). The likely mechanism for this action of resistin involves impaired insulin-stimulated phosphorylation of the insulin receptor substrates (Nakatani et al., 2005). Finally, PAI-1 is a pro-thrombotic adipocytokine that inhibits fibrinolysis and conversion of plasminogen, which leads to increased formation of fibrinogen and blood clots. Increase in PAI-1 raises a person's susceptibility to coagulation of blood and formation of thrombosis thereby causing atherosclerotic CVD. The amount of PAI-1 has also been found to be proportional to the degree of insulin resistance (Serrano Rios, 1998).

Also, hormone-sensitive lipase (HSL), which is controlled by cAMP and PKA, is the rate-limiting enzyme in the hydrolysis of stored TG in adipose tissue and lipolysis regulation (Stich & Berlan, 2004). In human adipocytes, the major hormones controlling the lipolytic are insulin (lipolysis inhibition) and catecholamines (lipolysis stimulation) (Lanfontan et al., 2000). Regulation of HSL involves binding of agonists to the β -adrenergic receptors coupled to adenylyl cyclase via stimulatory Gs protein, increasing cAMP production that leads to the activation of PKA and HSL phosphorylation, resulting in increased enzyme activity and NEFA released by fat cells (Langin et al., 1996).

1.5.3. Increased Ectopic Lipids and Lipotoxicity

Ectopic fat or lipids refers to the excessive deposition of TG within cells of non-adipose tissue that normally contain only small amounts of fat (Lettner & Roden, 2008). For example, in liver and muscle, TG content usually correlates with whole-body and tissue-

specific insulin sensitivity (Lay & Dugail, 2009). Recent studies have reported that obesity provokes metabolic disorders such as T2D, NAFLD and CVD. Recently, it has been observed that lipids and fats are commonly found to accumulate outside 'classical' adipose tissue depots, that is, in or around organs and tissues that constitute the lean body mass (e.g. skeletal muscle, heart, liver, pancreas, kidneys and blood vessels) (Dulloo et al., 2004). The metabolic and mechanical consequence of such ectopic fat storage ultimately leads to lipoapoptosis and gradual organ failure (Unger et al., 2010) and has also been recognised to be a contributing factor in the pathogenesis of T2D and CVD (Eldor, 2006; Toledo & Kelly, 2006).

With fat gain, lipid deposition can impair tissue and organ function by significantly increased fat pad size around key organs. This ultimately affects normal organ function either by simple physical compression or secretions of organ fat cells. Lipid accumulation can also occur in non-adipose cells and may lead to cell dysfunction or cell death. This is known as lipotoxicity (Montani et al., 2004; Kotani et al., 2004). Although not fully understood this is likely to involve alterations in adipocytokine levels, such as leptin, adiponectin and resistin (Montani et al., 2004).

Ectopic fat storage in the heart, blood vessels and kidneys can impair their function, contributing to the increased risk of CVD and obesity. There is also mounting evidence that ectopic fat accumulation inside the heart plays a role in cardiomyopathy, and eventually leads to heart failure (Sharma et al., 2004; Focheron et al., 2009). Similarly as in skeletal muscle and liver, it has been predicted over the years that metabolic dysfunction in lipid-overloaded hearts may induce insulin resistance (Van Herpen & Schrauwen-Hinderling, 2008). It is also noted that the accumulation of large amounts of TG may trigger a pathological signalling cascade resulting in apoptosis and systolic dysfunction. Studies have suggested that cardiac

lipotoxicity causes eccentric left ventricular remodelling, increased left ventricular pressure and decreased systolic performance leading to dilated cardiomyopathy (Van Gaal et al., 2006). Accumulation of large amounts of lipids in the heart may result in apoptosis and systolic dysfunction.

It has also been suggested that the eventual impairment of insulin secretion in subjects with T2D is related to ectopic fat accumulation inside the pancreas and high levels of FFA, which are both hallmarks of obesity (Van Herpen & Schrauwen-Hinderling, 2008). It has been identified that there is a significant relationship between pancreatic lipids, obesity, and hyperlipidaemia in relation to pancreatic islet function and the “lipotoxicity” associated with the onset of T2D (Pinnick et al., 2008).

In vivo studies have shown that pancreatic islet lipotoxicity occurs in Zucker diabetic fatty (ZDF) rats, which has been used commonly in research as it shares many common features of T2D in humans (Rasouli et al., 2007). There are several mechanisms that are involved and promote lipotoxicity in the pancreatic β -cell. Firstly, the deleterious effects of lipotoxicity are linked to elevated FFA, which up-regulates the activation of sterol regulatory element-binding proteins (SREBP)-uncoupling protein, subsequently influencing ATP production and insulin secretion (Fatehi-Hassanabad & Chan, 2005). Secondly, hyperlipidaemia that is associated with obesity is linked to the secretion of inflammatory cytokines such as TNF- α , IL-1 and IL-6. These cytokines accelerate inflammatory processes, leading to apoptosis in pancreatic β cells (Goldberg, 2009).

1.5.4. Abnormalities in Hepatic Glucose and Lipid Metabolism

The liver is a major organ that has the ability to consume, store, and produce glucose and lipids. Hepatic glucose metabolism includes the formation of glycogen (short-term energy

storage), generation of glucose from non-sugar carbon substrates and intracellular energy supply via glycolysis (Klover & Mooney, 2004). Fatty acid oxidation, de novo synthesis of fatty acids, cholesterol and bile acid synthesis, and lipoprotein assembly are the fundamental processes in lipid metabolism. The regulation of these metabolic pathways is important to maintain glucose and lipid homeostasis under physiological conditions (Raddatz & Ramadori, 2007). Consequently, the liver is a key target for the anabolic hormone insulin and its catabolic counterpart glucagon.

Impaired insulin sensitivity in the liver leads to increased hepatic glucose production and contributes significantly to T2D (Fritsche et al., 2008). IRS1 and IRS2 are complementary key players in the regulation of hepatic insulin signalling and expression of genes involved in gluconeogenesis, glycogen synthesis and lipid metabolism (Fritsche et al., 2008). Their expression levels and post-translational modifications regulate the function of IRS proteins. Dysfunction of IRS proteins in muscle initially leads to postprandial hyperglycaemia, increased hepatic glucose production, and dysregulated lipid synthesis, and is considered as a major pathophysiological mechanism for T2D (Simmgen et al., 2006).

In addition, ectopic fat accumulation in the liver, also known as hepatic steatosis, has been shown to have a direct link to T2D and diabetic complications. This disease includes a wide range of pathological abnormalities, from fatty liver without inflammation to steatohepatitis, which can ultimately lead to hepatic fibrosis and cirrhosis (Toledo & Kelly, 2006). Fatty liver disease is commonly found in T2D patients, with an estimated prevalence ranging from 21% to 78% and is also common to obese individuals (Toledo & Kelly, 2006). To date, the cause is not fully understood, however, studies have been carried out to identify and comprehend the pathogenesis and mechanisms involved. A study has found that fatty acids are the principal substrate oxidised by the liver. Increased fatty acid flux to the liver resulting

1 from impaired insulin-suppressed lipolysis in adipose tissue is thought to be a strong
2 contributor to hepatic steatosis. Another study has identified that the accumulation of TG in
3 hepatocytes reveals an imbalance between hepatic TG synthesis and utilisation. Utilisation
4 includes mitochondrial β -oxidation, production of ketone bodies, and secretion of TG in
5 VLDL particles (Yki-Jarvinen, 2002). Microvesicular steatosis, such as fatty liver during
6 pregnancy, Reye's syndrome and drugs or toxins, is a result of the impairment of
7 mitochondrial β -oxidation. Microvesicular steatosis is often accompanied by severe hepatic
8 dysfunction (Yki-Jarvinen, 2002) and it has been revealed that the direct causes of
9 macrovesicular steatosis include alcohol, NAFLD associated with features of insulin
10 resistance, total parenteral nutrition, and protein-calorie malnutrition (Yki-Jarvinen, 2002).

11 On a molecular level, increased hepatic glucose production involves changes in the activity of
12 key gluconeogenic enzymes. Phosphoenolpyruvate carboxykinase (PEPCK) catalyses the
13 conversion of oxaloacetic acid to phosphoenolpyruvate, a rate limiting step for
14 gluconeogenesis (Weickert & Pfeiffer, 2006). Dephosphorylation of glucose 6-phosphate to
15 free glucose is catalysed by glucose-6-phosphatase (G-6-Pase). The activity of PEPCK and
16 G-6-Pase, and gluconeogenesis, is normally inhibited by insulin, however this action is
17 restricted in insulin-resistant states. In addition to insulin, other hormones such as glucagon
18 and glucocorticoids interact with key glucogenic enzymes at the transcriptional level (Ferber
19 et al., 1994). Acute elevations of circulating glucose concentrations have also been reported
20 to significantly reduce expression of PEPCK and gluconeogenesis (Sun et al., 2002).

21 AMP-activated protein kinase (AMPK) belongs to a family of serine-threonine kinases and is
22 present in various organs, including the liver. When activated, AMPK initiates a series of
23 responses that protect the cell against ATP depletion, by stimulating fatty acid oxidation or
24 glycolysis and inhibiting ATP-consuming anabolic pathways such as gluconeogenesis,

1 protein and fatty acid synthesis (Kahn et al., 2005). AMPK is phosphorylated and thereby
2 activated by the protein–threonine kinase liver kinase B1 (LKB1), the major upstream
3 AMPK-activating factor. Activation of AMPK also results in inhibition of other lipogenic
4 factors such as sterol regulatory element binding protein-1c (SREBP-1c), fatty acid synthase
5 (FAS), and carbohydrate-responsive element-binding protein (ChREBP) (Weickert and
6 Pfeiffer, 2006). Activation of AMPK in liver has shown to also decrease PEPCK and G-6-
7 Pase transcription and further activate cAMP-response-element-binding protein (CREB),
8 leading to increased expression of PGC-1 α , thereby driving the expression of glucogenic
9 genes (Hardie et al., 2005).

10 Glycogen synthase kinase 3 (GSK3) has a clear role in opposing the effect of insulin, by
11 inhibiting the activation of glycogen synthase (GS) and the subsequent accumulation of
12 glycogen in adipose tissue and muscle (Yu et al., 2002; Cline et al., 2002). The binding of
13 insulin to its receptor activates the intrinsic tyrosine kinase of the receptor leading to
14 stimulation of PI3-kinase1 and other downstream kinases such as PKB/Akt, p70 S6 kinase,
15 and protein kinase C (PKC) ζ (Taha & Klip, 1999) and one target of PKB is GSK3. Two
16 isoforms of GSK3, α and β , are broadly expressed and play multiple regulatory roles in
17 development and metabolism (Orena et al., 2000).

18 Furthermore, a defect in insulin-stimulated liver glycogen synthesis is major factor
19 contributing to postprandial hyperglycemia in patients with T2D. In insulin-responsive tissue,
20 insulin exposure leads to a transient inhibition of GSK3 β via PKB/Akt phosphorylation of a
21 serine residue (Ser9). The inhibition of GSK3 β activity leads to net dephosphorylation and
22 activation of GS (Summers et al., 1999). Therefore, inhibition of GSK3 activity leads to
23 activation of GS activity and may enhance glycogen synthesis, glucose disposal and glucose
24 uptake in MetS and T2D patients (Cline et al., 2002).

1.6.Current Treatment and Therapies for Metabolic Syndrome

In response to the size of the growing problem, efforts to identify and develop new pharmaceutical agents for MetS have become crucial in the recent years. Currently, treatment options available to MetS patients are limited in efficacy and safety. At present, there are two main therapeutic approaches for MetS. One strategy is to identify and treat each risk factor of MetS separately, unrelated to its clustering with other risk factors. This strategy is currently viewed as the only convincing approach and widely implemented in MetS patients (Grundy, 2006). However, the alternative approach is to target all or multiple risk factors with single drug or therapy (Isomaa et al., 2001). This is highly necessary for successful treatment of MetS, for lowering costs of medications and preventing adverse and side effects associated with polypharmacy (Deedwania & Volkova, 2005; Grundy, 2006).

1.6.1. Conventional Medications

The main types of oral drugs used for MetS are described below.

1.6.1.1. Insulin-sensitising Medications

Biguanides, such as Metformin, has been utilised in the treatment of T2D for nearly 50 years. It acts as an insulin-sensitising agent, lowering fasting plasma insulin concentrations by inducing better peripheral uptake of glucose, as well as decreasing hepatic glucose output. More recently, the use of metformin has broadened, with evidence for its benefit in other insulin-resistant states. In polycystic ovary syndrome, it has been found to decrease insulin resistance, restore menstruation, facilitate conception, and reduce the rate of first-trimester spontaneous abortion (Glueck et al., 2002). Biguanides act on the liver to suppress gluconeogenesis by potentiating the effect of insulin or by reducing hepatic extraction of substrates and glycogenolysis, and it decreases the activity of glucose-6-phosphatase. It has

1 been found to also delay the progression to T2D in patients with impaired glucose tolerance.

2 In vitro experiments have shown that it also enhances insulin binding to hepatocytes of
3 insulin resistant mice, and stimulates tyrosine kinase activity. Glucose transport and glycogen
4 synthesis represent post-receptor targets of this drug (Iozzo et al., 2003).

5 Also, Rosiglitazone, the most common type of TZD, has been utilised as an agonist for
6 peroxisome proliferator activated receptor- γ (PPAR γ). This is a nuclear receptor mainly
7 expressed in adipose tissue which has been shown to increase insulin sensitivity both in the
8 liver and peripheral tissues (Yki-Jarvinen, 2002). PPAR γ activation stimulates a decrease in
9 insulin resistance, modification of adipocyte differentiation, inhibition of vascular endothelial
10 growth factor-induced angiogenesis, decrease in leptin levels (leading to increased appetite)
11 and an increase in adiponectin levels. A study published in 2006 found that the drug was
12 capable of the alteration of the expression of the genes which are involved in fatty acid
13 synthesis and storage, structural proteins, and genes involved in glucose transport and insulin
14 sensitivity. Results indicated that the drug group also changed the expression of genes
15 involved in fatty acid uptake, metabolism, and triacylglycerol synthesis in experimental
16 models (Kolak et al., 2006). However, weight gain is an undesirable consequence of TZD
17 therapy, although the underlying mechanism is not clear (Deedwania & Volkova, 2005).

18 **1.6.1.2. Anti-hyperlipidaemic Medications**

19 Hyperlipidaemia and dyslipidaemia are major components of MetS and CVD. One of the
20 main medications for dyslipidaemia is 3-hydroxy-3-methylglutaryl coenzyme A reductase
21 inhibitors (statins). Statins have been found to have direct LDL cholesterol-lowering effect
22 and have multiple indirect effects on the vasculature (Deedwania & Volkova, 2005). These
23 properties of statins have shown to reduce the risk of cardiovascular events in patients with

MetS and T2D. However, it has been reported that statins cause multiple side effects including gastrointestinal upset, muscle aches, sleep disturbances and hepatotoxicity (Bellosta et al., 2004).

Fibrates are also commonly used to target a range of hyperlipidaemic disorders, predominantly high cholesterol. They have also been shown to be effective in MetS by treating dyslipidaemia, lowering TG and LDL levels, increasing HDL and improving insulin sensitivity. Fibrates activate PPAR α , influencing carbohydrate metabolism and adipose tissue differentiation (Grundy et al., 2004). Also, omega-3 fatty acids from fish oil has shown to reduce the incidence of coronary heart disease and lower TG levels associated with MetS, however, exact mechanisms are not fully understood (Harris et al., 2008).

1.6.1.3. Anti-obesity Medications

The main pharmaceutical drugs for treating obesity are sibutramine, orlistat, lorcaserin, rimonabant, exantide and pramlintide. For example, sibutramine inhibits both noradrenergic and serotonergic reuptake in the hypothalamus to promote weight loss. However, sibutramine has been reported to increase blood pressure, which negatively impacts patients with MetS (Grundy, 2006). Orlistat acts on the intestines to inhibit pancreatic and gastric lipases, and therefore blocks lipolysis and the absorption of fat (Shi & Burn, 2004). However, orlistat has shown to cause steatorrhea, abdominal pain, and flatulence if used for extended periods (Deedwania & Volkova, 2005). Other agents under consideration increase energy expenditure, such as β 3-adrenoceptor agonists, thyroid hormone receptor β -subtype agonists, and PPAR γ agonists (Grundy, 2006).

1.6.1.4. Anti-hypertensive Medications

An integral component of MetS is a blood pressure greater than 130/85 mm Hg. The relationship between hypertension and MetS is emphasised by the fact that even lean hypertensive patients can manifest insulin resistance. It has been postulated that the direct effect of elevated insulin on sympathetic nervous system activity can lead to elevated blood pressure (Deedwania & Volkova, 2005). Diuretics and β -blockers, are two widely used conventional drugs for treating hypertension. Although both have been reported to worsen insulin resistance and dyslipidaemia, they are still the preferred treatment approach for MetS related hypertension due to their efficacy for preventing cardiovascular events (Grundy, 2006). Finally, angiotensin-converting enzyme inhibitors and angiotensin-II-receptor blockers have been reported to be effective in lowering blood pressure. In addition to their hypotensive effects, the two drugs have shown to also have beneficial actions on metabolism, inflammation and vascular biology to prevent diabetes and MetS (McFarlane et al., 2003).

1.6.2. Lifestyle Modifications

Lifestyle factors such as an unhealthy diet, alcohol consumption, cigarette smoking, and physical inactivity have been described to significantly increase an individual's susceptibility to MetS (Bhanushali et al., 2013). Studies have found that dietary patterns characterised by high consumption of fruit, vegetables, poultry, and legumes is associated with reduced risk of insulin resistance and MetS, as opposed to the western diet consisting of increased amounts of refined grains, red meats and saturated fats (Esmailzadeh et al., 2007). Further studies in the US have reported that alcohol consumption is associated with significant increases in lipids, waist circumference, and fasting insulin concentrations in compared to non-drinkers (Freiberg et al., 2004; Bhanushali et al., 2013). Smoking is also highly associated with increased lipids, hypertension and a greater risk for CVD (Slagter et al., 2013). Adhering to

life style modifications such as implementation of healthy diets as recommended by dietary guidelines, reduced consumption of alcohol and smoking and physical exercise can ultimately reduce inflammation, hypertension, insulin resistance and the onset and progression of MetS (Grundy, 2007; (Bhanushali et al., 2013).

1.6.3. Chinese Herbal Medicines

There have been records that suggest Chinese herbs have been used for symptoms similar to that of diabetes for thousands of years in Southeast Asia. A review carried about by Yin et al, has outlined 22 herbal agents that potentially have biochemical properties that may treat or be beneficial for metabolic syndrome (Yin et al., 2008). Some commonly used herbs are described in detail below.

Salvia Miltiorrhiza - Dan Shen

Dan Shen, with the pharmacological name Radix Salvia Miltiorrhizae, has been widely used in China, Japan, US and other European countries for treating cardiovascular and cerebrovascular diseases. Chemical constituents of the herb have been studied from as early as 1930s, with the main focus on lipophilic compounds. More than 30 diterpene compounds have been separated and identified from Dan Shen. Some examples of these are diterpene chinone compounds of Tanshinone group such as tanshinone I, IIA< IIB and cryptotanshinone (Lee et al., 2006).

In vitro and *in vivo* studies have suggested than Dan Shen has the chemical properties to improve microcirculation, dilate coronary arteries, inhibit platelet aggregation and oxidative modification of LDL leading to the prevention of the uptake of LDL by cultured macrophages.

In studies, a greater reduction of cholesterol and TG was found following Fufang Danshen Dripping Pill treatment of angina pectoris compared to isosorbide dinitrate (ISDN). The study also mentions that at least 4 other studies also showed that total cholesterol, TG and LDL cholesterol levels were significantly reduced by 28.3%, 34.3% and 29.9% respectively. In addition HDL cholesterol level was raised by 33.2% (Zhou et al., 2005). In another study comparing the Dan Shen injection with ISDN injection, the level of soluble intercellular adhesion molecule-1 and interleukin-6 was found to be lower in the Dan Shen group (Lee et al., 2006).

Rhizoma Coptidis - Huang Lian

Huang Lian (Rhizoma Coptidis) is widely used in traditional Chinese medicine as an anti-microbial and anti-tumour agent. It has been recently reported to alleviate cardiovascular disease by decreasing LDL and may relieve metabolic diseases such as T2D by altering cell signalling pathways (Ko et al., 2005). Recent research, such as the one mentioned above, has suggested that berberine (BBR), its active compound, is a highly effective insulin-sensitizing agent. BBR was reported to reduce body weight and improve glucose metabolism in animal models of metabolic syndrome (Yin et al., 2008). An *in vivo* study into BBR has found that BBR, the active compound, significantly reduces body weight with unaltered food intake. It also significantly lowers plasma TG of the high-fat-fed rats with no adverse pathology or inflammation in major organs (Lee et al., 2006).

The study also shows that BBR increases AMPK activity in multiple tissues and highlights the beneficial effects of berberine which may involve changes in gene expression to lessen insulin resistance, and the likelihood that berberine may contribute to reduced fat cell differentiation (Lee et al., 2006). Another study has revealed that BBR stimulates glucose

uptake and to a greater magnitude than insulin, by the activation of AMPK (Cheng et al., 2006).

In a study, significant results were attained in 3T3-L1 cells. Cells were incubated with 50 μ M BBR and stimulated with 0.2 nM insulin to attain a glucose uptake level augmented by 10 nM of insulin alone. It was established that this significance was associated with an increase in GLUT4 translocation into the plasma membrane by enhancing insulin signalling pathways and the IRS1 PI3-K-Akt (Kong et al., 2004).

BBR also increased glucose-stimulated insulin secretion and proliferation in Min6 cells via an enhanced insulin/insulin-like growth factor-1 signalling cascade. Similarly to the data above, this result suggests that the herb may be used as an effective insulin sensitising herb and optimally treat T2D. Furthermore, BBR was recently reported to alleviate cardiovascular disease by decreasing LDL via activating p44/p42 mitogen-activated protein kinase (Kong et al., 2004).

Another study found that BBR increases insulin stimulated glucose uptake as much as pioglitazone, a TZD with hypoglycaemic action, in a cell-based glucose uptake screening assay. Also associations with increases GLUT4 contents in the membrane via potentiating the IRS1 PI3-K Akt signalling cascade in 3T3-L1 adipocytes, were established. Unlike pioglitazone, BBR decreased TG accumulation and enhanced glucose-stimulated insulin secretion (Ko et al., 2005). The study also found that BBR remarkably decreased TG accumulation in 3T3-L1 cells in a dose dependent manner. Thus the study established that increased glucose uptake with BBR with 0.2nM insulin was associated with the enhancement of the IRS1, PI3-K Akt-GLUT4 translocation pathway.

More recently, research into dietary obese rats found that BBR increased insulin sensitivity after five-week administration. Fasting insulin and homeostasis model assessment of insulin resistance (HOMA-IR) were decreased by 46% and 48% in the rats, respectively. Also, in multiple cell lines including 3T3-L1 adipocytes, BBR was found to increase glucose consumption, 2-deoxy-glucose uptake and to a less degree 3-O-methyl-glucose uptake independently of insulin. The long-lasting phosphorylation of AMPK was found to be associated with persistent elevation in AMP/ATP ratio and the reduction in oxygen consumption and an increase in glycolysis were observed with a rise in lactic acid production. It exhibited no cytotoxicity, and protected plasma membrane in L6 myotubes. These results suggest that BBR enhances glucose metabolism by stimulation of glycolysis, which is related to inhibition of glucose oxidation in mitochondria (Yin et al., 2008).

Panax Notoginseng - San Qi

Recent studies have shown the effects of Notoginsenoside R1, the main ingredient of Panax Notoginseng in relation to cardiovascular activity. A study highlighted that notoginsenoside R1 significantly decreased TNF- α -induced plasminogen activator inhibitor-1 (PAI-1) mRNA, protein level and secretion in human aortic smooth muscle cells in a dose-dependent manner (Zhang & Wang, 2006). There is also evidence that notoginsenosides has anti-cancerous effects (Wang & Edens, 2006).

1.6.4. Natural Agents and Functional Foods

Common side effects, availability and cost of medications, therapeutic limitations of oral anti-diabetic and anti-obesity drugs are key reasons for the growing interest for natural agents and foods for use as treatment approaches for metabolic syndrome. Studies have suggested that nutritional supplementation with natural agents or botanicals may effectively address the

1 pathogenic mechanisms of MetS. The already widespread use of natural agents and
2 functional foods by the general public, in both western and Asian countries, represent an
3 attractive, novel and potentially effective approach to the global epidemic. Recent studies
4 have shown that natural agents such as BBR (*Coptis chinensis*), Resveratrol (grapes and
5 wines), Ginsenosides (Ginseng), Anthocyanin (black rice, purple grape, blackberry),
6 Triterpenoid (bitter melon), Curcumin (*Curcuma longa*) and teas may be used as potential
7 mutli-targeted drugs for MetS (Xia & Weng, 2010).

8 **1.7.Green Tea Polyphenols**

9 Green tea polyphenols (GTP) is a collective term for catechins, flavonoids, phenolic acids
10 and anthocyanidins. The most important component is catechins, which take up 60-80% total
11 volume of tea polyphenols. Tea polyphenols have been found to clear away free radicals,
12 lower blood pressure, fat and serum cholesterol, obesity, T2D, dilate blood capillaries to
13 prevent cardiovascular disorders (Cao et al., 2007).

14 Green tea is consumed worldwide, especially in the countries of East Asia. The main
15 constituents in Green tea include caffeine and polyphenolic compounds known as catechins.
16 Catechins primarily consist of epigallocatechin gallate (EGCG), epigallocatechin (EGC),
17 epicatechin gallate (ECG), and epicatechin (EC). Studies have found that the most abundant
18 catechin found in green tea is (-)-epigallocatechin-3-gallate (EGCG) (Cao et al., 2007),
19 which has been suggested to have positive effects on MetS (Bose et al., 2008).

20 The suppression of lipogenesis by green tea leaves in rats has been demonstrated by the
21 suppression of plasma TG, cholesterol, and LDL cholesterol in the experimental animals
22 (Kao et al., 2006) and it has been exposed that the consumption of green tea may possibly be

contrarily associated with liver damage and with the different markers of inflammation. In high fat– fed C57BL/6J and leptin-deficient *ob/ob* obese mice, green tea treatment reduced hepatic lipids and the markers that indicate liver damage (Bose et al, 2008).

A recent study showed positive results after long-term supplementation and on a hamster model of atherosclerosis with green tea. It was effective in inhibiting atherosclerosis with a dose of 0.0625% tea solution, decreasing it by 26–40%, and the high dose of 1.25% tea solution, decreasing it by 46–63%. The results of the study identified that atherosclerosis was inhibited by three significant mechanisms, hypolipidaemic, antioxidant, and anti-fibrinolytic (Kao et al., 2006).

A wide range of research has been carried out on GTP, however to date; the molecular mechanism by which tea catechins stimulate lipid metabolism is yet to be fully understood. It has become apparent that the expression of many lipid metabolising enzymes, including acyl-CoA oxidase and acyl-CoA dehydrogenase, is transcriptionally regulated by peroxisome proliferator-activated receptors (PPARs). It has been shown that catechins (EGCG, ECG, GCG, etc.) are not ligands for PPAR α by using a transient transfection assay (Kao et al., 2006).

In a study, green tea significantly stimulated the glucose uptake accompanied by a decrease in translocation of GLUT4 in adipose tissue, which in hand also stimulated glucose uptake within skeletal muscle. The compound also suppressed the expression of PPAR γ and the activation of SREBP-1 in adipose tissue. It was observed that green tea was capable of regulating the glucose uptake system in adipose tissue and skeletal muscle and suppressing the consequential transcription factors (Ashida et al., 2004).

It has been recognized to be a potent antioxidant and shown to have the ability to counteract the free radical damage associated with cardiovascular disease. It also strengthens resistance to illness and acts as an anti-inflammatory (Manach et al., 2004). These results indicate that GTP may have the potential to either treat or assist in the treatment of metabolic disorders, such as T2D, insulin resistance, and glucose intolerance.

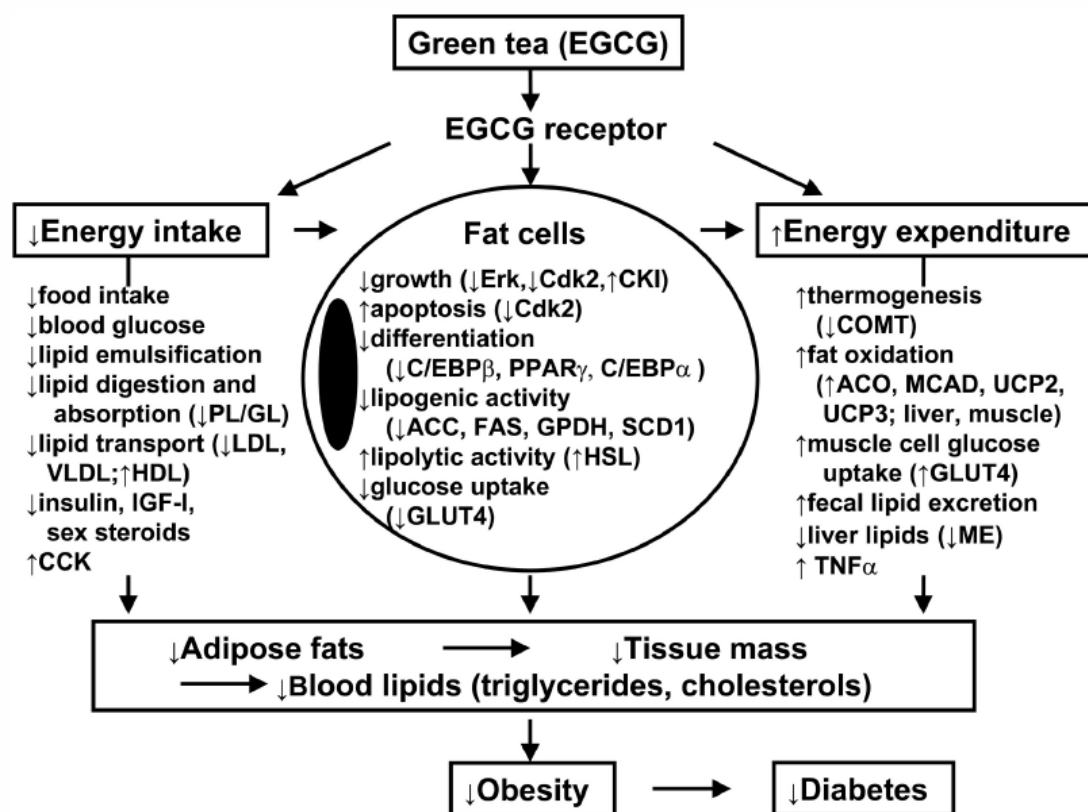
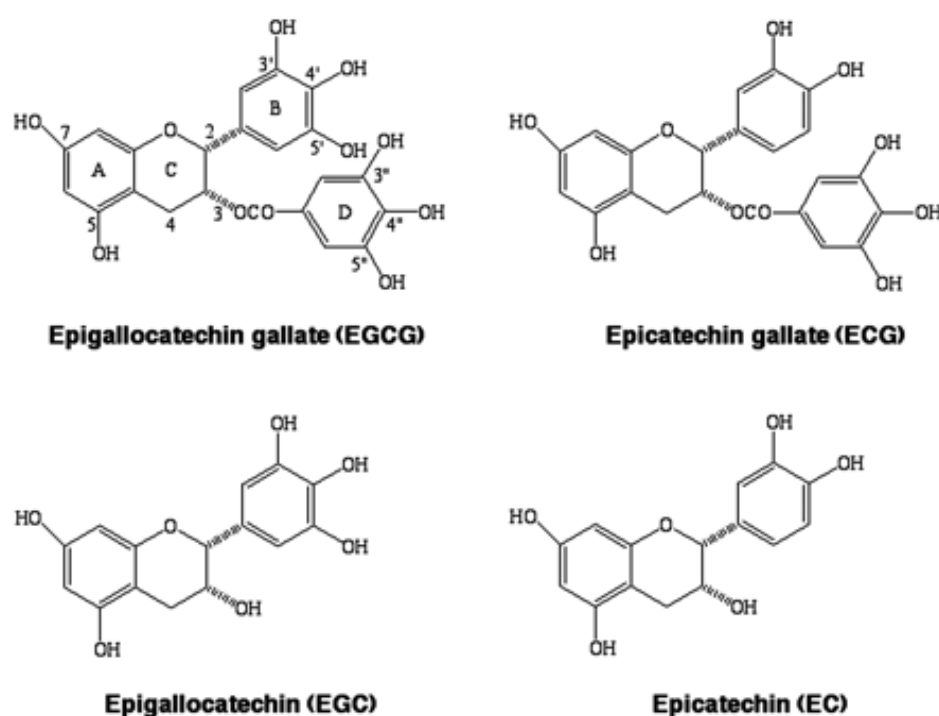


Fig 1.2. Proposed mechanisms of green tea for T2D and obesity (Kao et al., 2006).

1.7.1. Active Ingredients of Green Tea Polyphenols

As described above, green tea polyphenols is an herbal derivative of green tea leaves (*Camellia Sinensis*) containing mainly catechins. The four main catechin derivatives identified include epigallocatechin gallate (EGCG), epigallocatechin (EGC), epicatechin

1 gallate (ECG) and epicatechin (EC), of which, EGCG accounts for more than 40% of the
 2 total content.



3
 4 **Fig 1.3. Structure of green tea catechins** (Waltner-Law et al., 2002) The four main catechins are
 5 epigallocatechin gallate, (-)-epicatechin gallate, (-)-epigallocatechin, and (-)-epicatechin.

6 1.7.2. Pharmacological Actions of Green Tea Polyphenols

7 During the past decade, a number of *in vitro* and *in vivo* studies have suggested that green tea
 8 has strong anti-obesity, anti-inflammatory, anti-oxidative, anti-mutagenic, and anti-
 9 carcinogenic effects. (Hong-Li Jiao & Zhou, 2003; Coyle et al., 2008; Severino et al., 2009).
 10 For example, green tea contains an abundance of polyphenols that have anti-oxidative effects
 11 against free radicals and oxidative DNA damage and inhibits various transcriptional factors
 12 such as activator protein-1 (AP-1) (Wang et al., 2008). Green tea may also prevent
 13 carcinogenesis by several different mechanisms, including inhibiting angiogenesis, impairing
 14 cell cycle progression, inducing glutathione S-transferase and decreasing the production of

ROS (Waltner-Law et al., 2002). The pharmacological actions of GTP have been described in detail below.

1.7.3. Anti-oxidant Effects

Green tea has been proposed to be a dietary supplement in the prevention of cardiovascular diseases in which oxidative stress and pro-inflammation are the principal causes (Chan et al., 1997) and studies have shown that GTP has potent anti-oxidant effect (Salah et al., 1995). EGCG, the main polyphenol in GTP, can reduce the inflammatory response associated with local tissue injuries such as the hepatocellular necrosis in acute liver injury induced by carbon tetrachloride. The protective effect of EGCG is due to its ability to decrease lipid peroxidation, oxidative stress and the production of nitric oxide (NO) radicals by inhibiting the expression of inducible nitric oxide synthase (iNOS) (Chan et al., 1997).

A study has also found that EGCG protects cellular damage by inhibiting DNA damage and oxidation of LDL, through the protective properties of EGCG and its ability to scavenge free radicals (Surh et al, 2001). The anti-oxidant ability of green tea is mainly the result of inhibiting the reactive oxygen species (ROS) formation and enhancing the scavenging of free radicals (Tipoe et al, 2007). It has been also revealed that EGCG ameliorates the overproduction of pro-inflammatory cytokines and mediators, reduces the activity of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kappaB) and activator protein 1 (AP-1) and the subsequent formation of peroxynitrite with NO and ROS (Lin & Lin, 1997).

1.7.4. Metabolic Effects

Studies have demonstrated that green tea possess beneficial effects on metabolism. It has been shown that EGCG reduces food intake, plasma levels of glucose (Waltner-Law et al.,

2002; Collins et al., 2007; Tsuneki et al., 2004), body weight (Collins et al., 2007; Kao et al., 2000; Wolfram et al., 2006), oxidative stress (Mustata et al., 2005; Sabu et al., 2002), lower blood pressure and cholesterol (Kao et al., 2006) and reduce lipid content (Bose et al., 2008), all of which are important precursors of T2D. It has been found that EGCG inhibits proliferation and adipose differentiation in 3T3-L1 pre-adipocytes and induces apoptosis in 3T3-L1 adipocytes (Hung et al., 2005).

Recent studies have also suggested that EGCG may play an important role in modulating insulin secretion and insulin sensitivity (Collins et al., 2007; Potenza et al., 2007) possibly by inhibiting glutamate dehydrogenase (GDH) (Li et al., 2006). Clinical trials have shown that green tea, especially in high amounts, reduces body fat, cholesterol levels, and blood pressure in women and men without the need for any lifestyle changes. The ingestion of green tea at high concentrations may prevent T2D, obesity and decrease the risk of CVD (Nagao et al., 2007).

A wide range of research has been carried out on green tea, however to date; the molecular mechanism by which tea catechins stimulate metabolism is yet to be fully understood. Little is known about the underlying mechanisms of their action. Certain bases exist for the underlying molecular actions, such as decreasing digestive enzymatic activity, increasing lipolytic activity, decreasing lipogenic activity, increasing fat oxidation and thermogenesis, modulating lipoproteins, decreasing fat cells and decreasing hormone-stimulated proliferation of cells, decreasing GLUT4 activity, decreasing expression of gluconeogenic genes, increasing insulin sensitivity, increasing insulin-like activity, increasing protection of the liver and pancreatic β -cells (Kao et al., 2006).

1 The ability to clear away free radicals, lower blood pressure, and reduce fat and serum
2 cholesterol, whereby suppression of lipogenesis by green tea in rats has been identified by the
3 suppression of plasma TG, cholesterol, and LDL cholesterol in the experimental animals
4 (Kao et al., 2006; Reihl, 2005). It has been shown that the consumption of green tea may
5 have a positive effect on liver damage and on the different markers of inflammation and
6 transcriptional factors such as PEPCK and G6Pase (Waltner-Law et al., 2002; Zhang et al.,
7 2003). In high fat– fed C57BL/6J and leptin-deficient *ob/ob* obese mice with green tea
8 treatment, it was reported that there was a reduction of lipids in the liver and the different
9 markers that indicate liver damage (Bose et al, 2008). A study carried out to determine
10 whether GTP has an effect on oxidative stress and the formation of mitochondria-derived
11 superoxide, which has been proposed to play a key role in the pathogenesis of diabetic
12 complications. Results showed that green tea was successful in ameliorating oxidation end
13 points such as plasma hydroperoxides, erythrocyte glutathione and retinal superoxide
14 formation (Zhao, 2003).

15 A recent study also showed positive results after long-term supplementation and on a hamster
16 model of atherosclerosis with green tea. It was effective in inhibiting atherosclerosis with a
17 dose of 0.0625% tea solution, decreasing it by 26–40%, and the high dose of 1.25% tea
18 solution, decreasing it by 46–63%. The results of the study identified that atherosclerosis was
19 inhibited by three significant mechanisms, hypolipidaemic, anti-oxidant, and anti-fibrinolytic
20 (Kao et al., 2006). Also, a study revealed that green tea significantly stimulated glucose
21 uptake accompanied by a decrease in GLUT4 translocation in adipose tissue, which in hand
22 also stimulated glucose uptake within skeletal muscle (Ashida et al., 2004). Studies have also
23 shown that the expression of lipid metabolizing enzymes, such as acyl-CoA oxidase and acyl-
24 CoA dehydrogenase, is transcriptionally regulated by PPARs (Kao et al., 2006).

Green tea has also shown to enhance insulin sensitivity. Epididymal fat cell assay was used to demonstrate that EGCG induced 17-fold greater insulin activity than the control (Anderson & Polansky, 2002). Although many *in vitro*, *in vivo* and clinical studies have been undertaken over the years, the exact mechanism by which GTP enhances glucose and lipid metabolism and inhibits oxidation is not fully understood.

It is of great worth and importance to accurately elucidate not only the chemical structures, active compounds and pharmacological effects of green tea, but also the underlying mechanisms involved. Also, in recent years, there have been concerns about the inconsistent composition of green tea. Quality control of these teas has become crucial to ensure that consistency, safety and efficacy are maintained when these teas are employed as a treatment approach for a large number of metabolic diseases.

1.7.5. Cardiovascular Effects

The onset of CVD depends on many factors that are related to diet. In coronary artery disease, atherosclerotic plaques protrude from the inner surface of the arteries, narrow the lumen, and reduce blood flow. This may be caused by LDL cholesterol depositing at lesion sites of the arterial wall, which is subjected to oxidation when protectors are depleted. Also, oxidation of LDL causes significant changes in lipoproteins, stimulates inflammatory reactions, causes monocytes and monocyte-derived macrophages to accumulate in large amounts of oxidised LDL, and generates atherosclerotic plaques (Khan & Mukhtar, 2007). Over consumption of saturated fats, prolonged and excessive smoking has been found to accelerate these events. Tea polyphenols, especially in green tea have been found to be vasculoprotective, antioxidative, antithrombogenic, and have anti-inflammatory and lipid-lowering properties (Stangl et al., 2006).

GTP has shown to prevent development and progression of atherosclerosis by preserving arterial compliance and endothelial function (Khan & Mukhtar, 2007). A recent study showed positive results after long-term supplementation and on a hamster model of atherosclerosis with green tea. It was effective in inhibiting atherosclerosis with a dose of 0.0625% tea solution, decreasing it by 26–40%, and the high dose of 1.25% tea solution, decreasing it by 46–63%. The results of the study identified that atherosclerosis was inhibited by three significant mechanisms, hypolipidaemic, antioxidant, and antifibrinolytic (Kao et al., 2006). It has been shown that GTP decreases the absorption of TG and cholesterol by increases excretion of fat (Raederstorff et al., 2003) and it has been also proposed that EGCG, polyphenols and catechins may prevent or reduce cholesterol-related events that may lead to the onset of CVD (Khan & Mukhtar, 2007).

1.7.6. Anti-cancerous Effects

Accumulated evidence has shown that EGCG possesses inhibitory effect on carcinogenesis both *in vivo* and *in vitro*. It has been found to be a multi-potent chemopreventive and anti-cancer agent in several animal models, including leukaemia, lung, prostate, colon, and breast cancer. Accumulating evidence has shown that green tea catechins, like EGCG, have strong anti-oxidant activity and affect several signal transduction pathways relevant to cancer development (Khan & Mukhtar, 2007; Yu et al., 2014).

The prominent protective effects of EGCG found to date are the ability to inhibit the formation of free radicals and reduction of oxidative stress. Studies showed that EGCG could reduce oxidative DNA damage induced by tobacco-specific nitrosamine, UV light, pentachlorophenol and peroxynitrite. EGCG has also been shown to inhibit LDL oxidation (Yang & Wang, 1993; Mukhtar & Ahmad, 2000). This suggests that the protective action of

EGCG is partly attributed to the scavenging effect on the overproduction of free radicals during cellular damage.

A study demonstrated that oral administration of 1.5% green tea or other tea preparations as drinking fluid significantly reduced the incidence of oral dysplasia and carcinoma in Syrian golden hamsters. The treatment also reduced the frequency of micronucleated cells, the proliferation index and the level of epidermal growth factor (EGF) receptor expression in oral mucosal cells, thereby portrayed beneficial effects on gastrointestinal tract cancer (Li et al., 1999). Studies using human prostate cancer cells DU145 (androgen-insensitive) and LNCaP (androgen-sensitive) showed that EGCG induced apoptosis, cell-growth inhibition and cell-cycle dysregulation (Adhami et al., 2003). A later study also revealed that EGCG reduced the expressions of key enzymes that reduce the aggressiveness of prostate cancer (Pezzato et al., 2004). Also the co-administration of green tea catechins with anti-cancer drugs may increase the potency of anti-cancer medicines or prevent cytotoxicity of cancer treatment. Implementation of green tea to cancer treatment may also lower costs and economic burden for cancer patients.

1.8.Experimental Models for Investigating New Agents for Metabolic Syndrome

1.8.1. Cellular Models

1.8.1.1. 3T3-L1 Adipocytes

3T3-L1 adipocytes are a useful model commonly used *in vitro* studies of MetS and insulin resistance. 3T3-L1 pre-adipocytes, derived from mouse embryonic tissue, differentiate in culture from a fibroblast phenotype to an adipocytic phenotype under appropriate culture

1 conditions (Poulos et al., 2010; Zebisch et al., 2012). GLUT1 is present in both phenotypes
2 while GLUT4 is expressed only in the adipocytes phenotype. In the adipocytes, GLUT1 is
3 distributed between the plasma membrane and an intracellular vesicular storage site. GLUT4
4 under basal conditions resides almost exclusively intracellular but translocates to the plasma
5 membrane when cells are acutely stimulated with insulin (Thompson et al., 1997).

6 Recently, these 3T3-L1 adipocytes have been used to perform studies to evaluate the
7 properties and mechanisms of natural products in relation to their therapeutic effects on T2D
8 and insulin resistant states. A study has shown to support earlier hypotheses that 3T3-L1
9 adipocytes can serve as a model for studying the development of insulin resistance, a major
10 factor contributing to the onset of T2D. The study revealed that the cells develop insulin
11 resistance in response to physiological relevant concentrations of insulin (Thompson et al.,
12 1997).

13 Also, glucose deprivation, which prevents the development of insulin-resistant glucose
14 transport, also prevents the loss in GLUT4. Together, these data suggest that the loss of
15 GLUT4 protein underlies the inability of 3T3-L1 adipocytes to respond to insulin after
16 chronic exposure. This mimics the clinical manifestation of human obesity and non-insulin-
17 dependent diabetes where loss of GLUT4 protein has been observed in adipose tissue
18 (Thompson et al., 1997).

19 3T3- L1 adipocytes have been reported to have increasing activity of enzymes on pathway of
20 triacylglycerol synthesis during adipose conversion (Kuri-Harcuch, 1977), and among the
21 changes that take place during the adipose conversion are increases, in the activity of
22 lipogenic enzymes and increases in the responsiveness of the cells to hormones affecting
23 lipogenesis and lipolysis (Pairault & Green, 1979).

1 Recently, these 3T3- L1 adipocytes have been used to perform studies to evaluate the
2 properties and mechanisms of natural products in relation to their therapeutic effects on T2D
3 and insulin resistant states.

4 **1.8.1.2. HepG2 Cells**

5 The human hepatoma cell line HepG2 was isolated in 1979. It was established from human
6 liver tumour biopsies and was shown to have morphological characteristics and the epithelial
7 cell shape compatible with those of liver parenchymal cells. The HepG2 cell line was also
8 shown to synthesize and secrete various human plasma proteins into the medium. Various
9 studies have reported that this cell line, unlike most adipocytes and muscle cells is sensitive
10 to insulin with respect to glucose uptake (Mueckler et al., 1985). In hepatocytes, glucose
11 transporters are mostly located in the plasma membrane. A study showed that hepatocytes
12 express GLUT1 and GLUT2 glucose transports and that GLUT2 is stored in intracellular
13 organelles but lack the insulin-responsive glucose transporter translocation mechanism (Hah
14 et al., 1992). The Hep2G cell line is useful in exploring the effects and mechanisms ROS,
15 oxidative stress, mitochondrial membrane potential and liver toxicity (Vidyashankar & Mitra,
16 2010).

17 **1.8.1.3. L6 Cells**

18 The L6 cell line of skeletal muscle has been used widely in cellular experiments. The
19 myoblast-like cell line, originally derived from rat skeletal muscle, retains many
20 characteristics of skeletal muscle including differentiation into myotubes, display of electrical
21 and contractile activity and production of muscle-specific proteins. L6 myoblasts have been
22 found to fuse to form multi-nucleated and striated muscle fibres, whereby the fusion declines

1 following serial propagation. The increase in insulin-stimulated glucose transport in the cell
2 line is promoted by GLUT4 translocation to the cell membrane (Mitumoto et al., 1991).

3 These features of L6 myotubes are important in regards that GLUT4 is responsible for
4 insulin-dependent glucose uptake in mature skeletal muscle. In the myotube stage, GLUT4
5 coexists along with the housekeeping glucose transporter GLUT1 and the fetal muscle
6 transporter GLUT3. These cells have a fully functional insulin-signalling cascade including
7 robust activation of Akt.

8 The detrimental effects of exposing cells to high glucose concentrations (glucose toxicity)
9 include impaired glucose metabolism and insulin resistance. This is recognized in part to
10 increased metabolism of glucose via the hexosamine pathway (Maddux et al., 2001).
11 Although this pathway generally consumes only a small proportion (<3%) of glucose
12 entering cells, it provides an over-spill route for excess glucose that is not utilized via
13 glycolysis, glycogenesis or the pentose phosphate pathway (Marshall & Traxinger, 1991).
14 They also found that increased activity of the hexosamine pathway appears to serve as an
15 intracellular fuel sensor by generating signals within the cell to decrease glucose uptake,
16 particularly in insulin-sensitive cells such as skeletal muscle (Marshall & Traxinger, 1991).

17 **1.8.2. Animal Models**

18 **1.8.2.1. High Fat Diet Rodents**

19 High fat diet (HFD) fed rodent models have contributed significantly to the analysis of the
20 pathophysiology of MetS. Fat-enriched diets have been used to model obesity, dyslipidaemia
21 and insulin intolerance in a range of rats. It has been used extensively to this day in animal
22 experiments as it has been shown to closely resemble the metabolic disease states and related

1 cardiovascular complications in humans (Buettner et al., 2006). HFD fed male Wistar rats
2 develop an animal model that replicates the natural history and metabolic characteristics of
3 T2D and has been shown to be a valuable model for the screening of novel pharmacological
4 agents. A study showed that an animal model of diet-induced obesity in rats replicates the
5 main features of human obesity. It was found to be reproducible over several experiments and
6 well controlled. Statistics showed that HFD rats weighed more than and developed
7 substantially more adipose tissue than control rats and developed the insulin resistance and
8 hyperleptinaemia which is commonly associated with obesity (Woods et al., 2003).

9 The C57BL high fat fed mouse model is also a commonly used to investigate and evaluate
10 possible agents for MetS. A study that was carried out some time ago showed that C57BL/6J
11 mice became severely obese after HFD feeding, and the mice presented with clinical features
12 of hyperinsulinaemia, altered beta-cell function, and hypertension (Surwit et al., 1997).
13 Another study showed that HFD feeding in C5BL/6J mice induced hyperlipidaemia,
14 hyperleptinaemia and insulin resistance (Sumiyoshi et al., 2006).

15 Several studies showed that feeding rodents a HFD induced hyperinsulinaemia, and when
16 streptozotocin (STZ) was injected into these mice, they developed hyperglycaemia. It is
17 known that the murine models induced by STZ injection in combination with a high calorie
18 diet possess the characteristic of insulin resistance (Chen et al., 2007). A combination of HFD
19 and STZ injection in mice can be utilized to set up a T2D animal model (Leng et al., 2004;
20 Luo et al., 1998; Reed et al., 2000; Surwit et al., 1998).

21 The method of HFD feeding was developed in order to acquire more accurate models of
22 MetS in mice to mirror the human disease condition. Even though the genetically inbred
23 mice, such as ob/ob mice presented obesity and hyperinsulinaemia, the severity of these

features was far greater than those normally presented in the corresponding human condition. The clinical features of the HFD fed mice were similar to those of human, and compared to the genetically breeding method, the HFD feeding method was much easier in a practical sense (Luo et al., 1998).

1.8.2.2. Zucker Fatty and Zucker Diabetic Fatty Rats

Zucker fatty rats are genetically obese and they serve as a good model for MetS or the pre-diabetic stage of human disease. Thus, these rats are often used in research regarding the prevention of transitions of T2D from MetS (Marsh et al., 2007). The Zucker fatty rats are also both leptin deficient and resistant, which makes them hyperphagic (Bray, 1977). Genetically, Zucker diabetic fatty rats are similar to Zucker fatty rats but the former present with hyperglycaemia whereas the latter do not (Marsh et al., 2007).

1.8.2.3. Lep^{ob/ob} Mice

The Lep^{ob/ob} mouse model is also commonly used in studies for MetS. The Lep^{ob/ob} mice are usually obese by 4 weeks of age and on a chow diet, can weigh greater than 100 grams, which is four times that of their littermate controls. The genetic background is an extremely important consideration for the use of Lep^{ob/ob} mice in studying MetS. Generally, leptin deficiency results in hyperphagia, reduced energy expenditure and extreme obesity. On the C57BL/6J background, Lep^{ob/ob} mice have mild hyperglycaemia that is apparent transiently from 8 to 12 weeks of age. At this point, pancreatic β -cell compensation occurs and increased insulin levels bring glucose homeostasis under control (Kennedy et al., 2010). Alternatively, on the C57BL/KsJ background, Lep^{ob/ob} mice develop hyperglycaemia and diabetes, with blood glucose levels being sustained at about 400 mg/dl, accompanied by only temporary elevations in insulin levels and followed by β -cell failure (Kennedy et al., 2010).

1.9.Aims of Thesis

The global objective of study is to discover a natural agent for the management of MetS and prevention of T2D. As discussed above, MetS is a complex syndrome involving multiple disease states, namely, central obesity, insulin resistance, dyslipidaemia, hypertension, and pro-thrombotic and pro-inflammatory states. Its epidemiology as a global threat continues to rise due to the lack of knowledge about its exact pathogenesis, as well as a lack of effective multi-targeted drugs with minimal adverse effects. Despite many unanswered questions, research continues to uncover the various mechanisms of the multiple disease states of MetS.

Studies have identified that research into the area of natural agents may be highly valuable to our present society. Over the years natural agents and teas have been identified to have beneficial effects on the components and risk factors of MetS. The aim of the project is to determine the *in vitro* effects and mechanism of GTP on glucose and lipid metabolism and to utilise a rat model of MetS induced by genetic and dietary factors to determine the biochemical effects and molecular mechanisms of GTP in regulating glucose and lipid levels. GTP may prove to have beneficial effects for MetS and T2D prevention.

The specific aims of this thesis are:

- 1) Carry out an *in vitro* study to evaluate the metabolic effects of GTP on glucose and lipid metabolism. 3T3-L1 adipocytes and HepG2 cells will be utilised to explore the effects of GTP on insulin-stimulated glucose uptake, glycogen synthesis, lipogenesis and lipolysis and further investigate underlying molecular pathways.

- 1 2) Carry out an *in vivo* study to investigate whether GTP improves whole body insulin
- 2 sensitivity, circulatory lipid profiles, prevents onset and progression of fatty liver disease and
- 3 identify mechanisms of these actions.

4

CHAPTER 2: GENERAL MATERIALS AND METHODS

2. General Materials and Methods

2.1. Materials

2.1.1. General Materials and Reagents

Tea polyphenols (99% purity) were extracted from green tea leaves grown in Guizhou province, south-western China, by Zuyi Lushen Kangyuan Co (Guizhou, Meitan, China). 3T3-L1 adipocytes and HepG2 cells were supplied by American Type Culture Collection (ATCC) (VA, USA). Dulbecco's Modified Eagle Medium (DMEM), Fetal Bovine Serum (FBS), Penicillin-Streptomycin were supplied by GIBCO (Auckland, NZ) and Insulin was supplied by Eli Lilly Pty Ltd (NSW, Australia). Trypsin and tetrazolium salt, 3-(4,5-dimethylthiazol-2-yl)-5-(3,4-diphenyl tetrazolium) bromide (MTT) were purchased from Sigma-Aldrich (Irvine, Ayrshire, UK). Dimethyl sulfoxide (DMSO) was supplied by Amiresco (Solon, USA) and the chemicals NaCl, KCl, NaHCO₃, MgSO₄ and KH₂PO₄ were provided by BDH Chemicals (Kilsyth, Vic). Bovine serum albumin (BSA) was supplied by Fisher Scientific Ltd (Loughborough, UK). 2-DOG and [3H]2-Deoxyglucose were supplied by Sigma Chemicals Co (MO, USA). Optiphase 'Hisafe' 3 Scintillant (Fisher Chemicals, UK) and scintillation counter used was Beckman LS 7800 supplied by Beckman Scientific Instruments (Irvine, CA, USA). Triton X-100 was supplied by FISON Chemicals (Loughborough, UK), antibodies were supplied by Santa Cruz Biotechnology (Santa Cruz, CA, USA) and Cell Signaling Technology (MA, USA). Enhanced chemiluminescence (ECL) (Pierce, IL, USA). Cell culture flasks, plates and consumables were supplied by Sarstedt (NC, USA), Adipolysis and adipogenesis assay kits were purchased from Cayman Chemical Company (MI, USA).

Male Zucker fatty (fa/fa) rats (ZF) and lean rats, age at 5-7 weeks and approximately body weight 160 g, were supplied by the Monash Animal Research Platform (Monash University, Vic, Australia). Serum glucose concentrations were determined by a spectrophotometric kit (Dialab Ltd, Vienna, Austria). Serum NEFA concentrations were determined using an enzymatic colorimetric method (NEFA-C kit; Wako Pure Chemical Industries, Osaka, Japan), serum TG using an enzymatic colorimetric method (Triglycerides GPO-PAP reagent; Roche Diagnostics, IN, USA), serum ALT and AST assay kits obtained from Dialab (Vienna, Austria).

2.1.2. Identification of Chemical Composition of GTP

The composition of the GTP product was identified using liquid chromatography mass spectrometry procedure, where the polyphenols present in the GTP product was monitored by diode-array and mass spectrometry detection. The polyphenolic compounds identified using liquid chromatography-mass spectrometry (LC-MS) were 68% EGCG, 7% epigallocatechin (EGC), 1% epicatechin gallate (ECG), and 19% epicatechin (EC) in GTP (w/w), the structures of these polyphenols are shown in Figure 2.1. According to the literature, the most potent bioactive catechin in GTP is EGCG, followed by ECG, and EGC and EC with weak biological action (Muto et al., 2001). For all cellular studies, the molecular weight of EGCG was used to calculate a series of mole concentrations (0.01, 0.1, 1, and 10 μ M) to present the total GTP (1:0.68 GTP versus EGCG) and the term of GTP-EGCG was used to present all results from total polyphenols.

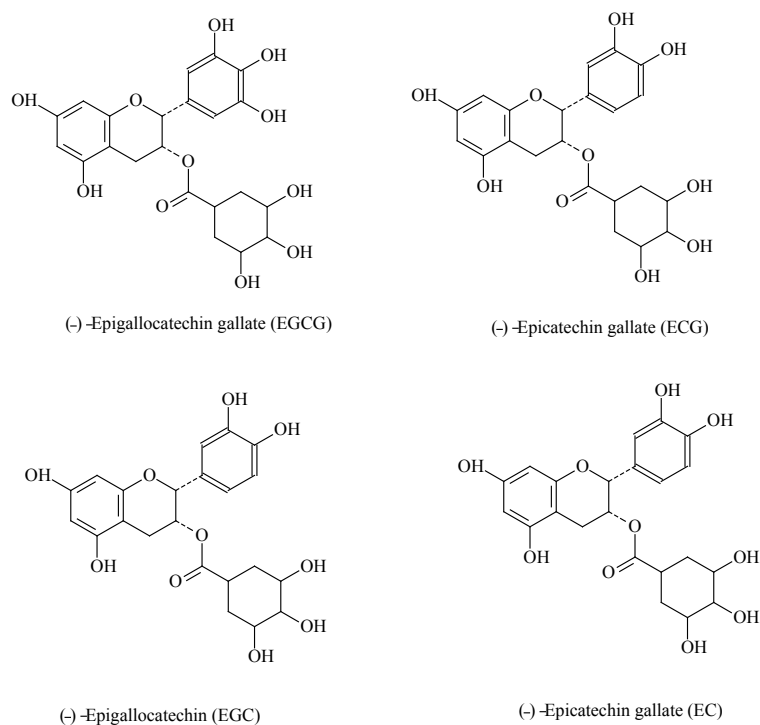


Fig 2.1. Structures for four polyphenols in green tea The main polyphenolic compounds identified in GTP product were (-)-epigallocatechin gallate (EGCG), (-)-epicatechin gallate (ECG), (-)-epigallocatechin (EGC), and (-)-epicatechin (EC).

2.2.Methods for Cellular Study

2.2.1. Cell Culture and GTP Treatment

2.2.1.1. Maintenance and Passaging of Cell Lines

Two cell lines were used for this study. 3T3-L1 adipocytes were supplied by American Type Culture Collection (ATCC) (VA, USA). 3T3-L1 mouse adipocytes were maintained in complete medium (CM) containing DMEM supplemented with 10% FBS and 1% penicillin in incubator (37°C and 5% CO₂). CM was replaced every second day until confluent.

Human hepatoma HepG2 cells (ATCC, VA, USA) were maintained in DMEM containing normal glucose (5.5 mM D-glucose) and supplemented with 10% FBS and kept in an

incubator at 37°C and 5% CO₂. HepG2 cells were cultured in complete medium with 10% FBS until 70-80% cell confluence. 24 h prior to all experimental procedures, appropriate glucose concentrations (5.5 mM or 30 mM D-Glucose) were added to the cell medium to mimic a normal physiological condition and diabetic condition.

Cells were subcultured at 70% confluency. Usually, 3T3-L1 adipocytes were subcultured every 4-5 days and HepG2 cells every 6-7 days. Media were aspirated and the cells were washed with 1 ml trypsin (Sigma, UK). Trypsin was added to the cell monolayer, which was incubated for 10 min in the incubator at 37°C until the cells detached from the cell culture flask as observed by microscopy. The cells were then resuspended in appropriate media and centrifuged at 500 g to collect cell pellets. Cells were counted using a haemocytometer (Improved Neubauer Counting Chamber, Germany) and seeded at the desired density.

2.2.1.2. GTP Treatment of Cells

GTP was always freshly prepared by dissolving in DMEM. The 1 M GTP-EGCG stock was further diluted in DMEM to obtain concentrations of 0.1 µM, 1 µM, 10 µM, 100 µM and 1 mM GTP-EGCG for experiments. Approximately 4 x 10³ cells were seeded to 24 well plates. When wells reached desired confluence, CM was discarded and starving medium (SM) containing 0.5% FBS and 1% penicillin was added and incubated for 6 h. After incubation, cells were treated with or without 100 nM of insulin, followed by concentrations of 0.1 µM, 1 µM, 10 µM, 100 µM and 1 mM GTP-EGCG. Plates were incubated for 24 h at 37°C and 5% CO₂. This treatment procedure was used as a standard for all experiments.

2.2.2. Cell Viability Assay

Cell viability was determined by MTT assay. MTT is a colourmetric assay that determines the viability of live cells. Yellow MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) is reduced to purple formazan in the mitochondria of living cells. The absorbance of the coloured solution is then quantified by measuring the samples at a certain wavelength, usually between 500-600 nm.

MTT was dissolved in sterile PBS to obtain stock solution of 5.0 mg/ml and filtered through 0.2 μ m filter to make sterile. 3T3-L1 cells were treated by standard GTP-EGCG treatment procedure and incubated for 4 h at 37°C to allow for colour development. The MTT media was then aspirated from wells and replaced with 100 μ L DMSO to allow for the releasing of formazan. The plate was incubated for 15 min to allow for equilibration and the absorbance was measured at 570 nm single fixed wavelength with a micro-plate reader (Bio-Tek Instruments Inc., USA).

Results were expressed as the mean percentage of control $\pm$ S.E. Viability of insulin-stimulated control cells was set as 100% and viability percentages for GTP-EGCG treated cells were corrected accordingly. Therefore, if the percentage of GTP-EGCG treated cells were significantly lower than the percentage of insulin-stimulated control cells, this was considered toxic.

2.2.3. Measurement of Glucose Uptake

2-deoxyglucose (2DOG) uptake was measured in cultured 3T3-L1 mouse adipocytes. The effect of GTP-EGCG on glucose uptake was determined by measurement of 2DOG, adapted from methodology described by Zhou et al (Zhou et al., 2007).

1 Approximately 4×10^3 cells were seeded to a 24-well flat bottom cell culture plate (Sarstedt,
2 NC, USA), containing 0.5 ml CM (10% FBS, 1% penicillin) until it reached 70% confluence
3 (time taken is usually 28-48 h). 1 ml SM (0.5% FBS, 1% penicillin) was added to each well
4 and incubated for 6 h at 37°C, 5% CO₂. 100 nM insulin and GTP-EGCG test samples (0.01
5 µM -10 mM) were added to the quiescent cells and placed in an incubator for 24 h. Following
6 treatment, wells were washed with Krebs buffer (114 mM NaCl, 5 mM KCl, 25 mM
7 NaHCO₃, 1.18 mM MgSO₄, 1.17 mM KH₂PO₄) at room temperature. Cells were then
8 incubated with 1 ml “Hot” Krebs Solution (Krebs Buffer + 0.2 µCi [³H]2-Deoxyglucose
9 (specific activity 10 Ci/mmol) + 0.1 mM 2-Deoxyglucose) for 10 min at 37°C, 5% CO₂. The
10 “Hot” Krebs Solution was discarded and wells were washed with 1 ml “Cold” Krebs Solution
11 (Krebs Buffer + 1.27M CaCl₂) twice. “Cold” Krebs Solution was discarded and 500 µl of 1M
12 NaOH was added to each well and incubated at room temperature for 3 h.

13 Samples in the wells were transferred to scintillation vials and 4.5 ml Optiphase Hisafe 3
14 Scintillant liquid (Fisher Chemicals UK) was added to each vial and vortexed thoroughly.
15 Radioactivity was determined with a scintillation counter (PerkinElmer Inc, MA, USA) and
16 analysis of data was performed using Graphpad Prism 6 software (GraphPad Inc, San Diego,
17 CA).

18 **2.2.4. Measurement of Insulin-stimulated Glycogen Synthesis**

19 HepG2 cells administered normal (5 mM D-glucose) or high glucose (30 mM D-glucose)
20 were used to determine the effect of GTP-EGCG on ¹⁴C-glucose incorporation into glycogen.
21 1 µCi ¹⁴C-glucose solution was added to GTP-EGCG treated HepG2 cells for 30 min at 37°C.
22 The reaction was stopped with 30% KOH and transferred into falcon tubes. 30% KOH with 6
23 mg/ml glycogen was the added and tubes were vortexed carefully and placed on a heating

block set at 100°C for 15 min and turned down to 85°C for a further 15 min. 95% ethanol was added to all tubes and vortexed gently until the samples turned uniformly cloudy. Tubes were returned to 85°C heating block for 30 min then into an ice bath. Tubes were left to chill for 15 min to completely precipitate the glycogen. Samples were centrifuged at 2,800 xg at 4°C for 10 min to pellet glycogen and then ethanol was aspirated and samples in deionised water were transferred to scintillation vials containing 5 ml scintillation liquid. The samples were counted using a scintillation counter (PerkinElmer Inc, MA, USA). Glycogen synthesis were attained by measuring the rate of incorporation of D-[U-¹⁴C]glucose into glycogen.

2.2.5. Lipolysis Assay

Lipolysis was carried out using the commercially available adipolysis assay kit for 3T3-L1 cells (Cayman Chemical Company, USA). Standard treatment of GTP-EGCG was carried out, 10 µM isoproterenol was used as a positive control and starving media as the negative control. The isoproterenol begins to induce adipolysis within 1 h and the amount of detectable free glycerol increases linearly afterwards. After 24 h, the cell culture supernatants from each well were transferred into glycerol-free containers and samples were immediately assayed.

For the assay, all samples were tested in duplicates. 25 µl of the standards were transferred (6 tubes, original stock is 125 µg/ml) into 96 well plates. 25 µl of each sample were then transferred to the corresponding wells on the test plate. Where the concentrations were too high and did not fall in the range of the standard curve, samples were diluted. 100 µl of diluted Free Glycerol Assay Reagent was added to each well and incubated for 15 min at room temperature. Finally, the absorbance was read at 540 nm single fixed wavelength with a micro-plate reader (Bio-Tek Instruments Inc., USA).

2.2.6. Lipogenesis Assay

Lipogenesis of GTP-EGCG treated HepG2 cells were tested by a commercially available kit (Cayman Chemical Company, USA). Following standard treatment of HepG2 cells with GTP-EGCG on coverslips in wells, the cell media was removed from the wells with a pipette for staining. 200 µl of Lipid Droplets Assay Fixative was added to each well and incubated for 15 min. The Lipid Droplets Assay Fixative was then discarded and wells were washed with 200 µl of Wash Solution twice for 5 min each. Wells were left to dry completely by placing the plate under a blowing hood.

Once completely dry, 200 µl of Oil Red O Working Solution were added to all wells including the background wells containing no cells and incubated for 20 min. The Oil Red O Solution was then discarded and wells were washed with distilled water 3 times until the water appeared to be clear (until any pink was not visible). Wells were washed twice with 300 µl wash solution for 5 min each. At this point, microscopic images were taken to visualise pink/red oil droplets staining in differentiated cells.

Wells were left to dry completely followed by adding 200 µl of dye extraction solution to each well. Wells were gently mixed for 20 min, transferred to a 96 well plate and the absorbance was read at 490 nm single fixed wavelength with a micro-plate reader (Bio-Tek Instruments Inc., USA).

2.2.7. Western Blotting

After treatment with GTP-EGCG for 24 h, cells were homogenised using lysis or RIPA buffer (50 mM Tris, 150 mM NaCl, 0.1% SDS, 0.5% Sodium Deoxycholate, 1% Triton X-100, 1 mM PMSF, Roche Complete Protease Inhibitor Tablet (Roche Diagnostics Corporation, IN, USA) and lysates were centrifuged at 14, 000 xg for 20 min at 4°C.

Supernatants were collected and protein concentrations were determined using the Bradford reagent.

The Bradford reagent was made up by dissolving 100 mg of Coomassie Brilliant Blue G-250 in 50 ml of 95% ethanol. 105 ml of 85% (w/v) phosphoric acid was added to the solution. The solution was diluted to 1 L by adding 845 ml distilled water and was mixed thoroughly. BSA standards were used to measure the concentrations of samples. BSA concentrations 0, 0.125, 0.25, 0.5, 1.0 mg/ml were diluted and 5 μ l of each standard was used added to each well on a 96-well plate. 5 μ l of test samples were then added followed by 250 μ l of Bradford Reagent. The absorbance was read at 595 nm single fixed wavelength with a micro-plate reader (Bio-Tek Instruments Inc., USA).

3T3-L1 and HepG2 lysates were subject to 7.5%-12.5% SDS-polyacrylamide gel electrophoresis then transferred to 0.45 μ M polyvinylidenedifluoride (PVDF) membrane and immunoblotted with primary antibodies IRS-1, PKB/AkT, GLUT4, PKA (Santa Cruz, CA, USA), phospho-GSK3 β (Ser9), phospho-GS (Ser641), phospho-AMPK α (Thr172), phospho-ACC (Ser79) (Cell Signaling Technology Inc, MA, USA) and β -actin (Santa Cruz, CA, USA) at 1:000 dilution and incubated overnight in a cool room at 4°C. Membranes were washed 3 times in 0.05% Tween 20 (PBS-T) while agitating, 10 min per wash, to remove residual primary antibody. Membranes were incubated with corresponding horseradish peroxidase-conjugated secondary antibodies (Santa Cruz, CA, USA) at 1:10000 dilution and blots were developed with enhanced chemiluminescence (ECL) (Pierce, IL, USA) according to manufacturer's instructions. The protein bands were visualized by ChemiDoc XRS systems (Bio-Rad Laboratories, CA, USA) and Quality One 4.6.1(Biorad) software and density of bands were quantified with the same analysis program.

2.3. Methods for Animal Study

2.3.1. Experimental Animals

Male zucker fatty (fa/fa) rats (ZF) and their lean littermates, aged 5-7 weeks and approximately 160 g body weight, were supplied by the Monash Animal Research Platform (Monash University, Vic, Australia). Rats were acclimatised in communal cages at 22 °C, with a 12 h light-dark cycle (lights on 0700 h) for 1 week and had *ad libitum* access to a standard chow diet (Gordon's Specialty Stock Feed, Sydney, Australia) and water.

2.3.2. Ethics Approval

All animal procedures described were approved by the Animal Care and Ethics Committee (ACEC # 2009-325A) of University of Technology, Sydney and were in accordance with the National Health and Medical Research Council of Australia Guidelines on Animal Experimentation.

2.3.3. High-Fat Diet Feeding

ZF rats (n=30) were fed a high-fat diet (HFD; 350 kJ/d) for 2 weeks. The energy percentage composition of the HFD was 59% fat, 20% carbohydrate, and 21% protein, with equal quantities of fibre, vitamins, and minerals to the standard chow diet (Kraegen et al., 1986). The HFD was replenished daily.

2.3.4. Treatment Protocol

After two weeks of HFD feeding, ZF rats (n=30) were divided into two groups. The control group (HFD-Con; n=15) was administered distilled water (5 ml/kg of body weight) and ZF rats in the GTP treatment group (HFD-GTP; n=15) was administered GTP at 200 mg/kg of

body weight daily for 8 weeks via oral gavage. HFD feeding was continued throughout the 8 weeks treatment period. Body weight and food intake were recorded daily.

Lean zucker rats (n=15) were fed a standard laboratory chow diet (Rat maintenance diet, Gordons Specialty Feeds, Sydney, Australia) containing 5% fat, 69% carbohydrate, and 21% protein plus fibre, vitamins, and minerals and received vehicle treatment (distilled water at 5 ml/kg of body weight) throughout the treatment period.

2.3.5. Body Weight and Food Intake

Body weights of rats were then measured on a daily basis throughout the treatment period. Daily food intake was calculated by measuring the amount of fresh food given then weighing the remaining food after each day.

2.3.6. Collection of Tail Vein Blood Samples

Blood was obtained at the end of the treatment, following an overnight fast (from 1600 h). Conscious rats were transferred from the animal house to the procedure room and allowed to settle for one hour before collection of blood samples. Tail vein blood samples were obtained by excising the tip of tail. Approximately 0.25 ml of blood was collected from the tail vein into eppendorf tubes. Serum was separated by rapid centrifugation at 4000 rpm for 10 min using a benchtop microcentrifuge (Sorvall MC 12V, CT, USA) and stored at -20°C for determination of serum glucose, insulin, triglycerides (TG), non-esterified fatty acids (NEFA), cholesterol, alanine transaminase (ALT) and aspartate transaminase (AST) concentrations.

2.3.7. Oral Glucose Tolerance Test

Oral glucose tolerance tests (OGTT) were performed on conscious rats following an overnight fast. Rats were transferred from the animal house to the procedure room at 0800 h and OGTT were commenced at 0900 h. Tail-vein blood samples were collected at baseline (0) and at 30, 60, 90 and 120 min after administration of 50% (w/v) glucose (Glucose Intravenous Infusion BP, AstraZeneca, Sydney, Australia) (2 g/kg of body weight) by oral gavage using a standard gavage needle attached to a syringe. Serum were separated by rapid centrifugation in a microcentrifuge at 4000 rpm for 10 min then frozen at -20°C for storage for the glucose measurement. The serum glucose concentrations were measured according to section 2.3.9.1.

2.3.8. Collection of Tissue Samples

At the end of the experiment, all rats were anaesthetised with an inhalation mixture of isoflurane, nitrous oxide and oxygen for collection of epididymal fat pad, inguinal fat, subcutaneous fat, liver, skeletal muscle, red quadriceps and red gastriceps. All tissues were rapidly excised, weighed and snap frozen in liquid nitrogen, and were stored at -80°C for subsequent use as required.

2.3.9. Laboratory Measurements

2.3.9.1. Glucose Concentration

Serum glucose concentrations were determined by the glucose oxidase-peroxidase (GOD-PAP) kit (D00221, Dialab Ltd, Vienna, Austria). The GOD-PAP method was applied, according to the manufacturer's instructions. The sample solution or standard (3 µl) was mixed with GOD-PAP enzyme solution (300 µl) in a 96 well plate and incubated at 37°C for

10 min. The optical density was determined at 500 nm using the Power Wave™ Microplate Spectrophotometer (Bio-Tek Instrument Inc., USA) The concentrations of glucose were determined by the glucose standard curve.

2.3.9.2. Insulin Concentration

Fasting serum insulin concentration was measured using an ELISA kit from Linco Diagnostic Services (MO, USA) according to manufacturer's instructions. Insulin molecules from samples were captured to the wells of microtiter plates coated by pre-titered amount of monoclonal mouse anti-rat insulin antibodies and the binding of biotinylated polyclonal antibodies to the captured insulin. Immobilised antibody-enzyme conjugates were measured spectrophotometrically by increased absorbency at 450 nm, corrected from absorbency at 590 nm, after acidification of formed products. The increase in absorbency is directly proportional to the amount of captured insulin in samples and determined from standard curve generated with standards of known concentrations of rat insulin.

2.3.9.3. Non-esterified Fatty Acids Concentration

Serum NEFA concentrations were determined using an enzymatic colorimetric method (NEFA-C kit; Wako Pure Chemical Industries, Osaka, Japan), according to the manufacturer's instructions. The assay was based on an acyl-CoA synthetase and acyl-CoA oxidase method and the amount of NEFA in sample was determined by measurement of optical density at 550 nm on the Power Wave™ Microplate Spectrophotometer (Bio-Tek Instrument Inc., USA). The amount of NEFA in samples was determined by the NEFA standard curve.

2.3.9.4. Triglycerides Concentration

Serum TG was also determined spectrophotometrically using an enzymatic colorimetric method (Triglycerides GPO-PAP reagent; Roche Diagnostics, IN, USA), according to the manufacturer's instructions. The assay was based on a lipase method, where TG were completely hydrolysed to glycerol and NEFA. The amount of glycerol in a sample (as a surrogate measure of triglyceride) was subsequently assessed from the optical density measured at 490nm on the Power Wave™ Microplate Spectrophotometer (Bio-Tek Instrument Inc., USA) compared to known amounts of glycerol on a standard curve.

2.3.9.5. Cholesterol Concentration

Serum cholesterol concentration was also determined spectrophotometrically using an enzymatic colorimetric method (total cholesterol: D95116 Dialab Ltd., Vienna, Austria), according to the manufacturer's instructions. In the assay, cholesterol ester was hydrolysed by addition of cholesterol esterase into cholesterol. Cholesterol was further oxidised by cholesterol oxidase to produce hydrogen peroxide. The hydrogen peroxide produced then reacted with 4-aminoantipyrine under the catalytic action of peroxidase to form a quinoneimine dye, to develop a pink colour, which was subsequently assessed by measuring the optical density at 500 nm with the Power Wave™ Microplate Spectrophotometer (Bio-Tek Instrument Inc., USA). Serum cholesterol concentrations of each sample were determined from the standard curve shown by the spectrophotometer.

2.3.10. Biochemical Assays

2.3.10.1. Western Blotting

Frozen tissue samples obtained as described in section 2.3.8 were minced with scalpel and scissors and then homogenised in appropriate volume of lysis buffer for 2 x 15 sec (setting 5) using a Polytron (Ultra-Turrax T8, IKA-Labortechnik, Staufen, Germany). The relative volumes of tissue to lysis buffer were 1:300 (mi: mg) for fat tissue and 1:100 for liver and skeletal muscle. The crude homogenate was spun at 400 xg for 15 min at 4°C. Supernatants were collected for total protein determination and immunoblotting analysis.

Protein (20 µg) was separated by SDS-PAGE (12.5%) and blotted onto PVDF membranes. Blotted membranes were blocked with 5% skim milk in PBS with 0.05% Tween 20 overnight and incubated with primary antibodies GLUT4, IRS-1 (Santa Cruz Biotechnology, CA, USA), AKT (Abcam, CA, USA), pSer612 IRS-1, pSer473 AKT (Cell Signaling, Technology Inc., MA, USA) PKCβ2, PKCθ, PKCε, PKCζ (Santa Cruz Biotechnology, CA, USA) and β-actin diluted at 1:1000 for 4 h. Membranes were then incubated with corresponding secondary antibodies (1:10000) for 2 h. Protein expressions were visualised by ECL (Pierce, IL, USA) and quantified with Quality One 4.6.1 software of ChemiDoc XRS system (Biorad Laboratories, CA, USA).

2.3.10.2. Determination of Alanine Transaminase and Aspartate Transaminase Concentrations

The concentrations of serum Alanine Transaminase (ALT) and Aspartate Transaminase (AST) were measured using serum ALT and AST assay kits obtained from Dialab (Vienna, Austria). In brief, a working reagent was prepared by four parts of reagent 1 (R1) mixed with one part of reagent 2 (R2) in a falcon tube and vortexed. In this enzymatic method, L-Alanine

1 and 2-Oxoglutarate produces Pyruvate and L-Glutamate by the catalytic action of ALT. L-
2 Aspartate and 2-Oxoglutarate produces Oxaloacetate and L-Glutamate by the catalytic action
3 of AST. The resultant pyruvate and oxaloacetate in addition to NADH are further oxidized by
4 lactate dehydrogenase to form nicotinamide adenine dinucleotide (NAD). A kinetic decreasing
5 occurs as NADH is oxidized to NAD. Decreases in absorbance at 340 nm are directly
6 proportional to the activity of ALT or AST in serum samples. The assay was carried out
7 using a 96-well microplate, 20 µl of blank and serum samples were transferred to plate in
8 duplicates. 100 µl of working reagent was added to each well and the absorbance was read at
9 a wavelength of 340 nm using the Power Wave™ Microplate Spectrophotometer (Bio-Tek
10 Instrument Inc., USA) precisely at 0, 1, 2 and 3 min (25°C). The serum ALT or AST
11 concentrations in each well were calculated from the absorbance displayed by the
12 spectrophotometer - the average difference of the first and last absorbance reading per
13 minute, multiplied by a factor of 952 (Δ Absorbance/ min x Factor).

14 **2.3.10.3. Tissue Triglyceride Content**

15 Triglycerides (TG) were extracted from tissues (liver and red quadriceps) using the method
16 outlined by Folch et al. (Folch et al., 1957) and quantified using an enzymatic colorimetric
17 method (Triglycerides GPO-PAP reagent; Roche Diagnostics, IN, USA). In brief, 4 ml of a
18 chloroform:methanol (2:1, v/v) solution was added to a known amount (approximately 50 mg
19 of tissue) of pulverised liver or red quadriceps in plastic tubes. The tubes were sealed and
20 rotated overnight at room temperature to extract the TG. The next day, 2 ml 0.6% NaCl was
21 added to each tube, the tubes were thoroughly vortexed and centrifuged at 850 xg for 10 min
22 at room temperature. The lower organic phase containing the TG was transferred to a glass
23 vial and dried under nitrogen gas. The TG extract was reconstituted in a known volume of
24 ethanol and samples (10 µl) were used to assay TG content spectrophotometrically by

measuring the absorbance at 490 nm with a microplate reader (Bio-Rad Laboratories, CA, USA).

2.3.11. Histological Analysis

2.3.11.1. Specimen Preparation

Following euthanasia, livers were rapidly removed from rats. From each rat, a small portion of the liver, approximately 2 mm x 8- 10 mm, was dissected carefully, freed from any visible fat and blood, embedded in Tissue-Tek OCT (optimal cutting temperature) compound (Lab-Tek Products, IL, USA) and rapidly frozen in liquid nitrogen-cooled isopentane. The samples were sectioned at 6 μ m with a cryotome (Shandon Cryotome E, Thermo Fisher Scientific Inc., MA, USA) and thaw mounted on uncoated pre-cleaned glass slides. Before processing or storage in -80°C, the samples were air-dried for 15 min. These samples were prepared for oil red o staining. The other part of liver was prepared for paraffin sectioning. Rat livers were removed and put in 4% formaldehyde in PBS over 60 h. Formalin-fixed livers were cut to relevant sizes keeping the desired thickness (approximately 10 mm x 10 mm x 3-5 mm). These samples were placed in specimen cases using the automated tissue processor (Thermo Fisher Scientific Inc., MA USA). The samples were dehydrated through a series of graded alcohols (50%, 70%, 95%, and 100%) and xylene, and then embedded in paraffin. Chilled paraffin blocks were cut to 5 μ m thick sections with the Microtome (Thermo Fisher Scientific Inc., MA, USA). The sections were floated onto glass slides, dried overnight, and then stained with haematoxylin and eosin (HE).

2.3.11.2. Haematoxylin and eosin Stain

Paraformaldehyde-fixed paraffin embedded liver specimens were stained with haematoxylin and eosin (HE) for determination of steatosis and nexro-inflammation. Liver samples were fixed in a formaldehyde solution (10 ml formaldehyde, 90 ml distilled water) then processed through a series of ethyl alcohol and xylene and embedded into paraffin blocks. HE staining was utilised to examine the general tissue composition of liver samples.

2.3.11.3. Oil red O Staining

Oil red O stain was performed to determine lipid content in rat liver. The frozen tissue sections were placed in 10% formaldehyde solution/1% deionised water. Liver sections were stained with a filtered solution of 0.25% oil red O (Sigma-Aldrich, MO, USA) in isopropanol (Sigma-Aldrich, MO, USA). Sections were then washed in deionised water for 30 sec three times. Sections were counterstained using Mayer's haematoxylin for 30 sec to visualise nuclei, then rinsed with running tap water and covered with a cover slip using clearmount solution (Invitrogen, CA, USA). Each sample was observed at 200× magnification. The quantities of lipid content were determined as the mean of 10 different fields in each slide.

2.4. Statistical Analysis

All results were presented as mean $\pm$ S.E. One-way analysis of variance (ANOVA) was used to test systemic differences among more than two means and significant differences between two groups were established by post-hoc analysis of Tukey's test or Dunnett's test using Graphpad Prism version 6 (GraphPad Inc, San Diego, CA). p -value < 0.05 was considered statistically significant.

2 **CHAPTER 3: EFFECTS AND MECHANISMS OF**
3 **GREEN TEA POLYPHENOLS ON INSULIN-**
4 **STIMULATED GLUCOSE UPTAKE AND LIPOLYSIS**
5 **IN 3T3-L1 CELLS**
6

3. Effects and Mechanisms of Green Tea Polyphenols on Insulin-stimulated Glucose Uptake and Lipolysis in 3T3-L1 Cells

3.1.Introduction

Obesity, a major component of MetS, has become a global public health problem that affects millions of people today. The World Health Organisation (WHO) predicts that being overweight and obesity may soon replace more traditional public health concerns, such as malnutrition and infectious diseases, as the most significant cause of poor health. The most common cause of obesity is the disequilibrium between energy intake and expenditure, and is highly likely to cause other chronic diseases such as heart disease, T2D, hypertension, stroke and some forms of cancer (Lee et al., 2009; Larsson et al., 1981).

It has been demonstrated that elevated non-esterified fatty acid (NEFA) levels, an increase in lipolysis and lipogenesis, in addition to insulin-resistant glucose uptake and intracellular glucose metabolism (such as glucose phosphorylation and glycogen synthesis) are early hallmarks of the insulin resistance that proceeds to the onset of obesity and T2D (Eriksson et al., 1999).

T2D and obesity are commonly accompanied with an inapt increase and acceleration of lipolysis in adipose tissue. This may occur in the presence of a defect in the regular action of insulin, thereby causing an elevation of circulating FFA level and subsequently increasing gluconeogenesis in the liver. In addition, increased FFA itself induces further insulin resistance directly in insulin sensitive tissues, which leads to the onset of hyperglycaemic state (Bays et al., 2005). Thus, investigation into new therapeutic agents that target abnormal

FFA release from adipose tissue have become of research interest, for both management of MetS, obesity and prevention of T2D.

Reduction in adipose tissue will lead to weight loss, which will in turn reduce blood pressure, overall lipid levels, and the incidence of T2D (Sheard, 2003). Therapeutic medications and agents that target a reduction in weight and fat accumulation are crucial to our society. Presently, natural agents such as herbal medicines and teas have received significant scientific attention, as they provide a much safer and natural approach for obesity and other related health problems. Herbal medicines and teas hold much value as they contain fewer side effects and are more readily available to our society than synthesised drugs.

A promising candidate for a glucose and lipid-lowering natural agent is the polyphenols extracted from green tea. Green tea is a readily available and widely consumed beverage and has been described by current research to have the ability to modulate serum lipid profiles (Makoto et al., 2005) and reduce body weight and adipose tissue weight in animal models (Murase et al., 2002). Based on latest research, the health promoting effects of green tea are mainly attributed to its polyphenol content and there is accumulating evidence which scientifically supports that green tea reduces body weight by eliminating fat (Wolfram et al., 2006). However, the effects and mechanisms of GTP on adipose dysfunction, including decreased glucose uptake and increased lipolysis still need clarification.

In cultured adipocyte models, green tea catechins have been found to robustly inhibit adipocyte differentiation. In a recent study, EGCG dose-dependently inhibited adipogenesis in C3H10T1/2 cells (Wolfram et al., 2006). Similarly, another study demonstrated that moderate concentrations of CG, EGC, ECG and EGCG suppressed lipid accumulation in 3T3-L1 cells. The study also suggested that two cell cycles control kinases, ERK and cyclin-

dependent kinase 2 (Cdk2) are responsible for these inhibitory effects. EGCG significantly lowered phospho-ERK1 and phospho-ERK2 which are the active forms of ERK. Cdk2 was also reduced by EGCG at both the protein and activity levels (Hung et al., 2005).

This chapter of the study has been carried out to evaluate the effects of GTP on insulin-stimulated glucose uptake and lipolysis in 3T3-L1 adipocytes. The study into the molecular mechanisms by which regulation of glucose uptake and lipolysis occurs with GTP treatment was also carried out.

3.2. Research Plan and Methods

Cell Culture and GTP Treatment

3T3-L1 adipocytes were maintained in DMEM and supplemented with 10% fetal bovine serum (FBS), and 100 U/mL penicillin (GIBCO, Auckland, NZ) in an incubator (37°C and 5% CO₂). Cells were grown in CM with 10% FBS until 80% cell confluence was reached. Approximately 4×10^3 cells were seeded to 24-well plates for all assays. When confluent, CM was discarded and then SM containing 0.5% FBS was added. After 6 h incubation with SM, 100 nM of insulin (Eli Lilly Pty Ltd, NSW, Australia) was added in each well, followed by adding a range of 0.1-10 µM GTP/EGCG into appropriate wells in duplicates. Plates were maintained for 24 hr in 5% CO₂ at 37°C. This treatment procedure was used for all experiments in this study.

MTT Assay

Cell viability of GTP/EGCG-treated 3T3-L1 adipocytes was determined by MTT assay as described in Chapter 2.2.2.

1 Cellular Assays

2 *Insulin-stimulated 2-Deoxyglucose uptake*

3 Insulin-stimulated 2-Deoxyglucose uptake measurement of GTP/EGCG-treated 3T3-L1
4 adipocytes was carried out according to Chapter 2.2.3. Following standard GTP-EGCG
5 treatment, wells were washed with Krebs buffer (114 mM NaCl, 5 mM KCl, 25 mM
6 NaHCO₃, 1.18 mM MgSO₄, 1.17 mM KH₂PO₄) at room temperature. Cells were then
7 incubated with 1 ml “Hot” Krebs Solution (Krebs Buffer + 0.2 µCi [³H]2-Deoxyglucose
8 (specific activity 10 Ci/mmol) + 0.1 mM 2-Deoxyglucose) for 10 min at 37°C, 5% CO₂. The
9 “Hot” Krebs Solution was discarded and wells were washed with 1 ml “Cold” Krebs Solution
10 (Krebs Buffer + 1.27M CaCl₂) twice. “Cold” Krebs Solution was discarded and 500 µl of 1M
11 NaOH was added to each well and incubated at room temperature for 3 h. Samples
12 transferred to scintillation vials and 4.5 ml Optiphase Hisafe 3 Scintillant liquid (Fisher
13 Chemicals UK) was added to each vial and vortexed thoroughly. Radioactivity was
14 determined with a scintillation counter (PerkinElmer Inc, MA, USA) and analysis of data was
15 performed using Graphpad Prism 6 software (GraphPad Inc, San Diego, CA).

16 *Lipolysis assay*

17 Lipolysis assay was undertaken via a commercially available adipolysis assay kit purchased
18 from Cayman Chemical Company (USA) outlined in Chapter 2.2.5. Following GTP-EGCG
19 treatment of cells, the assay was carried out where 25 µl of the standards were transferred (6
20 tubes, original stock is 125 µg/ml) into 96 well plates. 25 µl of each sample were then
21 transferred to the corresponding wells on the test plate. Where the concentrations were too
22 high and did not fall in the range of the standard curve, samples were diluted. 100 µl of
23 diluted Free Glycerol Assay Reagent was added to each well and incubated for 15 min at

room temperature. Finally, the absorbance was read at 540 nm single fixed wavelength with a micro-plate reader (Bio-Tek Instruments Inc., USA).

Western Blotting

Western blotting was carried out according to Chapter 2.2.7. 50 µg of protein were subjected to 7.5% SDS-polyacrylamide gel electrophoresis then transferred to 0.45 µM PVDF membrane and immunoblotted with primary antibodies of insulin receptor substrate 1 (IRS-1), protein kinase B (PKB/Akt), glucose transporter 4 (GLUT4), β-actin (Santa Cruz, CA, USA) protein kinase A (PKA) (Cell Signalling Technology Inc, MA, USA) at 1:1000 dilution then incubated with anti-rabbit or anti-goat IgG and corresponding horseradish peroxidase-conjugated secondary antibodies (Santa Cruz, CA, USA) at 1:10000 dilution.

Statistical Analysis

Data are presented as the means ± S.E. Comparisons across the variety of treatments were done using one-way ANOVA followed by post-hoc analysis of Tukey's test to determine significant differences between the two treatments using Prism version 6 (GraphPad Software Inc, CA, USA). *p*-value < 0.05 was considered statistically significant.

3.3.Results

3.3.1. Cell viability and optimisation of GTP-EGCG concentrations using MTT assay

MTT assay was used to determine cell viability of EGCG-treated 3T3-L1 cells. MTT assay results indicated that EGCG concentrations between 0.01-10 µM did not affect the viability of the cells. However, as shown in Figure 3.1, 100 µM EGCG significantly reduced cell

1 viability ($p < 0.01$). We can also note that 100 nM insulin stimulation for 24 h significantly
2 increased cell viability in 3T3-L1 adipocytes ($p < 0.05$). The results support that 0.01-10 μ M
3 EGCG do not exert toxic effects in 3T3-L1 cells, which is in agreement with previous studies
4 (Hung et al, 2005 and Lin et al, 2005). This confirms that it is feasible to undertake
5 experimentations with EGCG concentrations between 0.01-10 μ M.

6

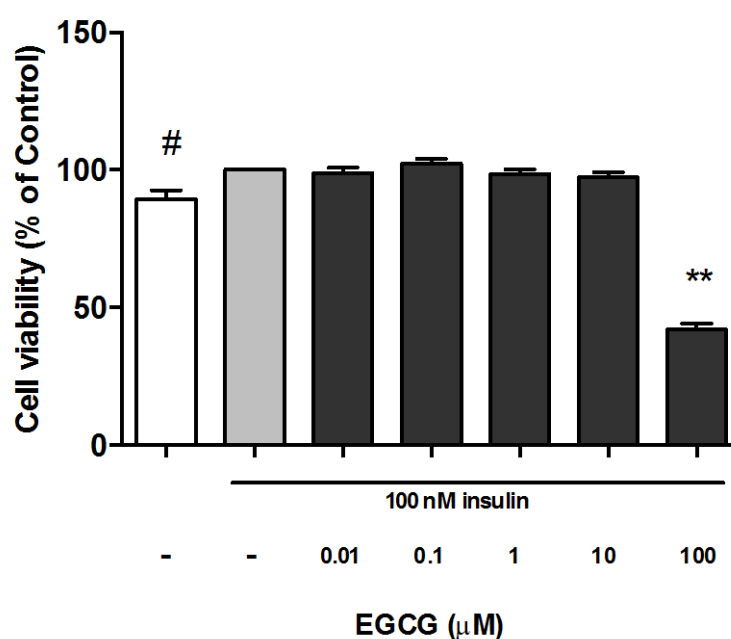


Fig 3.1. Viability of GTP/EGCG-treated 3T3-L1 cells MTT was dissolved at 5 mg/ml in PBS. Cell media containing MTT was added to each well for 4 h and incubated at 37°C, 5% CO₂. The MTT media solution was replaced with 1 ml of DMSO and optical density was measured at 570 nm. Data are means ± S.E. The data are from 5 separate experiments. #p<0.05 compared with insulin stimulation alone, **p<0.01 compared with insulin stimulation alone.

3.3.2. Effect of GTP-EGCG on insulin-stimulated glucose uptake in 3T3-L1 cells

The effect of GTP-EGCG administration on insulin-stimulated 2-deoxyglucose uptake in 3T3-L1 cells is shown in Figure 3.2. Results show that 100 nM insulin caused a slight increase in 2-deoxy-D-glucose uptake and 0.1, 1 and 10 μM EGCG significantly increased uptake of glucose by 133.3%, 147.5% (p<0.01) and 267.9% (p<0.001) respectively compared to control. This indicates that EGCG significantly increased glucose uptake in a dose-dependent manner.

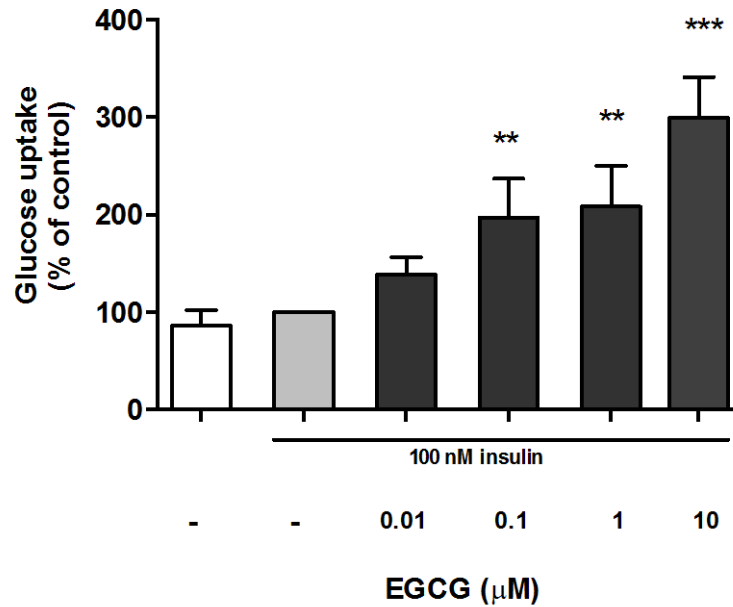


Fig 3.2. Effect of GTP/EGCG on glucose uptake in 3T3-L1 cells Glucose uptake was measured in 3T3-L1 adipocytes in the presence of without insulin stimulation, insulin (100 nM), and with insulin (100 nM) + EGCG concentrations of 0.1– 10 μM after 24 h. Cells were incubated with Krebs Solution containing 0.2 μCi [³H]2-Deoxyglucose (specific activity 10 Ci/mmol), for 10 min at 37°C, 0.5% CO₂. Radioactivity was determined via scintillation. Data are means ± S.E. from 5 separate experiments. **p<0.01, ***p<0.001 compared with insulin-stimulation alone.

3.3.3. Effect of GTP-EGCG on lipolysis in 3T3-L1 cells

Lipolysis is the process in which TG are hydrolysed into glycerol and FFA and released into the bloodstream. Obesity and T2D are associated with elevated lipolysis predominantly generated in adipocytes. 3T3-L1 adipocytes are a well-established cellular model for studying the uptake and release of FFA from adipocytes as this model shares similar morphology, gene expression and metabolism with adipocytes *in vivo*. FFA and glycerol can be measured by incubation with glycerol kinase, glycerol phosphate oxidase and horseradish peroxidase. The amount of glycerol released into the medium is proportional to the level of TG storage and lipolysis.

To examine the effect of GTP-EGCG on lipolysis, 3T3-L1 cells were treated with 0.1-10 μM EGCG and glycerol levels in the medium was measured using a glycerol reagent. Results in Figure 3.3 showed that 100 nM of insulin significantly decreased lipolysis by 43.7% ($p < 0.01$) and all EGCG concentrations (0.01, 0.1, 1 and 10 μM) further reduced lipolysis significantly by 78.3%, 85.1%, 82.9% and 68%, respectively (compared to insulin stimulation alone; $p < 0.001$); meaning that significantly less glycerol was released in 3T3-L1 cells with EGCG treatment than control cells.

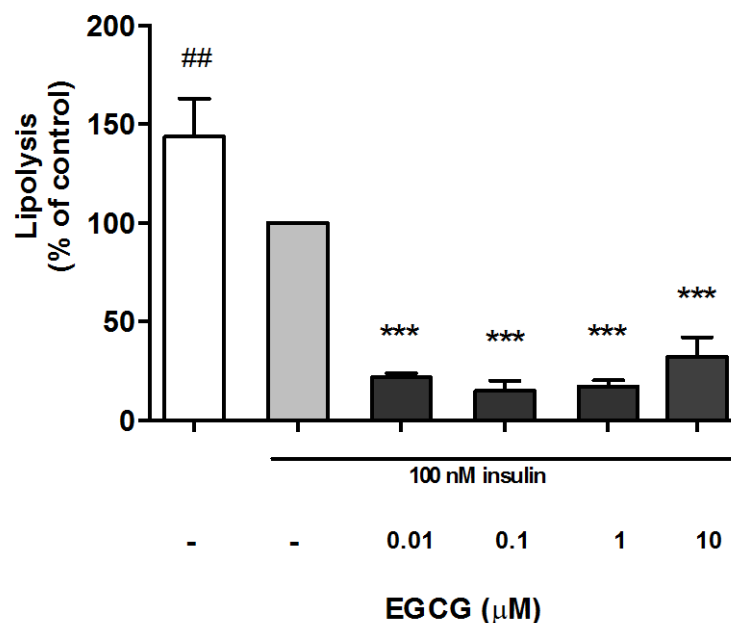


Fig 3.3. Effect of lipolytic activity in GTP/EGCG-treated 3T3-L1 cells The 3T3-L1 adipolysis kit was used to determine the amount of glycerol released from cells treated with EGCG. Cells were incubated with Free Glycerol Assay Reagent for 15 min at room temperature following EGCG treatment, and absorbance was measured at 540 nm with a micro-plate reader. Data was expressed as μM glycerol released and further converted as a percentage of the insulin stimulated cells. Data are means $\pm$ S.E from 5 independent experiments. ## $p < 0.01$ and *** $p < 0.001$ compared insulin stimulation alone.

3.3.4. Effects of GTP-EGCG on expressions of IRS-1, PKB/Akt and GLUT4 in 3T3-L1 cells

Previous studies have shown that significantly decreased expressions of the key enzymes of the insulin signalling pathway causes reduced glucose uptake in adipose cells and ultimately impairs glucose metabolism (Guilherme et al., 2008). In this study, significant changes in protein expressions of IRS-1, PKB/Akt and GLUT4 by western blotting were observed. Results showed that both low (0.1 μ M) and high dose (10 μ M) of EGCG significantly increased expression of IRS-1 ($p<0.05$). Furthermore, 0.1 μ M and 10 μ M EGCG significantly increased PKB/Akt and GLUT4 expressions ($p<0.05$ and $p<0.01$, respectively) in 3T3-L1 cells (Fig. 3.4C& D). The results demonstrate that EGCG promotes insulin-stimulated glucose uptake through increased IRS-1, PKB/Akt and GLUT4 protein expressions.

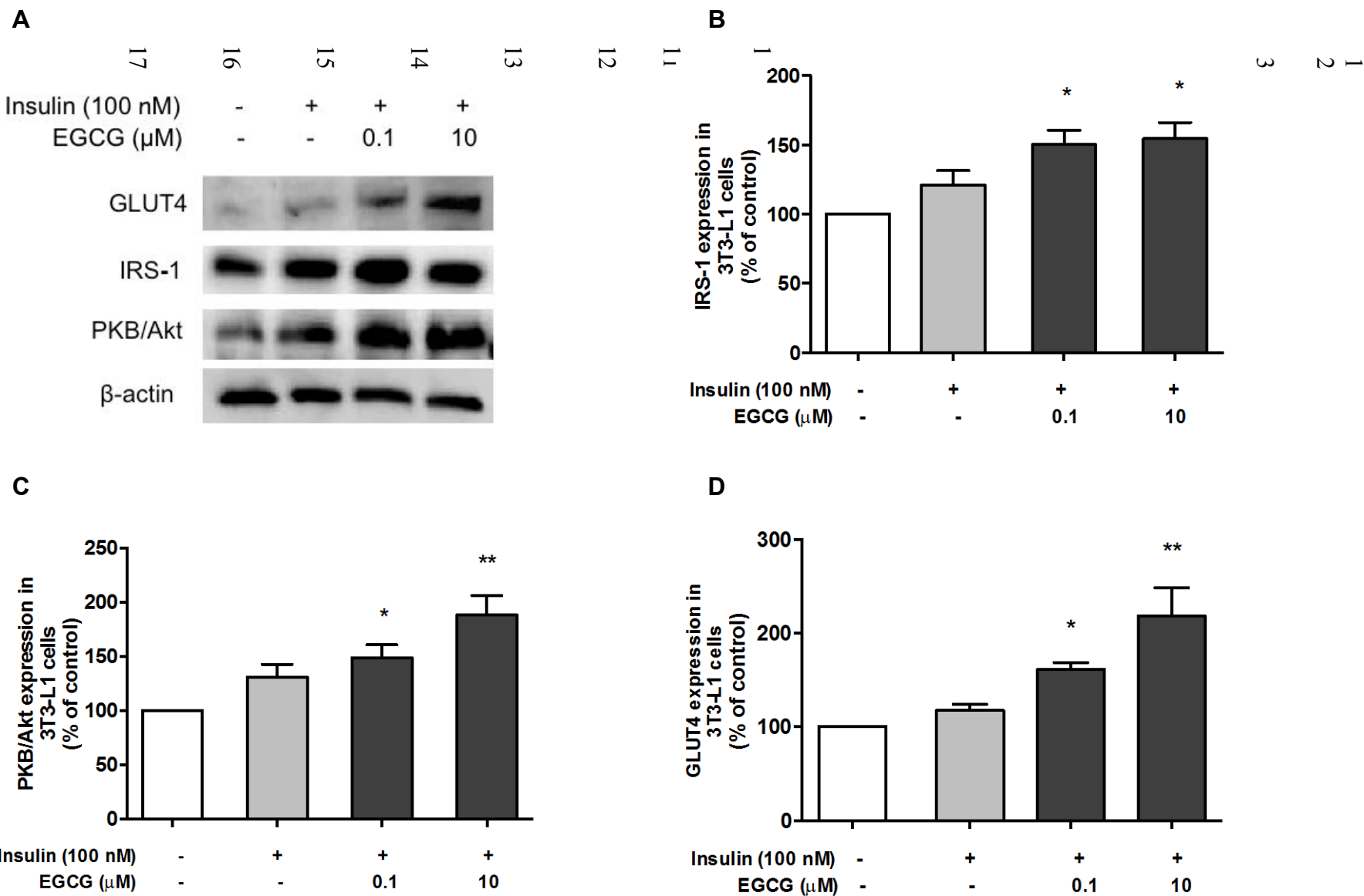


Fig 3.4. Effect of GTP-EGCG on IRS-1, PKB/Akt and GLUT4 expressions in 3T3-L1 cells Cells were quiesced in serum-free medium for 6 h and incubated in serum-free medium containing high (30 mM) concentration of D-glucose, with or without 100 nM insulin and 0.1 μ M and 10 μ M EGCG for 24 h. Representative western blot of IRS-1, PKB/Akt and GLUT4 are shown (A). Quantitative data are expressed as mean $\pm$ S.E. from 5 independent experiments shown on IRS-1 (B), PKB/Akt (C) and GLUT4 (D). * p <0.05, ** p <0.01 compared with insulin-stimulated control.

3.3.5. Effect of GTP-EGCG on expression of PKA in 3T3-L1 cells

To elucidate the mechanism by which EGCG inhibits lipolysis, the expression of PKA, a rate-limiting enzyme for lipolytic activity in adipocytes was determined via western blotting (Figure 3.5). Results showed that 100 nM insulin stimulation for 24 h inhibited PKA expression significantly ($p<0.01$) and 10 μ M EGCG further inhibited expression of PKA ($p<0.01$) by 51.1% compared to control (Figure 3.5B). This finding suggests that EGCG in 3T3-L1 cells inhibits lipolytic activity through a significantly lower expression of PKA.

A

Insulin (100 nM)	-	+	+
EGCG (10 μ M)	-	-	+



B

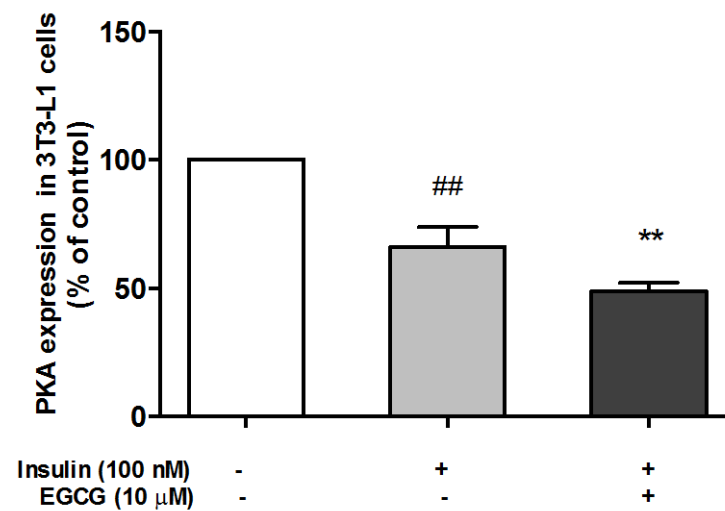


Fig 3.5. Effect of GTP-EGCG on PKA expression in 3T3-L1 cells Cell lysates was separated on 7.5% SDS-PAGE and incubated with 1:1000 PKA (Cell Signaling, USA) and β -actin was used as a loading control. (A) Representative western blot and quantitative analysis from 5 independent experiments is shown (B). Data are expressed as mean $\pm$ S.E. ($n = 5$), ^{##} $p < 0.01$ compared with control, ^{**} $p < 0.01$ compared with insulin-stimulated control.

3.4. Discussion

Previous studies have shown that tea consumption, especially green tea, is associated with lower incidence of metabolic disease (Kao et al., 2000; Wolfram et al., 2006; Collins et al., 2007). Although evidence shows that GTP-EGCG prevents and reverses the onset of insulin

1 resistance, T2D, obesity and other related disorders by improving lipid and glucose
2 metabolism in adipose tissue (Sinclair et al., 2009), the exact mechanisms of action are not
3 well known. This study explored the effects and mechanisms of actions of GTP-EGCG on
4 insulin-stimulated glucose uptake and lipolysis in 3T3-L1 adipocytes.

5 It has been well established that insulin resistance is reflected by decreased glucose uptake
6 via impaired activation of insulin receptor and IRS-1, PI3K and PKB/Akt-mediated signaling
7 in adipose cells (Saltiel & Kahn, 2001; Ouwens et al., 2005). Glucose uptake primarily occurs
8 through GLUT4, whereby GLUT4 mediates uptake of glucose in cells in response to insulin
9 stimulation. GLUT4 has been shown to be the predominant glucose transporter that accounts
10 for the major part of insulin-mediated glucose uptake in cultured adipocytes (Clarke et al.,
11 1994; , Holman et al., 1990). High fat-feeding of rodents also cause insulin resistance of
12 glucose transport, and is also associated with specific defects in the insulin signalling
13 pathway, including reduced insulin receptor and IRS-1 tyrosine phosphorylation, diminished
14 IRS-1 associated PI3-kinase activity and decrease in PKB/Akt activity, leading to a
15 significant decrease of insulin-stimulated translocation of GLUT4 protein to the cell surface
16 and an overall reduction in glucose transport activity (Jacques et al., 2001).

17 Results of this study revealed that 0.1- 10 μ M GTP-EGCG significantly increased insulin-
18 stimulated glucose uptake in 3T3-L1 cells. To elucidate the mechanism by which GTP-
19 EGCG enhanced glucose uptake, the key enzymes involved in the insulin-signalling pathway
20 were studied. Results showed that GTP significantly increased IRS-1 and PKB/Akt GLUT4
21 protein expressions in 3T3-L1 cells.

22 Also, 3T3-L1 adipocytes are not only a well-established cellular model for studying glucose
23 uptake but also FFA release from adipose cells as this model shares similar morphology, gene

1 expression and metabolism with adipocytes *in vivo* (Zebisch et al., 2012). FFA and glycerol
2 can be measured by incubation with glycerol kinase, glycerol phosphate oxidase and
3 horseradish peroxidase. The amount of glycerol released into the medium is proportional to
4 the level of TG storage and lipolysis. Obesity and T2D are associated with elevated lipolysis
5 predominantly generated in adipocytes due to increased circulating FFA levels, which
6 promotes insulin resistance (Ahmadian et al., 2009). Increased adipocyte size associated with
7 obesity may also contribute to increased lipolytic activity.

8 Adipocyte lipolysis is acutely regulated by hormones, neurotransmitters and other effector
9 molecules such as PKA and HSL (Holm, 2003; Arner, 2005). PKA has been found to a rate-
10 limiting enzyme for lipolysis in adipocytes, where reduced lipolysis is associated with
11 reduced expression of PKA (Duncan et al., 2007). It should also be noted that during fasting,
12 catecholamines stimulate lipolysis, promoting adenylyl cyclase activity and the production of
13 cAMP. cAMP binds to PKA, causing subsequent activation of catalytic subunits and
14 ultimately leading to increased expression of PKA in adipocytes (Duncan et al., 2007).
15 Following a meal, insulin stimulates uptake of nutrients such as glucose into specialized
16 tissues and potently inhibits lipolysis in adipocytes. It has been recently proposed that the
17 primary mechanism involved in inhibition of lipolysis is reduction in cAMP and PKA
18 expression levels (Choi et al, 2010). In insulin signalling pathway, phosphodiesterase 3b
19 (PDE3b) is activated through the phosphorylation of PKB/Akt. Upon activation by PKB/Akt,
20 PDE3b catalyses the hydrolysis of cAMP to 5'AMP, resulting in inhibition of PKA and
21 lipolysis in adipocytes (Choi et al., 2010).

22 This study showed that 0.01 - 10 μ M EGCG inhibited lipolysis significantly in 3T3-L1 cells.
23 Further to this, GTP-EGCG significantly down-regulated expression of PKA in 3T3-L1 cells.
24 As explained above, PKA is a rate-limiting target that regulates adipocyte lipolysis. Insulin

1 suppresses lipolysis through the activation of its downstream kinase, PKB/Akt, resulting in
2 the inhibition of PKA, the main positive effector of lipolysis (Savage et al., 2007; Choi et al.,
3 2010). During insulin resistance, this process is disrupted, leading to dyslipidaemia, impaired
4 insulin action and obesity (Duncan et al., 2007; Djouder et al., 2010). The result of this study
5 suggest that GTP-EGCG intake may reverse abnormal lipid metabolism through decreased
6 lipolysis and inhibited expression of PKA in adipocytes.

7 **3.5.Conclusion**

8 In summary, the results have shown that GTP-EGCG improved glucose uptake and inhibited
9 lipolysis in adipocytes. The results have demonstrated that GTP-EGCG increased insulin-
10 stimulated glucose uptake in a dose-dependent manner. It was revealed that GTP-EGCG
11 significantly increased glucose uptake by up-regulated expressions of IRS-1, PKB/Akt and
12 GLUT4 in 3T3-L1 adipocytes. Lipolysis was significantly inhibited in GTP-treated 3T3-L1
13 adipocytes through decreased expression of PKA. The results of this cellular study support
14 that GTP-EGCG may be a potential therapeutic agent for treating MetS and prevention of
15 obesity and T2D by improving insulin resistance in adipose tissue.

16

**CHAPTER 4: EFFECTS AND MECHANISMS OF
GREEN TEA POLYPHENOLS ON GLYCOGEN
SYNTHESIS AND LIPOGENESIS IN HEPG2 CELLS**

1
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3
4

4. Effects and Mechanisms of Green Tea Polyphenols on Glycogen Synthesis and Lipogenesis in HepG2 Cells

4.1.Introduction

As discussed in Chapter 1, MetS is a complex syndrome involving multiple metabolic and haemodynamic abnormalities, including central obesity, insulin resistance, dyslipidaemia, hypertension, and pro-thrombotic and pro-inflammatory states (Despres & Lemieux, 2006). Its epidemiology as a global threat continues to expand due to the lack of full knowledge about its pathogenesis, as well as a lack of effective multi-targeted drugs for MetS with minimal adverse effects. Disability and death are common outcomes if the disease is left undiagnosed and untreated (Daskalopoulou et al., 2004). It has become crucial to our society to implement both preventative measures and introduce proper treatment strategies to tackle this growing health problem. This has been deemed to be achieved with combined oral pharmaceuticals and dietary/lifestyle changes. As this complex disease progresses, oral interventions and treatment strategies are vital to prevent further cascading to diabetic/cardiovascular complications.

Having recognised the growing epidemic, a great deal of research has been undertaken globally, to develop new pharmaceuticals agents for MetS and to reach a full understanding of the disease. At present, the main therapeutic approach implemented for MetS is to identify and treat each risk factor of MetS separately, unrelated to its clustering with other risk factors. For example, insulin-sensitising agents such as Metformin lowers fasting plasma insulin concentrations by inducing better peripheral uptake of glucose, as well as decreasing hepatic glucose output. Statins and fibrates lower LDL cholesterol and to TG improve

dyslipidaemia, anti-obesity drugs include sibutramine and orlistat and diuretics and β -blockers are used to lower hypertension associated with MetS (Deedwania & Volkova, 2005; Grundy, 2006).

However, although these oral agents are useful in regulating the individual components of MetS to some degree, they also comprise of detrimental side effects such as weight gain, oedema and cardiac adverse events (Simpson et al., 2006). For example, although TZDs work effectively to improve insulin sensitivity and lower lipid levels, they may also induce lipid accumulation by their stimulatory effect on adipocyte differentiation, thereby increasing body weight (Lee et al., 2004; Deedwania & Volkova, 2005). Adverse effects of drugs are of growing concern, as multiple drugs are administered for long periods of time to achieve glucose and lipid levels in the normal range (Grundy, 2006; Simpson et al., 2006). Therefore, there is an urgent need for the development of safer pharmaceutical agents for managing Met, preventing T2D and metabolic complications. A novel agent that is capable of targeting multiple abnormalities is needed to minimise the potential adverse effects of polypharmacy (Grundy, 2006) and more importantly, to identify strategies and agents for the prevention of this complex disease.

There have been recent reports that GTP lowers blood pressure, fat and serum cholesterol, obesity, T2D, dilate blood capillaries to prevent the onset of cardiovascular disorders (Cao et al., 2007; Venables et al., 2008). Several lines of studies suggest that the anti-diabetic effects of green tea consumption are probably due to the effect of green tea (or more specifically, its active ingredients) in lowering central obesity—a major component of metabolic syndrome—by suppressing appetite and nutrient absorption (Sabu et al., 2002; Raederstorff et al., 2003). Yang et al. and Muramastu et al. (Yang et al., 2001; Muramastu et al., 1986) report that polyphenolic compounds found in green tea extract such as EGCG increase faecal lipid

1 content in high fat-fed rats. EGCG has also been shown to increase faecal cholesterol
2 excretion and faecal fat excretion in high fat, high cholesterol-fed rats when compared with
3 the control group (Raederstorff et al., 2003). Considerable amount of data demonstrate that
4 one mechanism by which tea polyphenols act against obesity and hyperlipidaemia, is by
5 modifying dietary fat emulsification in the gastrointestinal tract and inhibiting of
6 gastrointestinal lipolysis (Shishikura et al., 2006; Cha et al., 2012). However it has been
7 recently observed that polyphenols in green tea are also capable of accessing the bloodstream
8 through the intestinal epithelial outer cell membrane (Kidd, 2009). Due to a lack of study on
9 this observation, questions remain as to the effect of any direct actions and mechanisms of
10 green tea consumption on glucose and lipid metabolisms in insulin targeted tissues and
11 organs.

12 The liver is a major organ involved in glucose and lipid metabolism. Insulin resistance in the
13 liver leads to increased hepatic glucose production and lipogenesis, which contributes to
14 hyperglycaemia and lipotoxicity-induced pancreatic β -cell dysfunction (Bardini et al., 2012).
15 It is of interest to explore the metabolic effects and mechanisms of GTP, a naturally occurring
16 and readily available agent, for the treatment of MetS and related disorders. This chapter
17 focuses on studying the effects and mechanisms of GTP on glucose and lipid metabolism in
18 HepG2 hepatocytes. This chapter examined the dose-response effect of GTP on glycogen
19 synthesis and lipogenesis in HepG2 cells. HepG2 cells are considered suitable cellular
20 models for examining glycogen synthesis and lipogenesis in the liver (Wolfrum et al., 1999).

4.2. Research Plan and Methods

Cell Culture and Treatment

Human hepatoma HepG2 cells (ATCC HB 8065, ATCC, VA, USA) were maintained in Dulbecco's Modified Eagle Medium (DMEM) containing normal glucose (5 mM glucose), supplemented with 10% Fetal Bovine Serum (FBS) and 100 U/ml penicillin (GIBCO, Aukland, NZ) as described in Chapter 2.2.1. When confluent 0.01 – 10 μ M EGCG and insulin were added into appropriate wells in duplicates. Plates were maintained for 24 h in 5% CO₂ at 37°C.

Cellular Assays

Measurement of Insulin-stimulated Glycogen Synthesis

HepG2 cells administered normal (5 mM D-glucose) high glucose (30 mM D-glucose) were used to determine the effect of GTP on ¹⁴C-glucose incorporation into glycogen. 1 μ Ci ¹⁴C-glucose solution was added to EGCG-treated HepG2 cells then the reaction was stopped with 30% KOH and transferred into falcon tubes. 30% KOH with 6 mg/ml glycogen was the added and tubes were vortexed carefully and placed on a heating block set at 100°C for 15 min and turned down to 85°C for a further 15 min. 95% ethanol was added to all tubes and vortexed gently until the samples turned uniformly cloudy. Tubes were returned to 85°C heating block for 30 min then into an ice bath. Tubes were chilled for 15 min to completely precipitate the glycogen. Samples were centrifuged at 2,800 xg at 4°C for 10 min to pellet glycogen and then ethanol was aspirated and samples in deionised water were transferred to scintillation vials containing 5 ml scintillation liquid. The samples were counted using a

scintillation counter (PerkinElmer Inc, MA, USA). Glycogen synthesis were attained by measuring the rate of incorporation of D-[U-¹⁴C]glucose into glycogen.

Lipogenesis Assay

The effect of GTP on lipogenesis in HepG2 cells was tested by a colorimetric assay (Cayman Chemical Company, USA). Following standard treatment of HepG2 cells with EGCG on coverslips in 24 well plates, SM was removed from the wells with a pipette for staining. 75 µl of Lipid Droplets Assay Fixative was added to each well and incubated for 15 min. Wells were washed with Wash Solution twice for 5 min each and left to dry completely by placing the plate under a blowing hood. Oil Red O Working Solution were added to all wells including the background wells containing no cells and incubated for 20 min. Wells were washed with distilled water several times until the water appeared to be clear (until any pink was not visible). Microscopic images were taken to visualise pink/red oil droplets staining in differentiated cells. Dye extraction solution was added and wells were gently mixed for 20 min and the degree of lipogenesis was quantified from lipid droplets in cells by obtaining the absorbance at 490 nm single fixed wavelength with a micro-plate reader (Bio-Tek Instruments Inc., USA).

Western Blotting

Western blotting was carried out according to Chapter 2.2.7. 50 µg of protein were subject to 7.5% SDS-polyacrylamide gel electrophoresis then transferred to 0.45 µM polyvinylidenedifluoride (PVDF) membrane and immunoblotted with primary antibodies against phospho-GSK3β (Ser9), phospho-GS (Ser641), phospho-AMPKα (Thr172), phospho-ACC (Ser79) (Cell Signaling Technology Inc, MA, USA) and β-actin (Santa Cruz, CA, USA)

at 1:000 and incubated with anti-rabbit or anti-goat IgG and corresponding horseradish peroxidase-conjugated secondary antibodies (Santa Cruz, CA, USA) at 1:10000 dilution.

Statistical Analysis

Data are presented as the means $\pm$ S.E. Comparisons across the variety of treatments were done using one-way ANOVA followed by post-hoc analysis of Tukey's test to determine significant differences between the two treatments using Prism version 6 (GraphPad Inc, San Diego, CA). p -value < 0.05 was considered statistically significant.

4.3.Results

4.3.1. Effect of GTP-EGCG on glycogen synthesis in HepG2 Cells

To determine effect of GTP-EGCG on glycogen synthesis, we measured ^{14}C -glucose incorporation into glycogen in HepG2 cells pre-treated with high glucose (30 mM). Under 100 nM insulin stimulation, glycogen synthesis increased only 2% compared with HepG2 cells cultured with high glucose alone, indicating that high glucose treatment induced insulin resistance in HepG2 cells. Glycogen synthesis was further enhanced by 41%, 53% and 104% ($p < 0.01$) in HepG2 cells treated with 0.1, 1 and 10 μM of EGCG, respectively, indicating that EGCG increased glycogen synthesis in a dose-dependent manner (Figure 4.1).

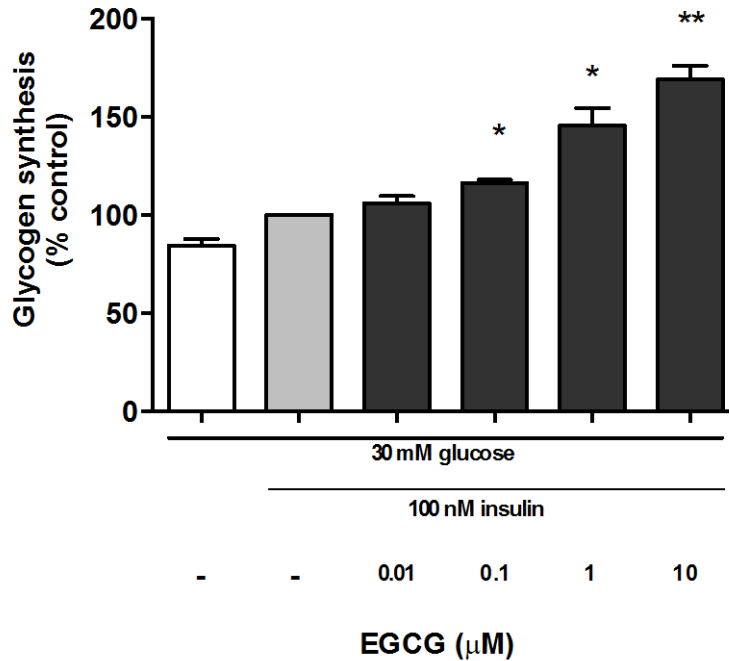


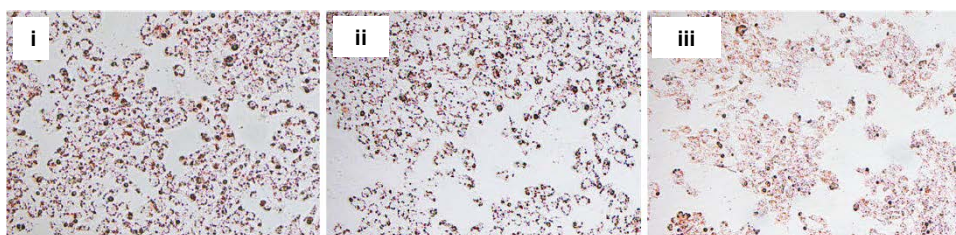
Fig 4.1. Glycogen synthesis in response to GTP-EGCG treatments in HepG2 cells The cells were pretreated with high glucose (30 mM) for 24 h then incubated with various concentrations of EGCG (0.1– 10 μM) with 100 nM insulin for 24 h. HepG2 cultured with high glucose without insulin was used to confirm that the HepG2 cells became insulin resistant. 1 μCi ¹⁴C-glucose solution was added to HepG2 cells for 30 min at 37°C. The results were attained by measuring the rate of incorporation of D-[U-¹⁴C]glucose into glycogen. Data are means ± S.E. The data are from 5 separate experiments. *p<0.05 and **p<0.01 compared with high glucose and insulin stimulated control.

4.3.2. Effect of GTP-EGCG on lipogenesis in HepG2 cells

Hepatic lipogenesis is the process by which ACC is converted to fats and involves sub-processes of fatty acid synthesis and subsequent TG synthesis in the liver. Increased liver fat and elevated hepatic lipogenesis have been demonstrated in obesity and insulin resistance status. To observe effect of GTP-EGCG on lipid deposition, Oil red O staining was used to view lipid droplets in HepG2 cells cultured in high glucose (30 mM) with different treatments. Figure 4.2A showed a slight decrease in lipid content in the cell culture with 100 nM insulin and a visibly greater reduction of lipid droplets in HepG2 cells treated with 100 nM insulin and 10 μM EGCG. To quantify de novo lipid synthesis, HepG2 cells exposed to

1 high glucose were used to determine hepatic lipogenesis with different treatments. In the
2 presence of 100 nM insulin, lipogenesis was reduced by 18% in high glucose treated HepG2
3 cells but the statistically significant difference was not achieved. GTP-EGCG treatments (0.1,
4 1 and 10 μ M) significantly inhibited lipogenesis in HepG2 cells by 31%, 39% (both $p < 0.05$),
5 and 65% ($p < 0.01$), respectively, compared with HepG2 cells treated with high glucose and
6 insulin (Figure 4.2B). These results indicate that GTP-EGCG improved insulin-medicated
7 lipogenesis in the hepatocytes exposed to high glucose.

A



B

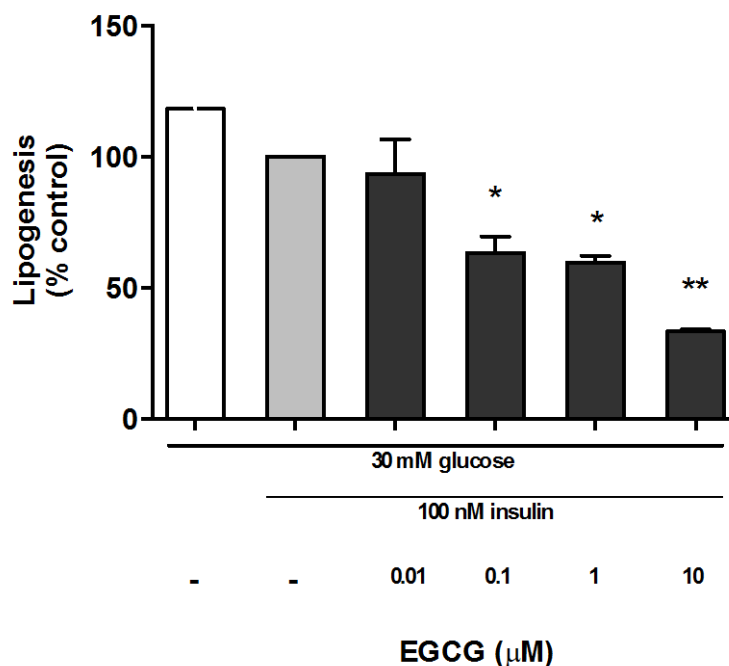


Fig 4.2. Effects of dose response to GTP-EGCG on lipogenesis in HepG2 cells (A) HepG2 cells were stained with oil Red O solution, and the dye was extracted from lipid droplets from cells. Images of cells were captured by microscope at 20 x original magnification showing lipid accumulation in cells stained by Oil Red O. (i) HepG2 cells cultured in 30 mM glucose, (ii) HepG2 cells cultured in 30 mM glucose with 100 nM insulin stimulation, and (iii) HepG2 cells cultured in 30 mM glucose with 100 nM insulin stimulation and 10 μM of GTP/EGCG. The degree of lipogenesis was quantified from lipid droplets in cells by measuring the absorbance at 490 nm. (B) The changes of lipogenesis by EGCG treatments were calculated as the percentage of insulin stimulation alone. Data are means ± S.E from 5 independent experiments. * $p < 0.05$ and ** $p < 0.01$ compared with high glucose and insulin stimulated control.

4.3.3. Effects of GTP-EGCG on expressions of Ser9 pGSK3 β and Ser641 pGS in HepG2 cells

Insulin plays an important role in hepatic glycogen synthesis and in insulin-resistant cellular models hepatic glycogen synthesis is markedly inhibited (Waltner-Law et al., 2002). GSK3 β is a rate-limiting enzyme, which acts as a downstream regulatory switch for numerous signalling pathways such as insulin action, hepatic glycogen synthesis and lipogenesis. Phosphorylation of GSK3 β not only activates target enzymes of the insulin-signalling pathway (Qin et al., 2010) but also regulates hepatic glycogen synthesis by increasing GS expression. Figure 4.3A and B showed that expression of phospho-GSK3 β (Ser79) was significantly reduced in high-glucose (30 mM) cultured HepG2 cells compared to HepG2 cells with normal glucose (5 mM). Expression of phospho-GS (Ser641) was also impeded by 23% with 30 mM glucose (Figure 4.3A & C).

Under 100 nM insulin stimulation, reduction of phosphorylation of GSK3 β in high-glucose treated HepG2 cells was improved and expression of phospho-GSK3 β (Ser9) was further enhanced by almost 2-fold by EGCG ($p < 0.01$) in HepG2 cells with 10 μ M EGCG treatment. A significant enhancement of expression of phospho-GS (Ser461) was also observed in EGCG treated HepG2 cells but insulin alone had no effect on expression of phospho-GS (Ser461) (Figure 4.3A & C).

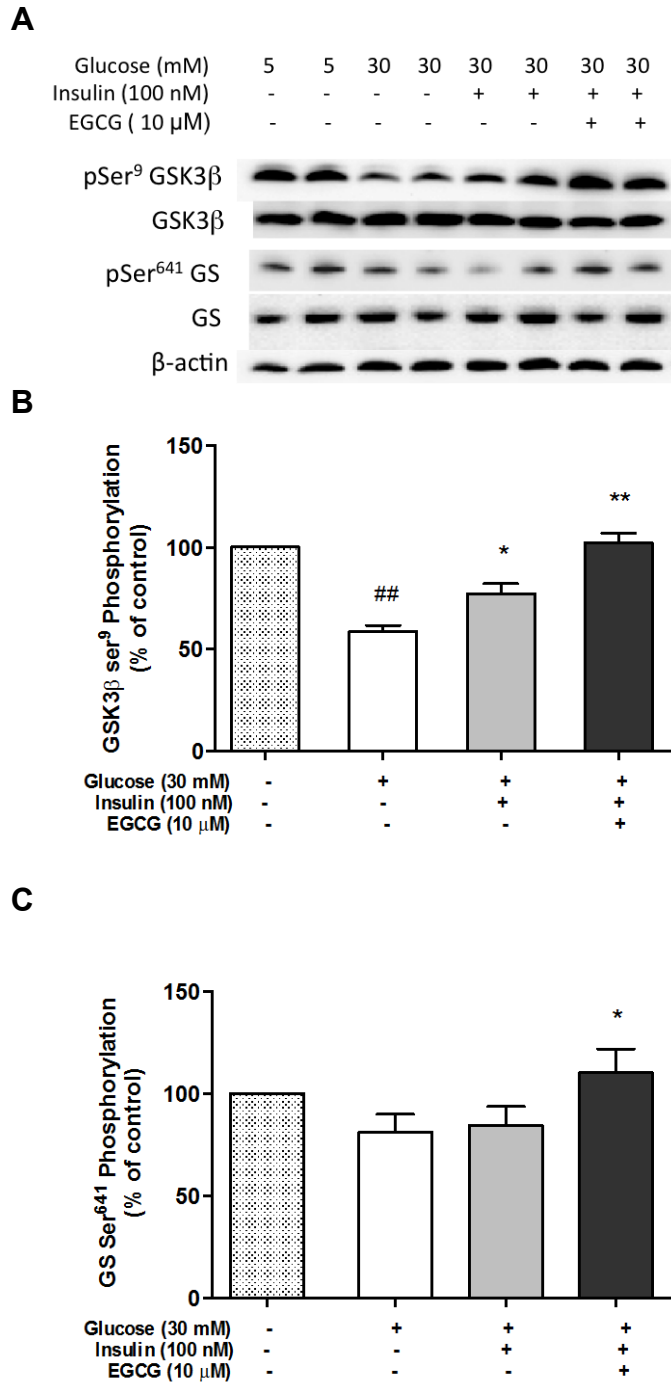


Fig 4.3. Effects of GTP-EGCG on expressions of phospho-GSK3 β (Ser9) and phospho-GS (Ser641) in HepG2 cells (A) The cell lysates were separated on 7.5% SDS-PAGE and incubated with antibodies against phospho-GSK3 β (Ser9) and phospho-GS (Ser641) as described. β -actin was used as a loading control. A representative western blot of phosphorylation of GSK3 β and GS from 5 independent experiments is shown. Quantitative data of (B) phospho-GSK3 β (Ser9) and (C) phospho-GS (Ser641) are expressed as means $\pm$ S.E. * p <0.05 and ** p <0.01 compared insulin-stimulated HepG2 cells with 30 mM glucose and ## p <0.01 compared with HepG2 cells with 5 mM glucose.

4.3.4. Effects on GTP-EGCG on expressions of Thr172 pAMPK α and Ser79 pACC in HepG2 cells

To understand the mechanism of GTP-EGCG on insulin-mediated hepatic lipogenesis, the effect of 10 μ M EGCG on expressions of phosphorylated AMPK α and ACC (two key enzymes involved in hepatic lipogenesis) in HepG2 cells were analysed with western blotting. Exposure of HepG2 cells to 30 mM glucose for 24 h slightly decreased expressions of phospho-AMPK α (Thr172) and phospho-ACC (Ser79) compared with HepG2 cells in 5 mM glucose. Under 100 mM insulin stimulation, expressions of phospho-AMPK α (Thr172) and phospho-ACC (Ser79) were markedly increased in HepG2 cells with normal glucose (5 mM) but insulin did not up-regulate expressions of phospho-AMPK α (Thr172) and pACC (Ser79) in HepG2 cells with high glucose (30 mM), indicating that insulin action was markedly stunted in HepG2 cells exposed to high glucose. Interestingly, the down-regulation of phospho-AMPK α (Thr172) and phospho-ACC (Ser79) in HepG2 cells were ameliorated by 10 μ M of EGCG, shown by significantly enhanced expressions of phospho-AMPK α (Thr172) and phospho-ACC (Ser79) in Figure 4.4.

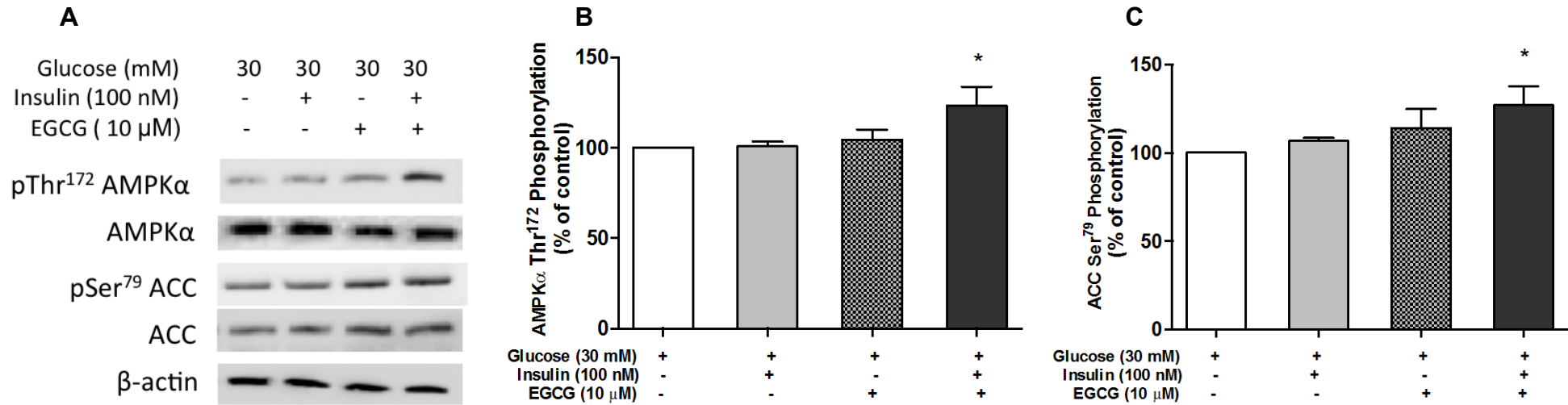


Fig 4.4. Effects of GTP-EGCG on expressions of phsopho-AMPK α (Thr172) and phospho-ACC (Ser79) in HepG2 cells Cell lysates was separated on 7.5% SDS-PAGE and incubated with 1:1000 phospho-AMPK α (Thr172) and phospho-ACC (Ser79) (Cell Signaling, USA) and β -actin was used as a loading control. A representative western blot analysis from 5 independent experiments is shown (A). Data (B, C) are expressed as means $\pm$ S.E. ($n = 5$), * $p < 0.05$, compared with insulin-stimulated HepG2 cells with 30 mM glucose.

4.4. Discussion

In vivo studies have postulated that polyphenolic compounds in green tea reduce body weight, prevent MetS and fatty liver disease through blocking lipid absorption (Hsu et al., 2008; Fukino et al., 2008; Rains et al., 2011). To understand direct effects of GTP and its major active compounds, such as EGCG, on glucose and lipid metabolism, insulin-mediated glycogen synthesis and *de novo* lipogenesis experiments were carried out on HepG2 cells. HepG2 cells were firstly exposed to high-glucose to induce insulin resistance, and this was shown by reduced insulin-stimulated glycogen synthesis and elevated lipogenesis. 10 μ M EGCG under 100 nM insulin-stimulation significantly increased rate of glucose incorporation into glycogen by 104% in HepG2 cells. This study also demonstrated that at presence of 10 μ M of EGCG the elevated lipogenesis were normalized in insulin resistant HepG2 cells. The findings of this chapter indicate the beneficial effects of GTP-EGCG against MetS and diabetes and are not only secondary to inhibiting lipid absorption or antioxidant actions (Murase et al., 2002) but also through its direct action to enhance glycogen synthesis and decrease lipogenesis in insulin targeted tissues.

Liver is a major organ involved in insulin-mediated glucose and lipid metabolism. Under insulin resistant state, hepatic glycogen synthesis is diminished and is also associated with increased lipogenesis, which leads to hyperglycaemia and contribute to the development of MetS, T2D and liver disease (Henriksen & Dokken, 2006). In this study, high glucose cultured HepG2 cells were used to mimic a hepatic insulin-resistant state. The treatments with GTP-EGCG ameliorated the diminished glycogen synthesis indicate that GTP-EGCG is capable of controlling hyperglycaemia through reduction of hepatic glucose production.

To understand the molecular mechanism of GTP-EGCG enhancement of glycogen synthesis expressions of phospho-GSK3 β (Ser9) and phospho-GS (Ser641) were detected. GSK3 is a rate-limiting enzyme which acts as a downstream regulatory switch for inactivation of GS leading to reduction of glycogen synthesis (Henriksen & Dokken, 2006; Orena et al., 2000). Insulin promotes glycogen synthesis through enhancing expressions of phospho-GSK3 β (Ser9) and phospho-GS (Ser641). Treatment with GTP-EGCG enhances phospho-GSK3 β , which mimics insulin's inhibitory effects on GSK3 β , enhances activity of GS and subsequently increased glycogen synthesis in HepG2 cells.

Moreover, GTP-EGCG treatment significantly increased phospho-AMPK α (Thr172) and phospho-ACC (Ser79) expressions in HepG2 cells. AMPK and ACC are key enzymes that regulate lipogenesis in the liver (Huang et al., 2009) and contribute significantly to overall metabolism of lipids. Insulin activates AMPK by promoting its phosphorylation at Thr172 (Huang et al., 2009) and by direct activation via an allosteric AMP site. Evidence shows that phosphorylation of Thr172, the major stimulatory phosphorylation site of α subunit, is essential for AMPK activity (Zang et al., 2004). An increase in AMPK α phosphorylation in the liver leads to phosphorylation and inactivation of ACC resulting in decreased lipid synthesis, through the biosynthesis of malonyl-CoA from acetyl-CoA, and this may lead to decreased lipid synthesis and regulation of fatty acid oxidation (Zhou et al., 2001).

4.5.Conclusion

This study found that GTP-EGCG has direct effects on regulation of glucose and lipid metabolism in high glucose treated HepG2 cells. Results demonstrate that hepatic glycogen synthesis was significantly up-regulated in HepG2 cells with GTP-EGCG treatment through increased phosphorylation of GSK3 β and GS, which are critical elements in the regulation of

1 hepatic glycogen synthesis *in vivo*. GTP-EGCG also inhibited hepatic lipogenesis in cells
2 through increased expressions of phospho-AMPK α (Thr 172) and phospho-ACC (Ser79). In
3 conclusion, the findings showed the beneficial effects of GTP-EGCG against MetS through
4 direct enhancement of glycogen synthesis in the liver and decreased hepatic lipogenesis.

**CHAPTER 5: DOES GREEN TEA POLYPHENOLS
IMPROVE INSULIN RESISTANCE IN HIGH FAT FED
OBESE ZUCKER RATS?**

5. Does Green Tea Polyphenols Improve Insulin Resistance in High Fat Fed Obese Zucker Rats?

5.1.Introduction

The prevalence of MetS and T2D is continuing to escalate worldwide (Ford, 2005). Epidemiological studies estimate that one-quarter of the world's adult population suffer from MetS (Kaur, 2014). Obesity, a major component of MetS and risk factor for cardiovascular disease, is continuing to rise; an estimated 64% of adults in the US are either overweight or obese (Lau et al., 2006). However, at present, oral medications have shown to have limited efficacy, fail to be multi-targeted, cause severe side effects and are extremely costly. Studies have found that the use of certain medications may in fact increase the risk of the development of the MetS by either promoting weight gain or altering lipid/cholesterol or glucose metabolism (Deedwania & Volkova, 2005; Grundy, 2006). Thus, it is essential to develop new therapeutic agents that improve insulin sensitivity, regulate lipid and glucose metabolism to lower hyperglycaemia and hyperlipidaemia, inhibit weight gain and that are safe to administer for long-term use.

A large number of studies suggest that insulin resistance and central obesity are key underlying factors that contribute to the onset MetS and T2D (Gastaldelli et al., 2002; Despres & Lemieux, 2006; Gustafson et al., 2007; Petersen et al., 2007). Insulin resistance in skeletal muscle ultimately affects whole body glucose metabolism, and in particular, may promote the onset of atherogenic dyslipidaemia by diverting ingested carbohydrate away from muscle glycogen storage and into hepatic lipogenesis, resulting in hypertriglyceridaemia (Petersen et al., 2007).

Under normal physiological conditions, insulin binds to its receptor and leads to IR tyrosine kinase activation that phosphorylates the downstream IR substrates, such as IRS-1. IRS-1 then continues to activate a cascade of phosphorylation-dephosphorylation reactions, including PI3K-p85 and serine/threonine kinases PKB/Akt, leading to GLUT4 translocation and intracellular glucose metabolism (Shepherd & Kahn, 1999; Guo et al., 2013). Defects in the IRS/PI3K/GLUT4 insulin-signalling pathway are commonly seen in skeletal muscle of individuals with insulin resistance and MetS (Guo et al., 2013). Therefore, the insulin-signalling pathway in skeletal muscle is a promising therapeutic target that may promote muscle glucose homeostasis and effectively treat insulin resistance, T2D and MetS (Zaid et al., 2009).

GTP has become widely popular due to its well-known antioxidant and weight loss effects, and more recently its metabolic and cardiovascular effects. GTP is known to be rich in flavonoids and may become an important functional food/natural agent to improve or prevent MetS and related disorders. Evidence from literature and previous studies suggest that polyphenols or catechins from green tea have multi-targeted effects on free radicals, blood pressure, fat and serum cholesterol, obesity, T2D, and CVD (Cao et al., 2007; Bose et al., 2008). However, whether GTP is capable of reversing dietary and genetic factors induced by obesity and insulin resistance needs to be elucidated. *In vitro* results in chapters 3 and 4 of this thesis have demonstrated the role of GTP in regulating glucose and lipid metabolism in 3T3-L1 and HepG2 cell lines, therefore it is of great interest to extend this research to an animal model.

At present, the most suitable animal model for studying MetS is the obese Zucker rat model. These animals are mainly used as experimental models for obesity, however they also portray changes similar to those seen in human MetS, and are now commonly implemented in MetS

studies (De Artiñano & Castro, 2009). ZF rats demonstrate a marked state of insulin resistance, dyslipidaemia, mild glucose intolerance, renal damage and hyperinsulinaemia. Hyperinsulinaemia is detectable at 3 weeks and continues throughout life (Zucker & Zucker, 1962; De Artiñano & Castro, 2009).

The purpose of this chapter was to investigate the effects of GTP treatment on the metabolic profiles and insulin resistance, and identify the underlying mechanisms of GTP action using HFD fed Zucker fatty rats.

5.2. Research Plan and Methods

Animals and treatment

The animals outlined below were used for chapters 5 and 6 of this thesis. Male Zucker fatty (fa/fa) rats (ZF) and their lean littermates, aged 5-7 weeks and approximately 160 g body weight, were supplied by the Monash Animal Research Platform (Monash University, Vic, Australia). Rats were acclimatised in communal cages at 22 °C, with a 12 h light-dark cycle (lights on 0700 h) for 1 week and had *ad libitum* access to a standard chow diet (Gordon's Specialty Stock Feed, Sydney, Australia) and water. ZF rats (n=30) were fed a high-fat diet (HFD; 350 kJ/d) for 2 weeks. The energy percentage composition of the HFD was 59% fat, 20% carbohydrate, and 21% protein, with equal quantities of fibre, vitamins, and minerals to the standard chow diet. The HFD was replenished daily. After two weeks of HFD feeding, ZF rats (n=30) were divided into two groups. The control group (HFD-Con; n=15) was administered distilled water (5 ml/kg of body weight) and ZF rats in the GTP treatment group (HFD-GTP; n=15) was administered GTP at 200 mg/kg of body weight daily for 8 weeks via oral gavage. HFD feeding was continued throughout the 8 weeks treatment period. Body

weight and food intake were recorded daily. Lean Zucker rats (n=15) were fed a standard laboratory chow diet throughout the treatment period as outlined in Chapter 2.3.4.

At the end of treatment, oral glucose tolerance test was performed as described previously in Chapter 2.3.7 and blood samples at time 0 were used for the following biochemical assays.

Biochemical Assays and the homeostasis model assessment of insulin resistance

Fasting blood samples obtained from each experimental rat were separated by centrifugation (400 Xg for 10 min). Serum samples were collected for measurements of glucose, insulin, NEFA, TG and cholesterol concentrations - described in Chapter 2.2.10.

Whole-body insulin sensitivity was estimated using the homeostasis model assessment of insulin resistance (HOMA-IR) by using the formula: [fasting serum glucose (mmol/L) times fasting serum insulin (mU/L)]/22.5. Because HOMA is negatively correlated with insulin sensitivity, low HOMA-IR values indicate high insulin sensitivity, whereas high HOMA-IR values indicate insulin resistance.

Western blotting

The total proteins or cytosol and membrane fractions from red quadriceps (RQ) were extracted by the following procedure. In brief, 100 mg RQ were homogenised in 1 ml lysis buffer. To separate the cytosol and membrane fractions, samples were spun at 400 xg for 15 min. The supernatant was collected then ultracentrifuged at 105,000 xg for 60 min then removed and the sediment (membrane fraction) was resuspended in 0.5 ml lysis buffer. Protein (20 µg) was separated by SDS-PAGE (12.5%) and blotted onto PVDF membranes. Blotted membranes were blocked with 5% skim milk in PBS with 0.05% Tween 20 overnight

and incubated with primary antibodies GLUT4, IRS-1 (Santa Cruz Biotechnology, CA, USA), AKT (Abcam, CA, USA), pSer⁶¹² IRS-1, pSer⁴⁷³ AKT (Cell Signaling, Technology Inc., MA, USA) PKC β 2, PKC θ , PKC ϵ , PKC ζ (Santa Cruz Biotechnology, CA, USA) and β -actin diluted at 1:1000 for 4 h. Membranes were washed 3 times in 0.05% Tween 20 (PBS-T) while agitating, 10 min per wash, to remove residual primary antibody. Membranes were incubated with corresponding horseradish peroxidase-conjugated secondary antibodies (Santa Cruz, CA, USA) at 1:10000 dilution and blots were developed with enhanced chemiluminescence (ECL) (Pierce, IL, USA) according to manufacturer's instructions.

Statistical Analysis

Data are presented as the means $\pm$ S.E. Analysis was done using one-way ANOVA followed by Dunnett's test to determine significant differences between groups using Prism version 6 (GraphPad Software Inc, CA, USA). p -value < 0.05 was considered statistically significant. The total area under the curve was calculated using the trapezoidal rule. Differences between groups with p -value < 0.05 was considered statistically significant.

5.3.Results

5.3.1. Effect of GTP on body weight gain in HFD ZF rats

Results in Figure 5.1 showed that 8 weeks of GTP treatment significantly inhibited body weight gain in ZF HFD rats compared to the ZF HFD control group (125.06 ± 12.94 g vs 75.57 ± 18.06 g; $p < 0.05$).

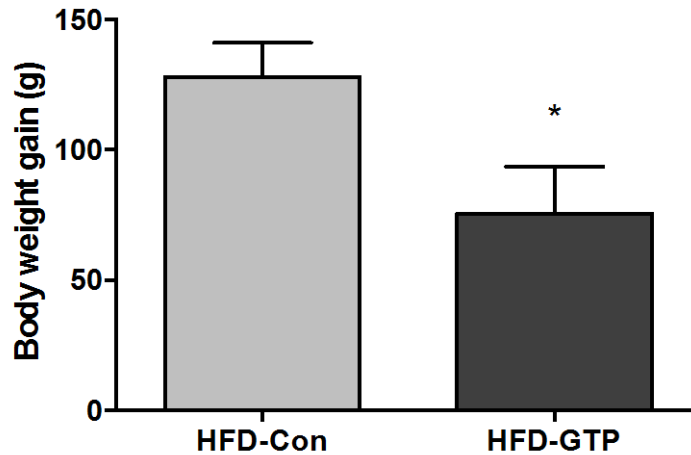


Fig 5.1. Body weight gain in ZF HFD rats Body weight gain of high-fat red rats after 8 weeks of daily treatment with saline (5 ml/kg; Con.) or GTP (200 mg/kg; GTP). Data are means $\pm$ S.E., n = 15 rats/group. *p<0.05 vs HFD-Con.

5.3.2. Effects of GTP on serum glucose and whole body insulin resistance

Results in Figure 5.2 show that serum glucose and insulin levels were significantly increased in ZF HFD control rats compared to lean rats (4.38 ± 0.54 mmol/L; $p < 0.01$). HFD feeding also augmented hyperinsulinaemia, an indicator for insulin resistance. Serum insulin levels in HFD-ZF rats were also almost 135-fold higher than in the lean rats (1863.38 ± 685.08 vs 13.71 ± 1.69 mU/mL; $p < 0.001$). GTP treatment for 8 weeks significantly reduced serum glucose levels compared with vehicle treated HFD-ZF rats (6.6 ± 1.22 vs 7.54 ± 1.70 mmol/L; $p < 0.05$) and serum insulin levels by 51% in high-fat fed ZF rats ($p < 0.05$). HOMA-IR values were further calculated to measure the degree of insulin resistance in Fig 5.2C. Results show that the HOMA-IR value was significantly increased in HFD-ZF rats compared to lean rats (0.65 ± 0.21 vs 0.0027 ± 0.0004 ; $p < 0.001$). GTP treatment significantly lowered HOMA-IR (0.25 ± 0.22 ; $p < 0.01$) compared with HFD-ZF rats.

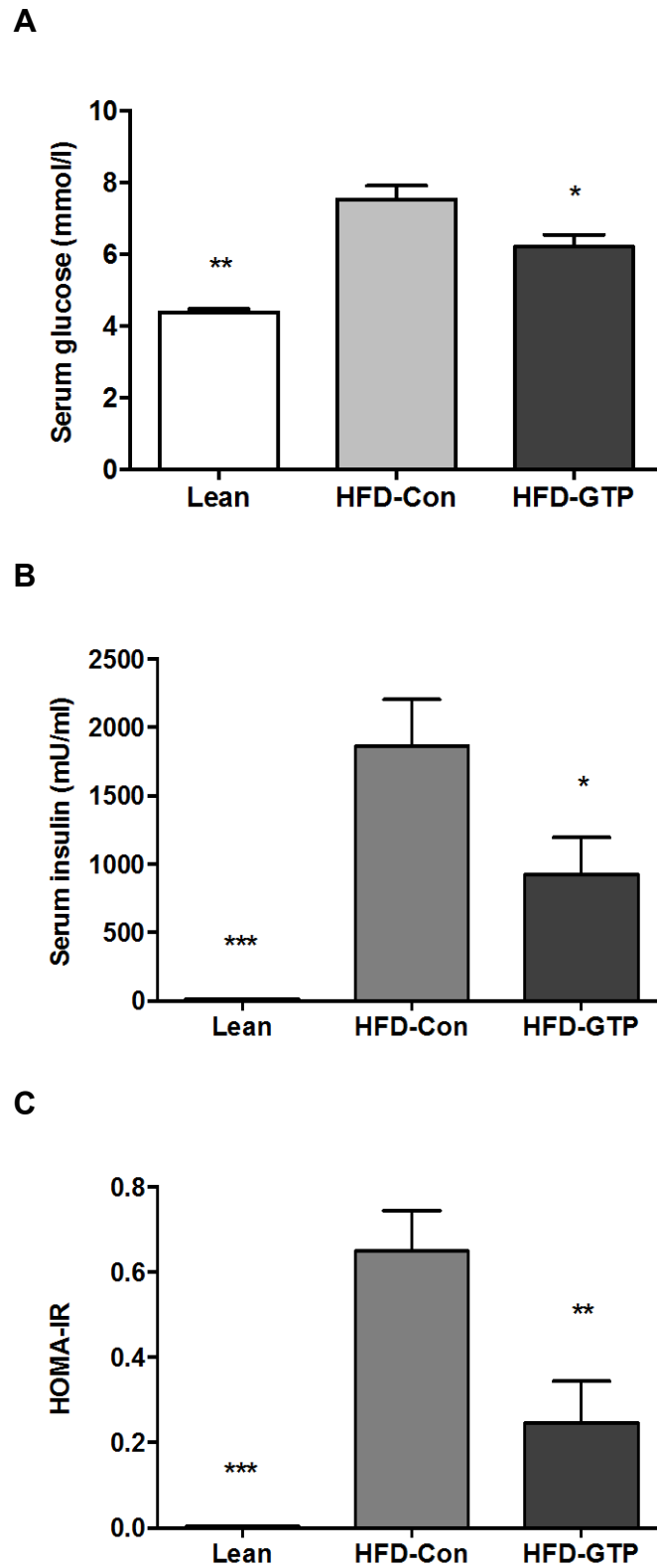


Fig 5.2. Serum glucose (A) insulin (B) levels and (C) HOMA-IR in HFD ZF rats Serum glucose and insulin levels were measured after 8 weeks of daily treatment with saline (5 ml/kg; HFD) or GTP (200 mg/kg; GTP). Data are means $\pm$ S.E., n = 15 rats/group. * p <0.05 vs HFD-Con rats; ** p <0.01 vs HFD-Con rats; *** p <0.001 vs HFD-Con rats.

5.3.3. Effects of GTP on blood lipid profiles HFD ZF rats

Serum TG, NEFA and total cholesterol concentrations were measured to identify the effects of GTP treatment in ZF rats. TG, NEFA and cholesterol levels were all significantly higher in HFD fed ZF rats compared to lean rats (0.11 ± 0.01 vs 0.75 ± 0.01 mmol/L, 0.76 ± 0.04 mmol/L vs 1.29 ± 0.03 mmol/L, 0.21 ± 0.01 mmol/L vs 0.64 ± 0.02 mmol/L, respectively). GTP treatment for 8 weeks significantly decreased serum TG by 34% ($p < 0.01$), NEFA by 21% ($p < 0.05$) and cholesterol by 25% ($p < 0.01$) compared with high-fat fed ZF control rats (Figure 5.3).

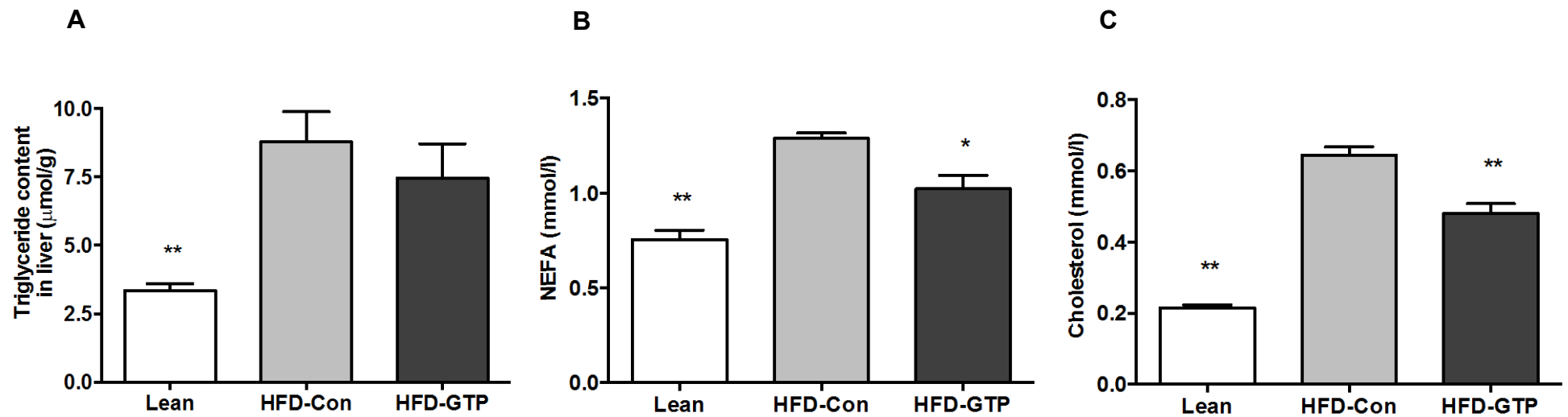


Fig 5.3. Serum TG (A) NEFA (B) and cholesterol (C) in HFD ZF rats Serum TG, NEFA and cholesterol concentrations were measured after 8 weeks of daily treatment with saline (5 ml/kg; HFD) or GTP (200 mg/kg; GTP). Data are means $\pm$ S.E., n = 15 rats/group. (A)** $p < 0.01$, *** $p < 0.001$ vs HFD-Con rats (B) * $p < 0.05$, ** $p < 0.01$ vs HFD-Con rats (C) ** $p < 0.01$, ** $p < 0.01$ vs HFD-Con rats.

5.3.4. Effects of GTP on oral glucose tolerance in HFD ZF rats

Oral glucose tolerance test was performed after 7 weeks GTP treatment on chow fed rats (Lean) and HFD rats (HFD-Con; HFD-GTP). The serum glucose responses after oral glucose loading (2 g/kg of body weight) are shown in Figure 5.4. Results show that vehicle treated HFD ZF rats had higher glucose levels compared with the lean control after glucose loading. GTP reduced glucose levels by 6.08 ± 2.19 % at 0 min, 7.56 ± 4.46 % at 30 min, 18.89 ± 4.41 % at 60 min ($p < 0.05$), 26.57 ± 4.04 % at 90 min ($p < 0.01$) and 12.79 ± 3.99 % at 120 min, compared to HFD-Con group. AUC showed that GTP significantly improved glucose tolerance in HFD rats ($p < 0.05$).

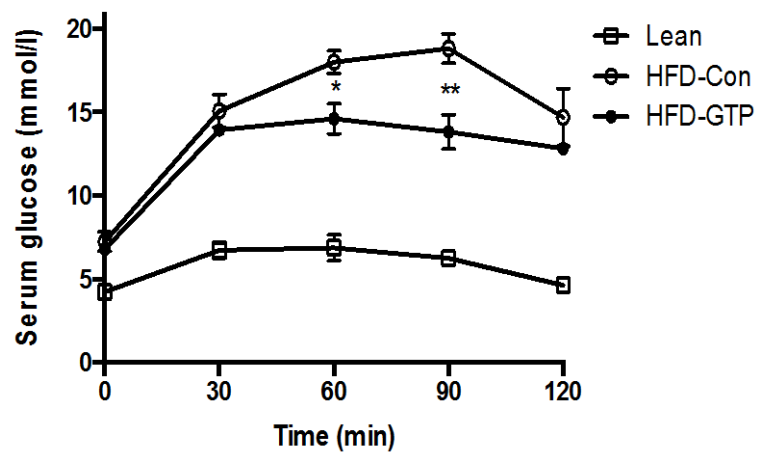
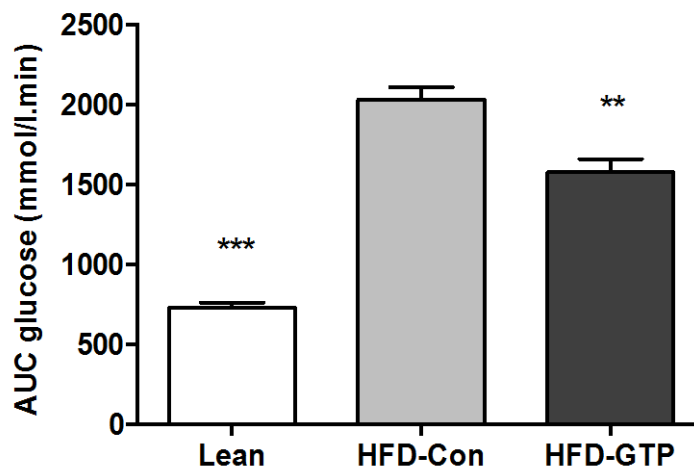
A**B**

Fig 5.4. Effect of GTP treatment on serum glucose during oral glucose tolerance test (OGTT)
 (A) Glucose (2 g/kg) was administered by gavage (at 0 min) to overnight-fasted ZF rats treated with GTP (200 mg/kg of body weight). Tail-vein blood samples were collected at baseline (0) and at 30, 60, 90 and 120 min (B) Area under curve (AUC) is shown. Data are means $\pm$ S.E., n = 15 rats/group. **p<0.01 vs HFD-Con rats; ***p<0.001 vs HFD-Con rats.

5.3.5. Effect of GTP on skeletal muscle in HFD ZF rats

TG content in skeletal muscle (red quadriceps) was significantly increased in high-fat fed rats by 3.6-fold compared to lean rats ($4.84 \pm 1.19 \mu\text{mol/g}$ vs $17.51 \pm 0.78 \mu\text{mol/g}$; p<0.01). GTP

significantly decreased TG content in skeletal muscle by 50% in HFD ZF rats (17.51 ± 0.78 $\mu\text{mol/g}$ vs 8.76 ± 1.07 $\mu\text{mol/g}$; $p < 0.01$) (Figure 5.6).

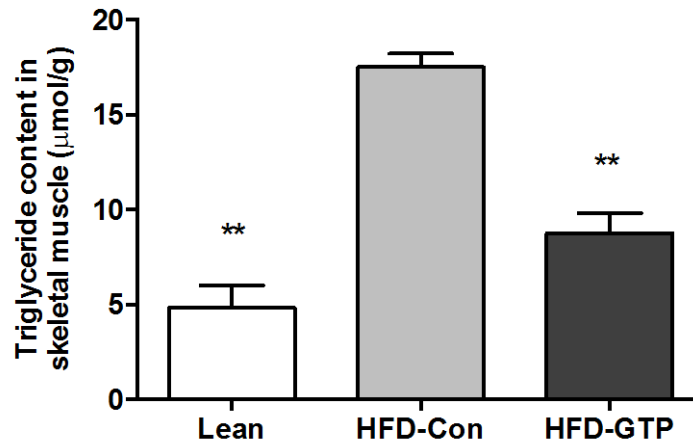


Fig 5.6. TG content in red quadriceps TG content in red quadriceps were measured after 8 weeks of daily treatment with saline (5 ml/kg; HFD) or GTP (200 mg/kg; GTP). Data are means $\pm$ S.E., $n = 15$ rats/group. ** $p < 0.01$ vs HFD-Con rats.

5.3.6. Increased GLUT4 translocation in skeletal muscle of GTP-treated HFD ZF rats

To elucidate the molecular mechanisms for enhanced glucose disposal with GTP treatment, GLUT4 translocation in red quadriceps skeletal muscle was determined by immunoblotting. Results in Figure 5.7 showed a significant diminution in GLUT4 protein expression and translocation from cytosol to membrane in high-fat fed ZF rats compared to lean rats (membrane fraction of lean rats vs high-fat fed rats; $p < 0.01$). In high-fat fed rats, GTP treatment up-regulated inhibited GLUT4 translocation and significantly increased expressions of GLUT4 in both cytosol and membrane fractions ($p < 0.05$ vs high-fat rats cytosol and membrane).

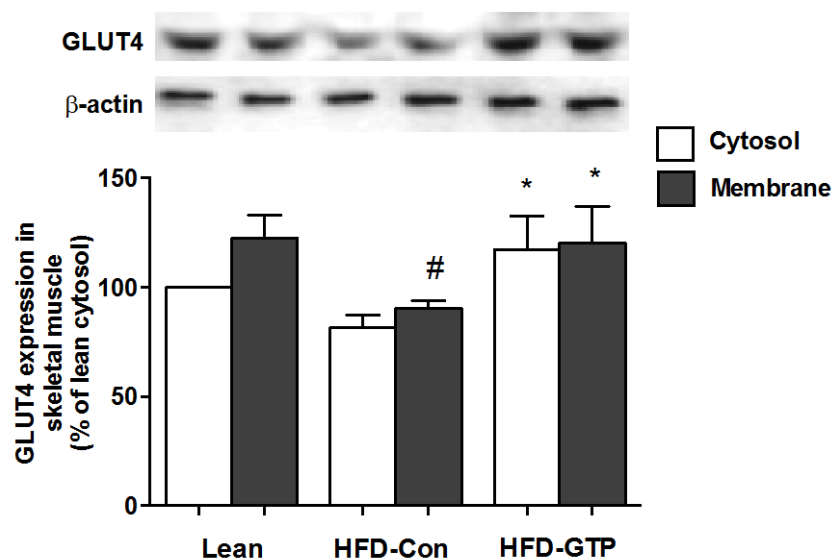


Fig 5.7. The effects of GTP on GLUT4 protein expression in HFD ZF rats Red quadriceps (skeletal muscle) were separated into cytosol and membrane fractions. Data are means $\pm$ S.E., 6-8 rats per group. # $p<0.01$, * $p<0.05$ vs Lean membrane vs HFD-Con cytosol and membrane.

5.3.7. Phosphorylation of IRS-1 and PKB/Akt in skeletal muscle of GTP-treated HFD ZF rats

To study the molecular mechanisms for reduction of hyperglycaemia in high-fed rats with GTP treatment, key proteins of the insulin-signalling pathway were determined by western blotting. It has been proposed that increased NEFA delivery or decreased intracellular metabolism of fatty acids results in an increase in the intracellular content of fatty acid metabolites such as diacylglycerol (DAG), fatty acyl-coenzyme A (fatty acyl-CoA) and ceramides, which in turn activate a serine/threonine kinase cascade leading to serine/threonine phosphorylation of IRS-1 and IRS-2, and a reduced ability of these molecules to activate PI(3)K. As a result, events downstream of insulin-receptor signalling are diminished. Results in Figure 5.8 indicated that there were no significant differences in both phosphorylated and total IRS-1 expressions between lean, high-fat fed and GTP-treated HFD rats however interestingly, phosphorylation of PKB/Akt was diminished in HFD rats

compared to lean rats ($p<0.01$). Results further revealed that the impediment of PKB/Akt phosphorylation in HFD rats was significantly up-regulated with GTP treatment ($p<0.05$).

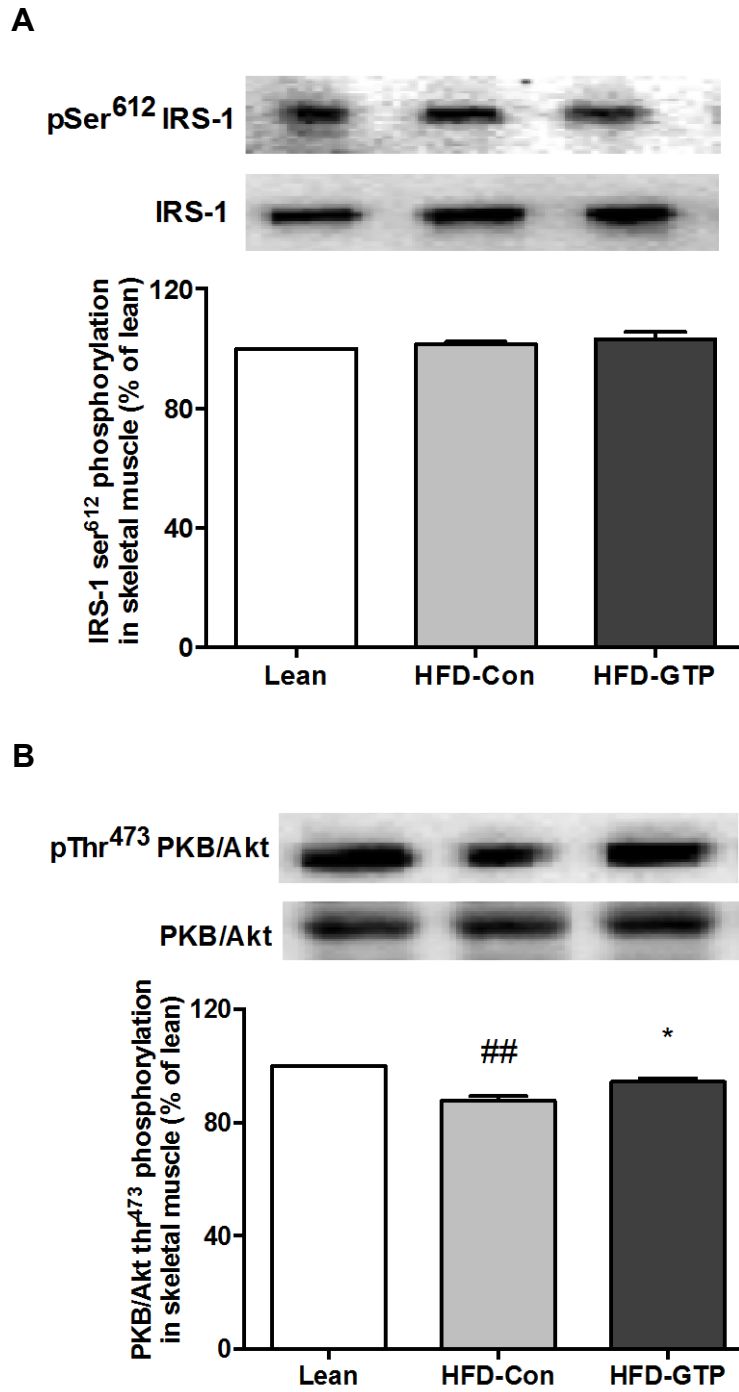


Fig 5.8. Determination of (A) IRS-1 (Ser612) and (B) AKT (Ser473) phosphorylation in skeletal muscle of GTP-treated ZF rats Data are means $\pm$ S.E. of 5 rats per group. Representative immunoblots of PKB/Akt phosphorylation are shown ^{*} $p < 0.05$ vs HFD-Con, ^{##} $p < 0.01$ vs Lean.

5.3.8. Translocation and expressions of PKC isoforms in skeletal muscle of GTP-treated HFD ZF rats

Previous studies have shown that protein kinase C (PKC) may play a significant role in the phosphorylation and inhibition of insulin-mediated pathways. Increased PKC expression has been associated with insulin resistance (insulin-stimulated glucose transport) and impaired glycogen synthesis. Western blotting was carried out to evaluate the expressions of PKC isoforms and their subcellular distribution between the cytosol and membrane fractions in skeletal muscle of HFD ZF rats. Results were expressed as membrane/cytosol ratio and significant changes in translocation of PKC θ and PKC ζ were observed in rats. Marginal translocation of PKC θ and PKC ζ was evidenced by low cytosol/membrane ratios in lean ZF rats. PKC θ and PKC ζ translocation was significantly increased in high-fat fed rats shown by the significantly higher cytosol/membrane ratios compared to lean ZF rats. It was observed that GTP treatment in high-fat fed rats significantly inhibited translocation of both PKC θ and PKC ζ ($p < 0.01$), with expressions that were similar to lean rats (Figure 5.9). No significant differences were found in PKC $\beta 2$ and PKC ϵ membrane/cytosol ratios.

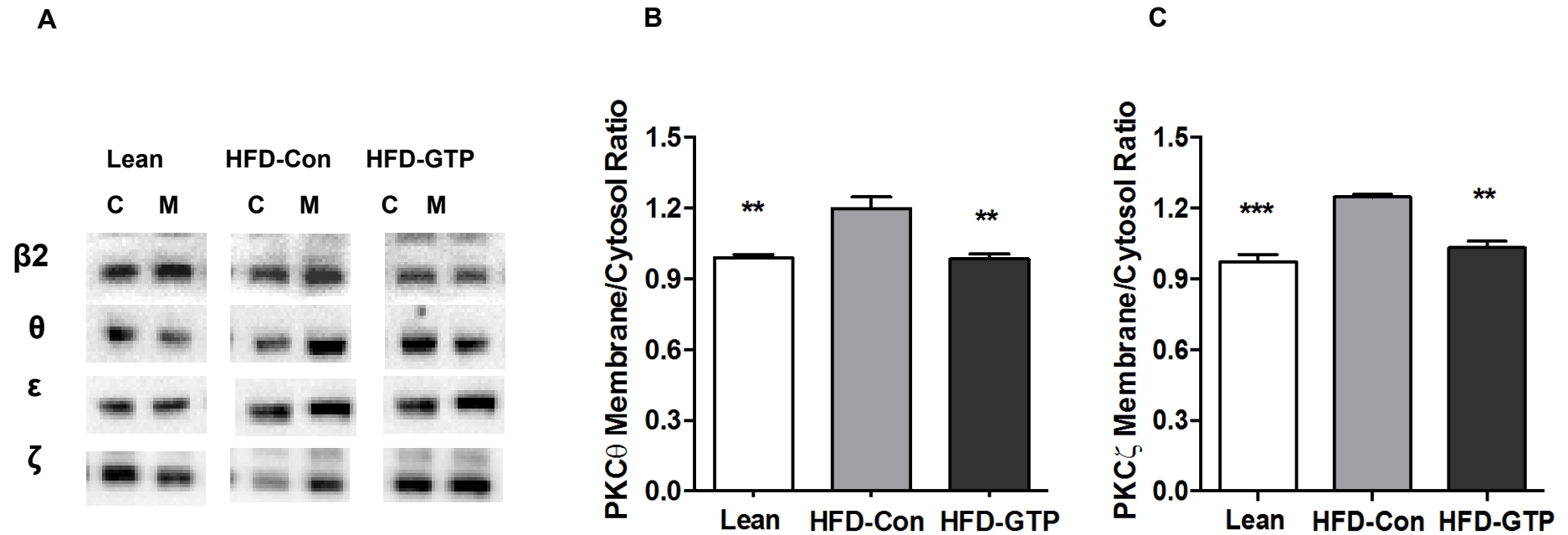


Fig 5.9. Determination of PKC isoforms in red quadriceps (skeletal muscle) of GTP-treated ZF rats (A) Representative Western blots show cytosol and membrane fractions for PKC- $\beta 2$, θ , ϵ and ζ isoforms. Densities of membrane fractions were compared with the cytosol fractions to determine PKC activation. PKC membrane/cytosol ratios were calculated. Values represent the means $\pm$ S.E. of 5 animals per group. (B) ** $p < 0.01$ vs HFD-Con (C) ** $p < 0.01$, *** $p < 0.001$ vs HFD-Con.

5.4.Discussion

The findings of this chapter revealed that GTP has positive effects in the HFD Zucker fatty rat model. High-fat feeding of ZF rats caused a state of insulin resistance and obesity and this animal model was used to examine the effects of GTP on key metabolic parameters of MetS and T2D. Based on the results outlined above, GTP treatment for 8 weeks lead to decreased weight gain and lowered glucose, TG, NEFA and cholesterol levels in high-fat fed rats.

The high prevalence and complexity of MetS gives rise to the urgent need for new therapeutic strategies in our present society. MetS involves insulin resistance, obesity, hypertension and NAFLD, and progression to cardiovascular complications is often rapid and at times fatal (Lau et al., 2006). Not only are the numbers increasing but also the age of onset for MetS associated T2D is falling, where diagnosis of T2D has been reported to be increasing in children and adolescents all over the world (Zimmet et al., 2007). Also, MetS was once a threat to only developed countries however, is now becoming widespread in developing countries as well. This is also problematic as oral medications are costly to manufacture and are not readily available to everyone. There is no doubt that MetS has become a global public health problem, and it is crucial that MetS and its risk factors are prevented or treated efficiently and effectively.

The most commonly implemented therapeutic strategies at present are a combination of lifestyle coaching (ie increased exercise and dietary changes) and oral medications. However, the safety and efficacy of oral medications used in MetS treatment has raised concerns in recent years. For example, insulin-sensitising agents used for the treatment of MetS, have been reported to cause weight gain, fluid retention, and cardiovascular events such as

myocardial infarction and heart failure (Nesto et al., 2004, Shah, 2010). Therefore, this study was carried out to elucidate a natural/readily available and multi-targeted agent for the treatment and/or prevention of MetS.

It was demonstrated in this chapter that GTP significantly reduced serum glucose, TG, NEFA and cholesterol levels in high-fat fed rats, which are key biochemical features of MetS. The Zucker fatty rat mimics most of the clinical features of MetS, including, obesity, hyperlipidaemia and hyperinsulinaemia with euglycaemia. In this study, HFD feeding exacerbated mild hyperglycaemia evidenced by significantly increased in serum glucose, compared to the lean rats. Thus, HFD-ZF rats can be used to investigate whether GTP reverses insulin resistance and prevents the development of T2D. The results showed that 8-weeks GTP treatment significantly reduced not only serum NEFA, TG and cholesterol concentrations, but also reversed hyperinsulinaemia and normalised circulating glucose levels, suggesting GTP has potent hypolipidaemic and hypoglycaemic effects. HOMA-IR values that determine insulin resistance were calculated as described previously (Matthews et al., 1985). Low HOMA-IR values indicate high insulin sensitivity, whereas high HOMA-IR values indicate insulin resistance (Matthews et al., 1985). Results showed that GTP treatment significantly increased insulin sensitivity in HFD ZF rats.

Also, an interesting observation was that GTP treatment significantly inhibited body weight gain in ZF HFD rats compared to the ZF HFD control group. This is highly beneficial in MetS and T2D patients as weight gain/obesity is a key risk factor for disease progression and debilitation and a common side effect reported in conventional oral medications.

OGTT results also indicated that GTP treatment improved glucose intolerance and insulin sensitivity in HFD ZF rats. The underlying mechanisms for the increased glucose disposal

with GTP were further studied and results suggested that GTP enhanced insulin-mediated glucose transport in skeletal muscle. This was evidenced by increased protein expressions of PKB/Akt phosphorylation and GLUT4 translocation in red quadriceps (skeletal muscle) in GTP-treated HFD ZF rats. Increased GLUT4 expression in muscle is indicative of increased insulin-mediated glucose transportation from cytosol to membrane (Henriksen et al., 1990; Abel et al., 2001; Zaid et al., 2009). In this study, GLUT4 expression and translocation was significantly impeded in high-fat fed rats compared to lean rats. GTP treatment significantly increased GLUT4 cytosol and membrane expressions, indicating marked increase in insulin action on glucose transport in insulin-resistant HFD rats.

PKB/Akt is a serine/threonine kinase that is thought to be a component of the insulin-stimulated GLUT4 translocation pathway. PKB/Akt undergoes serine/threonine phosphorylation upon insulin stimulation (Taniguchi et al., 2006). These events are downstream of and dependent on PI 3-kinase and PI-dependent kinase 1 (PDK1) (Chan et al., 1999). Results in this study showed that PKB/Akt phosphorylation was significantly reduced in high-fat fed rats compared to lean rats. Administration of GTP led to a substantial increase in PKB/Akt phosphorylation, suggesting that GTP treatment improved insulin-signalling. IRS-1 is a well-known substrate of the insulin receptor that, after tyrosine phosphorylation, associates with and activates PI 3-kinase. No changes in IRS-1 phosphorylation was seen in this current study, however, increased GLUT4 translocation coupled with increased PKB/Akt phosphorylation, provides evidence that GTP may be beneficial for insulin resistance by improving glucose intolerance and insulin sensitivity through increased glucose transport and insulin-signalling in HFD ZF rats.

In addition, previous studies have shown that PKC may also play a significant role in the phosphorylation and inhibition of insulin-mediated pathways. Increased PKC expression has

been associated with insulin resistance (impaired insulin-stimulated glucose transport) in muscle and impaired glycogen synthesis. Also, the DAG-PKC pathway is one of the most investigated pathways in cellular signaling induced by diabetes. In diabetes and other metabolic abnormalities, DAG levels have been found to be elevated in vascular tissues, such as the retina, aorta, heart, and renal glomeruli, and in non-vascular tissues, such as liver and skeletal muscles (Geraldes & King, 2010).

There are many isoforms of PKC that function in a wide variety of biological systems. The conventional PKC (cPKC) isoforms (ie. PKC- α , - β 1, - β 2, and - γ) are activated by phosphatidylserine, calcium, and DAG or phorbol esters such as phorbol 12-myristate 13-acetate (PMA), whereas novel PKCs (nPKCs) (ie. PKC- δ , - ϵ , - θ , and - η) are activated by phosphatidylserine, DAG or PMA (Steinberg, 2008; Geraldes & King, 2010). In this study, immunoblotting was carried out to evaluate the expressions of PKC isoforms and their subcellular distribution between the cytosol and membrane fractions in skeletal muscle of HFD ZF rats. No significant changes of expression were noticed in PKC β 2 and PKC ϵ membrane/cytosol ratios, but interestingly, marked changes in translocation of PKC θ and PKC ζ were observed in rats. GTP treatment in high-fat fed rats significantly inhibited translocation of both PKC θ and PKC ζ , evidenced by lower membrane/cytosol ratio than untreated rats. This observation suggests that GTP enhances insulin-signalling and insulin-stimulated glucose transport through reduced translocation of PKC isoforms in insulin-resistant HFD ZF rats.

5.5. Conclusion

In conclusion, the findings of this chapter show a marked state of insulin resistance in HFD ZF rats, which is associated with various defects in the insulin-signalling/glucose transport

pathway. GTP significantly improved serum parameters such as glucose, insulin, TG, NEFA and cholesterol, and improved insulin sensitivity and glucose intolerance in HFD ZF rats. The likely molecular mechanisms involved in the regulatory processes are insulin-signalling targets such as PKB/Akt, GLUT4 translocation and regulation of PKC translocation in skeletal muscle of HFD ZF rats. It is postulated that the decreased insulin-signalling in skeletal muscle of rats may be, in part, caused by serine/threonine phosphorylation by PKC. Therefore, the results of the study strongly support that GTP may be implemented as a natural multi-targeted therapy for treatment and prevention of MetS and reduction of cardiovascular risk.

**CHAPTER 6: EFFECTS OF GREEN TEA
POLYPHENOLS ON NON-ALCHOLIC FATTY LIVER
DISEASE INDUCED BY HIGH-FAT DIET IN ZUCKER
FATTY RATS**

6. Effects of Green Tea Polyphenols on Non-alcoholic Fatty Liver Disease Induced By High-fat Diet in Zucker Fatty Rats

6.1.Introduction

Non-alcoholic fatty liver disease (NAFLD) describes a spectrum of disorders, increasingly recognised as a major health burden especially in developed countries. NAFLD is defined by hepatic fat infiltration > 5% hepatocytes, as assessed by liver biopsy, in the absence of excessive alcohol intake, viral, autoimmune and drug-induced liver disease. It encompasses a wide spectrum of liver diseases ranging from simple steatosis to non-alcoholic steatohepatitis (NASH), which, in turn, can evolve into cirrhosis and end stage liver disease (Toledo & Kelly, 2006; Paschos & Paletas, 2009). NAFLD has become the most common form of liver disease, affecting 20-30% of the US population (Kim, 2008). Studies have shown that NAFLD is highly associated with the features of MetS. Insulin resistance and obesity are key pathogenic factors in both NAFLD and MetS (Marchesini et al., 2001).

Obesity, in particular central adiposity and insulin resistance, are considered as the main factors related to NAFLD. Increased adipose energy storage present in obesity causes an escalation in FFA flux and TG storage in tissues such as the liver, which in this case promotes the onset of hepatic insulin resistance. The onset of NAFLD is even more common in people who are morbidly obese, i.e., who have a body mass index greater than 40 kg/m². Studies have revealed that in morbidly obese patients, NASH was not predicted by age or body mass index, but it was more common in subjects with diabetes and/or insulin resistance (Machado et al., 2006). Because of the rise in the prevalence of childhood obesity, NAFLD

is becoming one of the most important chronic liver disease among children (Schwimmer et al., 2006).

The pathophysiology of NAFLD is not fully understood. However, research has identified that insulin resistance and obesity, which are key features of MetS, play a fundamental role in the pathogenesis of fatty liver disease (Ballestri et al., 2014). Although prognosis of simple steatosis is good and often reversed by weight loss and dietary modifications, NASH can progress to cirrhosis or hepatic carcinoma in approximately 10-15% of patients (Kim, 2008). Therefore, implementation of a multi-targeted drug that is safe and effective for NAFLD is crucial. At present, although many treatments are used for NAFLD, there is no consensus. The typical first-line strategy is weight loss and exercise and then to further target each component of MetS with oral medication as it appears, such as hyperglycaemia and hyperlipidaemia. Typical oral medications used include insulin-sensitisers (ie. biguanides and glitazones), lipid-lowering drugs such as statins and gemfibrozil (Lopid), pentoxifylline (Pentoxil, Trental), probiotics, and angiotensin-converting enzyme inhibitors. However, there have only been limited studies into the effectiveness of these drugs and common side effects including weight gain and hepatotoxicity have been reported (Bugianesi et al., 2005; Adams & Angulo, 2006).

The previous chapters of this thesis outlined the beneficial effects of GTP on the metabolic parameters and pathways *in vitro* and *in vivo*, showing significant outcomes on abnormalities of glucose and lipid metabolism, and improving insulin resistance. Therefore, it was of interest to study the possible effects of GTP on improving high-fat diet induced fatty liver in rats. Short term high fat feeding in rats leads to a state of hepatic fat accumulation and provides a model of NAFLD, useful in studying the mechanisms of hepatic insulin resistance (Samuel et al., 2004).

The aim of this study was to investigate the effects of GTP on NALFD induced by HFD feeding in ZF rats and to identify the possible mechanisms involved.

6.2. Research Plan and Methods

Animals

The animals used for this chapter is outlined in Chapter 5.2. Male Zucker fatty (fa/fa) rats (ZF) and their lean littermates, aged 5-7 weeks and approximately 160 g body weight were acclimatised in communal cages at 22 °C, with a 12 h light-dark cycle (lights on 0700 h) for 1 week and had *ad libitum* access to a standard chow diet (Gordon's Specialty Stock Feed, Sydney, Australia) and water. ZF rats (n=30) were fed a high-fat diet (HFD; 350 kJ/d) for 2 weeks. The energy percentage composition of the HFD was 59% fat, 20% carbohydrate, and 21% protein, with equal quantities of fibre, vitamins, and minerals to the standard chow diet. The HFD was replenished daily. After two weeks of HFD feeding, ZF rats (n=30) were divided into two groups. The control group (HFD-Con; n=15) was administered distilled water (5 ml/kg of body weight) and ZF rats in the GTP treatment group (HFD-GTP; n=15) was administered GTP at 200 mg/kg of body weight daily for 8 weeks via oral gavage. HFD feeding was continued throughout the 8 weeks treatment period. Lean Zucker rats (n=15) were fed a standard laboratory chow diet throughout the treatment period.

Biochemical Assays

At the end of the experiment, all rats were anaesthetised according to Chapter 2.3.6, and blood serum samples were collected as described in Chapter 2.3.7. Determination of ALT and AST concentrations were carried out using assay kits according to manufacturer's instructions detailed in Chapter 2.3.10.2.

Tissue Triglyceride Content in Liver

Triglycerides (TG) were extracted then quantified using an enzymatic colorimetric method (Triglycerides GPO-PAP reagent; Roche Diagnostics, IN, USA). TG content was determined spectrophotometrically by measuring the absorbance at 490 nm with a microplate reader (Bio-Rad Laboratories, CA, USA).

Western Blotting

Frozen liver samples were obtained, minced and homogenised according to Chapter 2.3.10.1. The homogenate was spun at 400 xg for 15 min at 4°C. Supernatants were collected for total protein determination and immunoblotting analysis. Protein (20 µg) was separated by SDS-PAGE (12.5%) and blotted onto PVDF membranes. Blotted membranes were blocked with 5% skim milk in PBS with 0.05% Tween 20 overnight and incubated with primary antibodies phospho-GSK3β (Ser9), phospho-GS (Ser641), phospho-AMPKα (Thr172), phospho-ACC (Ser79) (Cell Signaling Technology Inc, MA, USA), PEPCK (Santa Cruz Biotechnology, CA, USA) and β-actin diluted at 1:1000 for 4 h. Membranes were washed 3 times in 0.05% Tween 20 (PBS-T) while agitating, 10 min per wash, to remove residual primary antibody. Membranes were incubated with corresponding horseradish peroxidase-conjugated secondary antibodies (Santa Cruz, CA, USA) at 1:10000 dilution and blots were developed with enhanced chemiluminescence (ECL) (Pierce, IL, USA) according to manufacturer's instructions.

Histological Analysis

The histological analysis of livers was undertaken as shown in Chapters 2.3.11.1, 2.3.11.2 and 2.3.11.3, covering sample preparation, tissue sectioning and staining procedures. HE

staining was utilised to examine the general tissue composition of liver samples and oil red o staining was performed to determine lipid content in rat liver samples.

The liver was weighed following a midline laparotomy to remove the whole liver. Liver tissue from rats were quickly placed into liquid nitrogen then stored at -80°C. A small portion of frozen tissue was cut and embedded with pre-cooled optimal cutting compound (Torrance, CA, USA) for cryostat sectioning at 6 µm. The sections were mounted on Fisherbrand Superfrost Plus slides (Thermo Fisher Scientific Inc., MA, USA) then fixed with 10% formaldehyde solution for 48 hr. After washing with PBS 3 times, slides were stained with haematoxylin and eosin (HE) and oil red o (ORO) to investigate the structure and fat deposition of hepatic tissue. In ORO stained images, the respective areas were quantified using Image-Pro 6.2 (Media Cybernetics Inc., MD, USA) under light microscopy (Olympus BX51). For each group, liver from 8 rats were prepared and stained, then 6 fragments from each liver were further analysed. Morphometric results are presented as area fractions – the percentage of specific counts in relation to total number of counted points.

Statistical Analysis

Data are presented as the means $\pm$ S.E. Analysis was done using one-way ANOVA followed by Dunnett's test to determine significant differences between groups using Prism version 6 (GraphPad Software Inc, CA, USA). *p* -value < 0.05 was considered statistically significant.

6.3.Results

6.3.1. Effects of GTP on liver enzymes ALT and AST levels in HFD ZF rats

Serum ALT and AST concentrations in HFD ZF rats were significantly higher than in chow fed rats (29.20 ± 4.01 U/L vs 189.89 ± 20.69 U/L and 54.71 ± 17.88 U/L vs 183.14 ± 13.91 U/L, respectively; $p < 0.01$). As shown in Figure 6.1, GTP treatment significantly lowered both ALT and AST concentrations in HFD ZF rats (71.19 ± 10.23 U/L vs 189.89 ± 20.69 U/L and 82.25 ± 7.51 U/L vs 183.14 ± 13.91 U/L, respectively; $p < 0.01$).

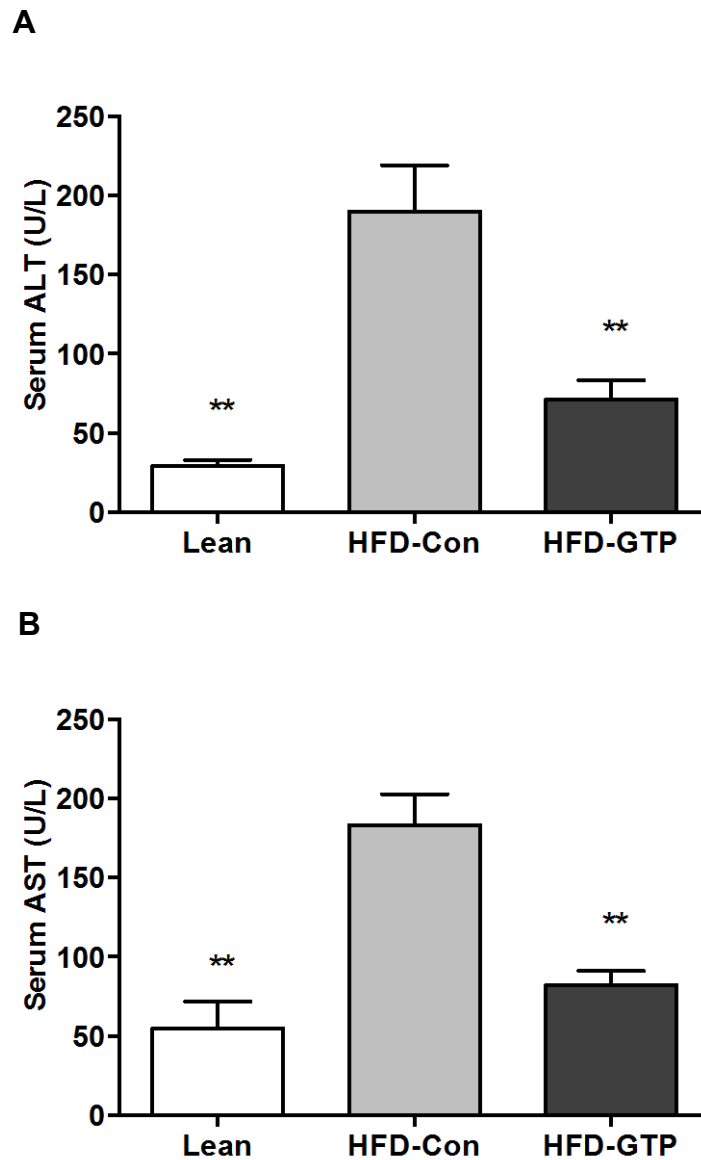


Fig 6.1. Effect of GTP on alanine transaminase (ALT) (A) and aspartate transaminase (AST) (B) in HFD ZF rats Serum ALT and AST concentrations were measured after 8 weeks of daily treatment with saline (5 ml/kg; Lean, HFD-Con) or GTP (200 mg/kg; HFD-GTP). Data are means $\pm$ S.E., n = 15 rats/group. **p<0.01 vs HFD-Con rats.

6.3.2. Effect of GTP on TG deposition in the liver of HFD ZF rats

Measurement of TG content in the liver showed that HFD ZF rats presented significantly higher hepatic TG by 4-fold compared to lean rats (13.65 ± 4.51 vs 3.36 ± 0.72 $\mu\text{mol/g}$;

$p < 0.01$). Results in Figure 6.2 showed that GTP treatment significantly reduced hepatic TG content in HFD fed ZF rats ($9.05 \pm 2.87 \mu\text{mol/g}$ vs $13.65 \pm 4.51 \mu\text{mol/g}$; $p < 0.01$).

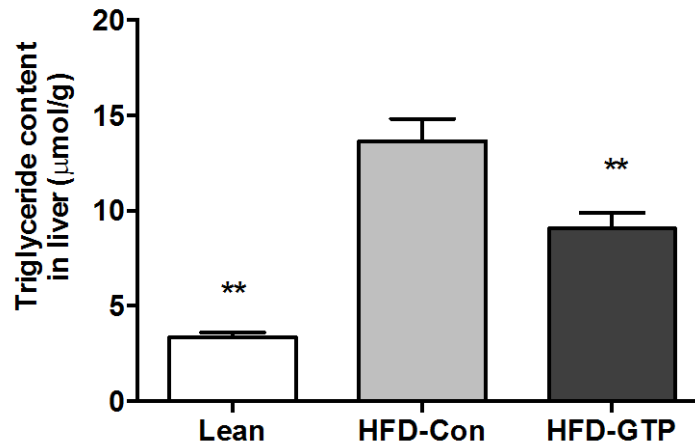


Fig 6.2. TG content in liver of HFD ZF rats TG content in liver was measured after 8 weeks of daily treatment with saline (5 ml/kg; Lean, HFD-Con) or GTP (200 mg/kg; HFD-GTP). Data are means $\pm$ S.E., $n = 15$ rats/group. ** $p < 0.01$ vs HFD-Con rats.

6.3.3. Effect of GTP on lipid deposition in the liver and hepatic steatosis of HFD ZF rats

Lipid accumulation in the liver impairs hepatocyte function leading to fatty liver or macrovesicular steatosis. Liver tissue was analysed under microscope after HE staining and ORO staining. Lipid accumulation was elevated in the HFD ZF rats as indicated by the unstained area in hepatocytes under HE staining (Figure 6.3B). Slight reduction in lipid droplets was seen with GTP treatment (Figure 6.3C) in HFD ZF rats.

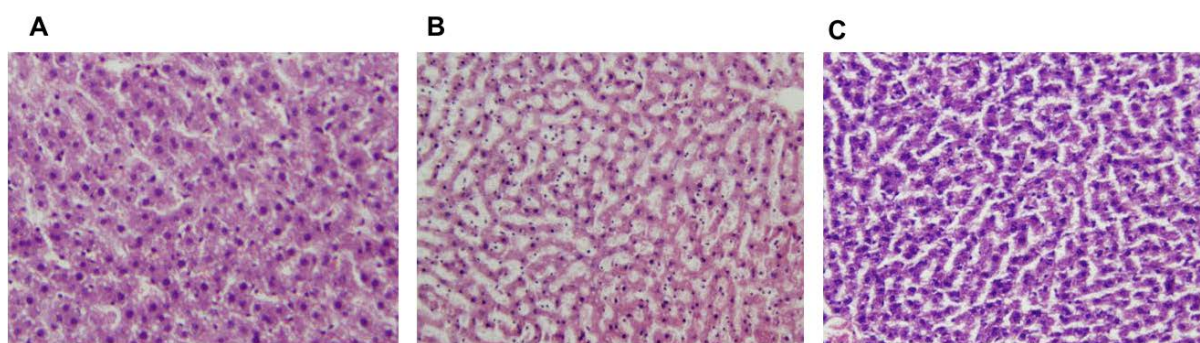


Fig 6.3. Representative haematoxylin and eosin staining of livers from (A) lean (B) HFD-Con and (C) HFD-GTP rats Images were stained with haematoxylin and eosin to visualise hepatic histology. Microscopic images were attained from liver tissue with 20 x magnification.

Oil red O staining in frozen liver tissues was carried out to observe the liver droplets in rats, microscopically. Many lipid droplets were observed in livers of HFD ZF control rats (stained red), whereas no lipids were visible in lean-chow fed rats (Figures 6.4A and B). GTP administration markedly reduced lipids droplets in HFD ZF rats, as perceived in figure 6.4C. Quantitative analysis, carried out by blindly scored liver sections (Figure 6.4D), showed a significant increase in lipids of HFD ZF control rats than lean rats, and this increase was significantly attenuated with GTP treatment ($p < 0.01$).

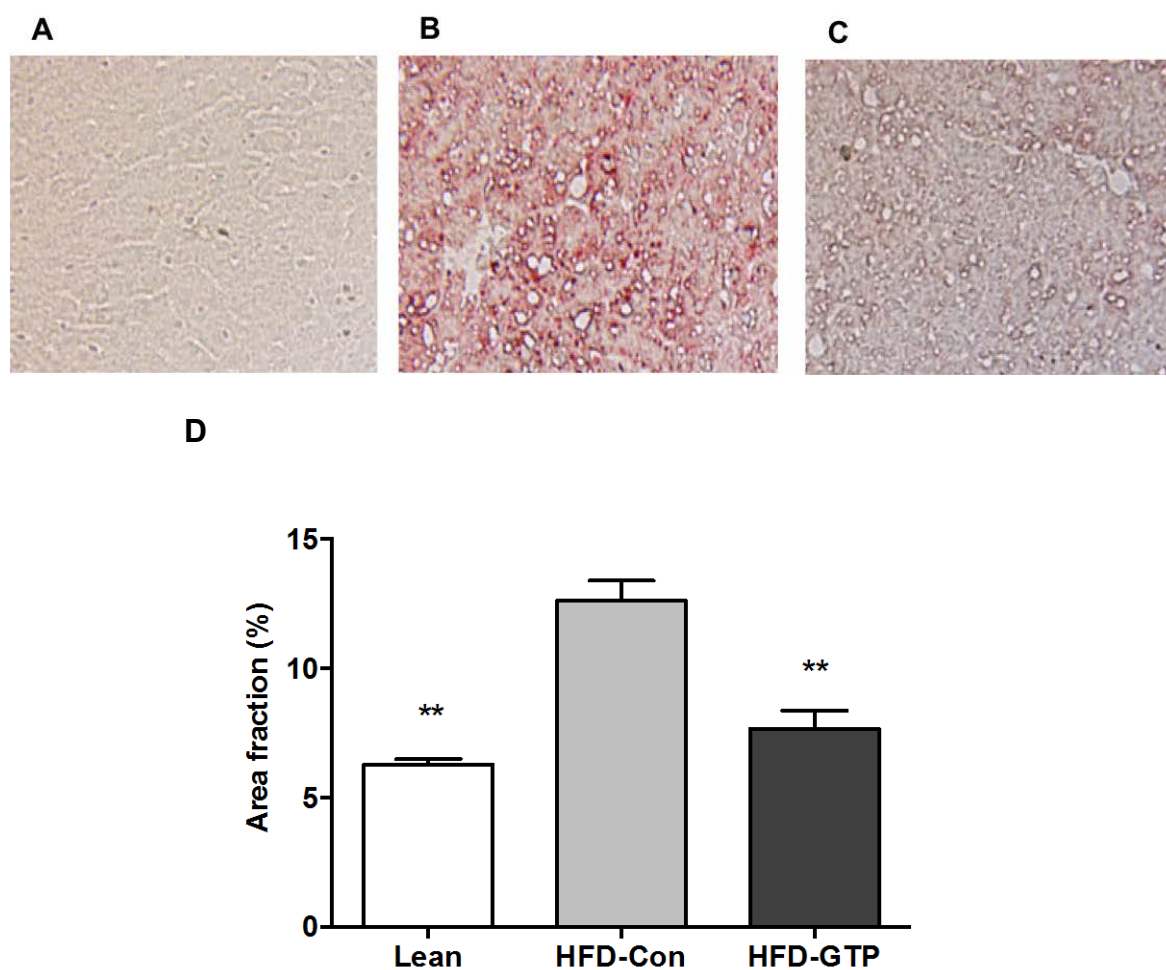


Fig 6.4. Representative oil red O staining images of livers from (A) lean, (B) HFD-Con and (C) HFD-GTP rats Images were attained from liver tissue with 20x magnification. The oil red O stains were quantitatively measured (D) Data are means $\pm$ S.E. (n=5) **p<0.01 vs HFD-Con rats.

6.3.4. Effects of GTP on Expressions of Ser9 pGSK3 β and Ser641 pGS in Liver of HFD ZF rats

Glycogen synthase kinase 3 β (GSK3 β) is a rate-limiting enzyme, which acts as a downstream regulatory switch for numerous signalling pathways, in particularly glycogen synthesis and *de novo* lipogenesis. Results indicate that both phosphorylation of GSK3 β and GS were reduced in HFD fed ZF rats, where GS phosphorylation was significantly down-regulated compared to lean rats (p<0.01). Figure 6.5 shows that GTP treatment in high-fat fed ZF rats

significantly increased phosphorylation of GSK3 β and GS ($p<0.05$ and $p<0.01$, respectively), thereby inhibiting GSK3 β and increasing GS in the liver. This is the likely mechanism by which hepatic insulin-signalling, glycogen synthesis and *de novo* lipogenesis is enhanced by GTP in HFD ZF rats.

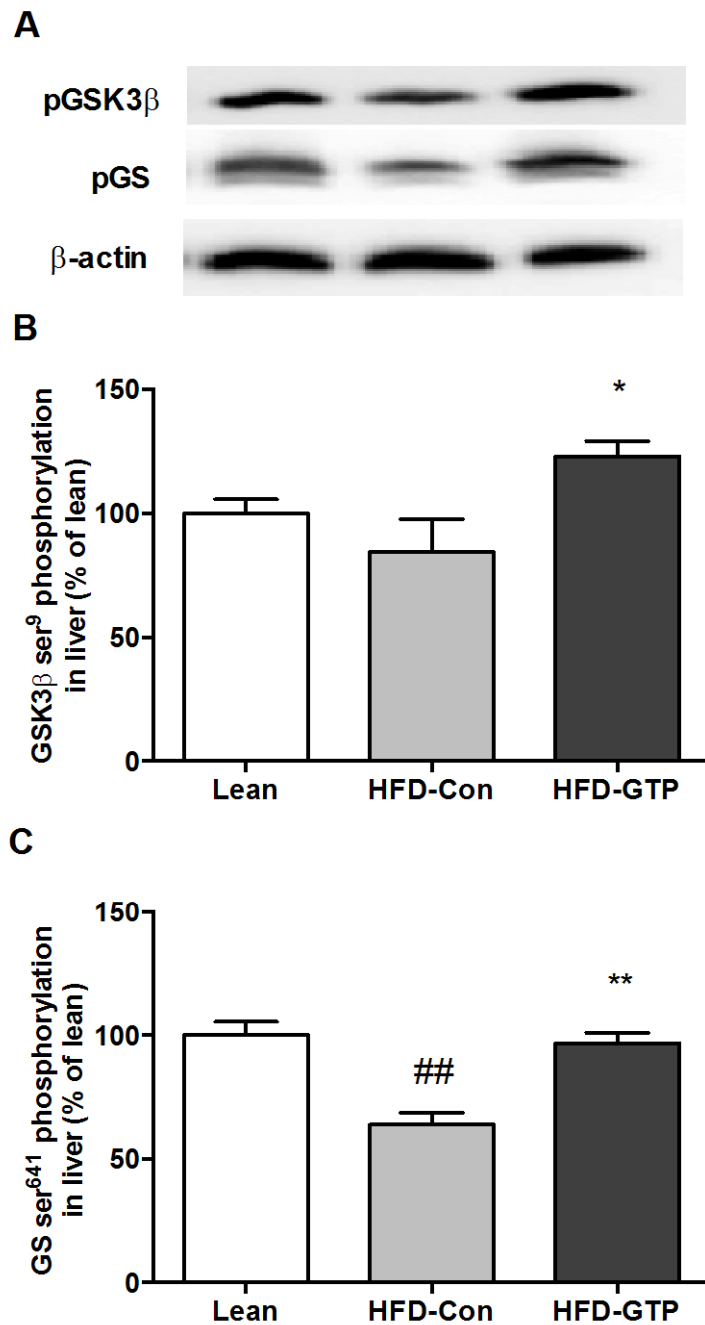


Fig 6.5. Determination of GSK3 β (Ser9) and GS (Ser641) phosphorylation in the liver of ZF rats
 Immunoblots (A) and quantitative analysis of (B) GSK3 β (Ser⁹) and (C) GS (Ser⁶⁴¹) in liver tissue of chow fed lean rats and HFD fed ZF rats treated with saline (5 ml/kg of body weight) and HFD ZF rats treated with GTP (200 mg/kg of body weight). Data are expressed as means $\pm$ S.E. of 5 rats per group. *p<0.05 vs HFD-Con, ##p<0.01 vs Lean, **p<0.01 vs HFD-Con.

6.3.5. Effects of GTP on Expressions of Thr172 pAMPK α and Ser79 pACC in the Liver of HFD ZF rats

AMPK and ACC are key enzymes that regulate *de novo* lipogenesis in the liver (Huang et al., 2009) and contribute considerably to overall metabolism of lipids. Results in Figure 6.6 show that HFD fed ZF rats significantly lowered levels of phospho-AMPK α (Thr172) compared with the lean control rats ($p < 0.05$), and GTP administration significantly increased expression of phospho-AMPK α (Thr172) ($p < 0.05$) and normalised phosphorylation of AMPK α in HFD ZF rats. A slight reduction of ACC (Ser79) phosphorylation was observed in the liver of HFD ZF rats, and this regulated in a similar manner to that of phospho-AMPK α (Thr172).

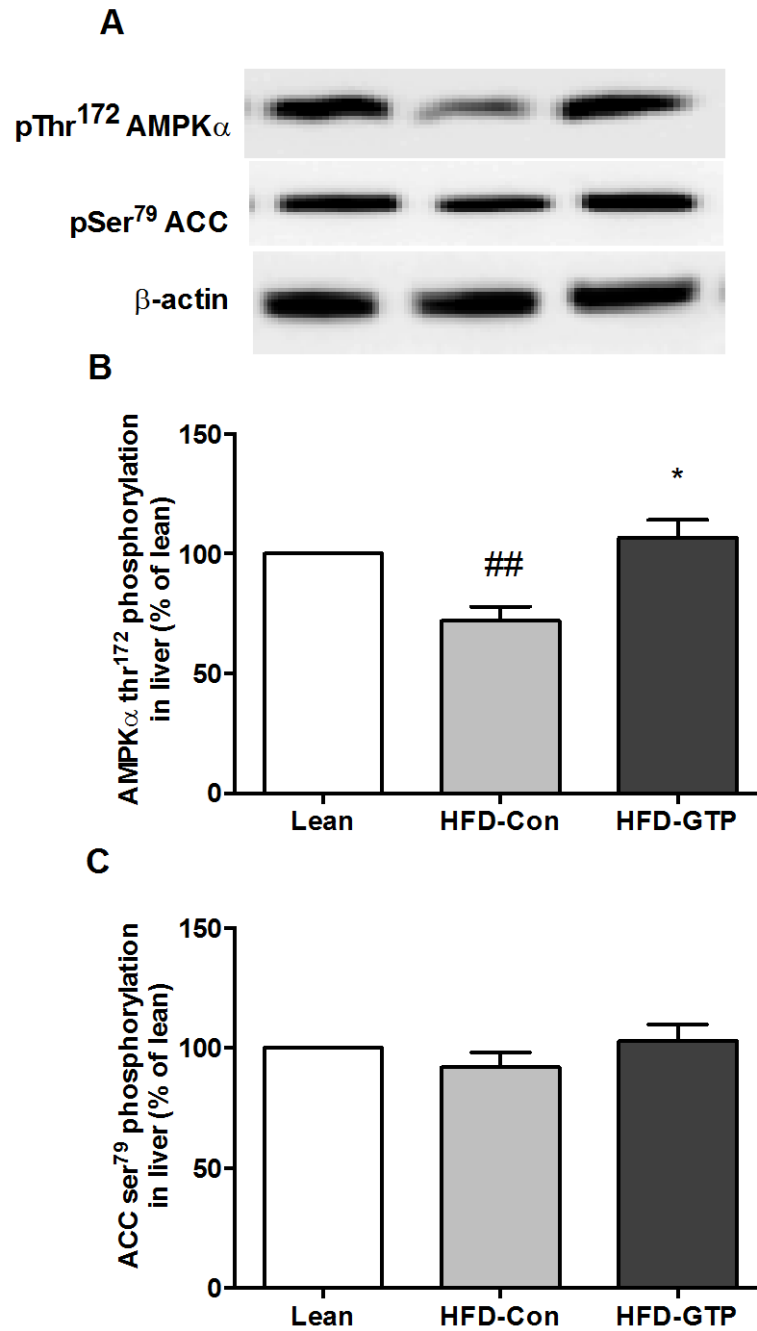


Fig 6.6. Determination of AMPK α (Thr172) and ACC (Ser79) phosphorylation in the liver of ZF rats Immunoblots (A) and quantitative analysis of (B) AMPK α (Thr¹⁷²) and (C) ACC (Ser⁷⁹) in liver tissue of chow fed lean rats and HFD fed ZF rats treated with saline (5 ml/kg of body weight) and HFD ZF rats treated with GTP (200 mg/kg of body weight). Data are expressed as means $\pm$ S.E. of 5 rats per group. ^{##}p < 0.01 vs Lean, ^{*}p < 0.05 vs HFD-Con.

6.3.6. Effect of GTP on PEPCK expression in Liver of HFD ZF rats

It has been observed that overexpression of PEPCK results in the onset of T2D and NAFLD due to its rate-limiting regulatory functions on glucose synthesis in liver tissue. Results demonstrate that PEPCK expression was significantly increased in HFD ZF rats ($p<0.01$). This elevation of PEPCK expression was significantly reduced with GTP administration in HFD ZF rats ($p<0.05$), shown in Figure 6.7.

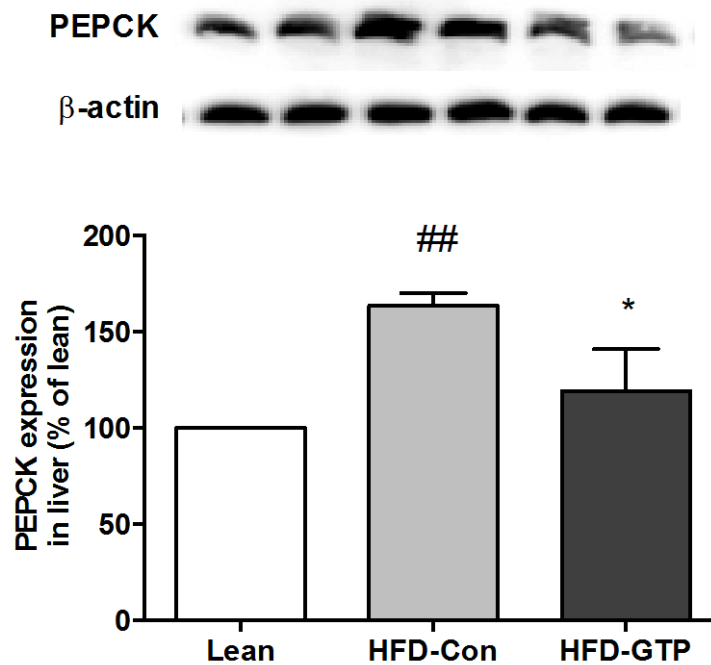


Fig 6.7. Determination of PEPCK expression in the liver of ZF rats Immunoblot and quantitative analysis of PEPCK in liver tissue of chow fed lean rats and HFD fed ZF rats treated with saline (5 ml/kg of body weight) and HFD ZF rats treated with GTP (200 mg/kg of body weight). Data are expressed as means $\pm$ S.E. of 5 rats per group. ## $p<0.01$ vs Lean, * $p<0.05$ vs HFD-Con.

6.4. Discussion

The results of this chapter showed that GTP administration improved NAFLD induced by HFD in ZF rats. This was evidenced by significantly improved hepatic enzymes AST and ALT levels and significantly lower TG content in the liver of GTP-treated HFD ZF rats. Histological analysis showed that GTP also markedly reduced lipid droplets in the liver of HFD ZF rats. Western blotting revealed the underlying mechanisms involved are likely to include upregulation of GSK3 β and GS expressions, enhanced phosphorylation of AMPK α and inhibited expression of PEPCK with GTP administration in HFD ZF rats.

NAFLD is strongly associated with the clinical features of MetS, especially, obesity and insulin resistance that are the main risk factors for NAFLD. The previous *in vitro* and *in vivo* studies in this thesis demonstrated the beneficial effects of GTP on body weight, glucose and lipid metabolism as well as improving insulin sensitivity. It has been observed that over nutrition or inappropriate diet lead to chronic elevated plasma concentrations of glucose, insulin and FFA, which significantly contribute to the development of NAFLD (Gaemers & Groen, 2006), in particular for those who have tendency for obesity genetically. Therefore, it was of interest to study the possible effects and underlying mechanism of GTP, naturally occurring catechins and widely consumed beverage, on NAFLD using a genetically obese rodent model fed by HFD.

GTP treatment significantly prevented weight gain and normalised blood lipids levels. The results of this chapter show that GTP significantly improved hepatic enzymes AST and ALT levels and lowered TG contents in the liver of HFD ZF rats. Serum transaminases (ALT and AST) play a key role in animal amino acid metabolism. Elevated levels of these enzymes

frequently indicate liver damage. High serum ALT concentrations are associated with increased hepatic fat fraction and increased intra-abdominal visceral adipose tissue. Therefore, serum ALT has been used as a surrogate marker for fatty liver disease in population studies (Pratt & Kaplan, 2000). This study showed that serum ALT and AST concentrations in HFD ZF rats were significantly higher than in chow fed rats and GTP administration in HFD ZF rats significantly lowered both ALT and AST concentrations, indicating GTP protects lipotoxicity-induced liver damage.

Results attained from histological experimentation further supports the protective effects of GTP on liver damage related to lipid deposition. In oil red O staining, GTP administration markedly reduced lipids droplets in the liver of HFD ZF rats. Quantitative analysis showed a significant increase in lipids of HFD ZF control rats compared to lean rats, and this increase was significantly attenuated with GTP ($p < 0.01$).

To elucidate mechanisms of GTP treatment for improvement of NAFLD, key protein targets of liver glycogen synthesis and lipogenesis were investigated. GTP significantly increased phosphorylation of GSK3 β and GS ($p < 0.05$ and $p < 0.01$, respectively), thereby inhibiting GSK3 β and increasing GS in the liver. Activation of GS through inhibition of GSK3 represents a potential new therapeutic target for MetS. GSK3 is a rate-limiting enzyme which acts as a downstream regulatory switch for inactivation of GS leading to reduction of glycogen synthesis has been found to also increase hepatic glucose uptake and insulin sensitivity (Henriksen & Dokken, 2006; Orena et al., 2000). Studies into inhibitors of GSK3 have been utilized to investigate the contribution of GSK3 to glucose metabolism and insulin resistance. Administration of a GSK3 inhibitor to ZF rats improved glucose tolerance and elevated GS activity in liver and muscle accompanied by increased hepatic glycogen and enhanced glucose sensitivity. GSK3 inhibition also improved whole body glucose tolerance

and insulin sensitivity (MacAulay & Woodgett, 2008). Therefore, GTP may be useful in enhancing glycogen synthesis, and regulating lipogenesis in patients with NAFLD.

AMPK and ACC are key enzymes that regulate *de novo* lipogenesis in the liver (Huang et al., 2009) and contribute significantly to overall metabolism of lipids. Insulin activates AMPK by promoting its phosphorylation at Thr172 (Huang et al., 2009). An increase in AMPK α phosphorylation in the liver leads to phosphorylation and inactivation of ACC resulting in decreased lipid synthesis, through the biosynthesis of malonyl-CoA from acetyl-CoA, and this may lead to decreased lipid synthesis and regulation of fatty acid oxidation (Zhou et al., 2001), key mechanisms for the treatment and prevention of not only T2D, but NAFLD. GTP administration significantly increased phosphorylation and activity of AMPK which in turn stimulated phosphorylation of acetyl coenzyme A carboxylase (ACC), possibly promoting fatty-acid oxidation and glucose uptake in liver of HFD ZF rats.

Inactivation of ACC (ACC1 and ACC2) also reduces hepatic insulin resistance in NAFLD and T2D by inhibiting PEPCK. This has been observed in the hyperinsulinaemic phase of the hyperinsulinaemic-euglycemic clamp and PKC ϵ membrane translocation (Musso et al., 2009). Phosphoenolpyruvate carboxykinase (PEPCK) is an enzyme in the metabolic pathway of gluconeogenesis and glucose regulation. It has been observed that overexpression of PEPCK results in the onset of T2D, due to its rate-limiting regulatory functions of glucose synthesis in the liver. In the case of liver insulin resistance, glucagon indirectly elevates the expression of PEPCK by increasing the levels of cyclic adenosine monophosphate (cAMP) (via activation of adenylyl cyclase), which subsequently leads to the phosphorylation of cAMP response element-binding protein (CREB). CREB then binds upstream of the PEPCK gene at CRE (cAMP response element) and induces PEPCK transcription (Ferber et al., 1994; Weickert & Pfeiffer, 2006). In this study, PEPCK expression was significantly increased in

the liver of HFD ZF rats compared to lean rats ($p<0.05$). This elevation of PEPCK expression was significantly reduced with GTP administration in HFD ZF rats ($p<0.05$). Decreased PEPCK in liver may be responsible for inhibiting gluconeogenesis, which inhibits hepatic glucose formation and stimulates glucose uptake by cells, in GTP-administered HFD ZF rats.

6.5. Conclusion

Taken together, the data above strongly supports the beneficial effects of GTP in reducing hepatic fat accumulation and insulin resistance. On the basis of these results, GTP seems to be a promising candidate for both the treatment and prevention of NAFLD and other related hepatic disorders. This is evidenced by significant reduction of serum ALT and AST levels and hepatic TG content in GTP-treated HFD ZF rats. Mechanisms are likely to include upregulation of GSK3 β and GS expressions thereby enhancing glycogen synthesis and down-regulating *de novo* lipogenesis, through enhancing AMPK α and ACC mediated fatty-acid oxidation and glucose uptake in the liver and reducing PEPCK to inhibit hepatic glucose production.

CHAPTER 7: FINAL DISCUSSION, CONCLUSION AND FUTURE DIRECTIONS

7. Final Discussion, Conclusion and Future Directions

7.1.Final Discussion and Conclusion

This thesis was carried out to determine the effects and mechanisms of GTP, a widely consumed tea and natural agent, on experimental models of MetS, which may ultimately prevent the onset or treat MetS and other related disorders such as T2D and obesity. Significant alterations in glucose and lipid metabolism may cause the onset of metabolic diseases and it is crucial for implementation of early and successful treatment strategies to prevent complications such as cardiovascular disease or death. Due to the therapeutic limitations, toxicity, side effects and high-costs of oral drugs currently used, it is essential that novel agents are developed for both management and prevention of MetS and related disorders. GTP is a popular beverage consumed all over the world and recently reported of possible hypoglycaemic and lipid-lowering effects. The aims of this thesis were to identify active compounds of GTP, establish an effective and safe dose range for GTP and to elucidate the *in vitro* effects and mechanisms of GTP on glucose and lipid metabolism. A HFD rat model was further utilised to determine the biochemical effects and molecular mechanisms of GTP administration in regulating metabolism of glucose and lipids.

The key findings of this thesis are summarised below:

GTP increased insulin-stimulated glucose uptake by up-regulated expressions of IRS-1, PKB/Akt and GLUT4 and inhibited lipolysis through decreased expression of PKA in 3T3-L1 adipocytes.

Results from this chapter showed that GTP-EGCG improved glucose uptake and inhibited lipolysis in adipocytes. GTP-EGCG increased insulin-stimulated glucose uptake in a dose-dependent manner. The likely molecular mechanisms involved include up-regulated expressions of IRS-1, PKB/Akt and GLUT4 in 3T3-L1 adipocytes. Lipolysis was significantly inhibited in GTP-treated 3T3-L1 adipocytes through decreased expression of PKA. These results support that GTP-EGCG may be a potential therapeutic agent for treating MetS and prevention of obesity and T2D by improving insulin resistance in adipose tissue.

GTP regulated hepatic glycogen synthesis through increased phosphorylation of GSK3 β and GS and inhibited hepatic lipogenesis through increased phosphorylation of AMPK α and ACC in HepG2 cells.

Results demonstrated that hepatic glycogen synthesis was significantly up-regulated in HepG2 cells with GTP-EGCG treatment through increased phosphorylation of GSK3 β and GS, which are critical elements in the regulation of hepatic glycogen synthesis *in vivo*. GTP-EGCG also inhibited hepatic lipogenesis in cells through increased expressions of phospho-AMPK α (Thr 172) and phospho-ACC (Ser79). The findings of this chapter revealed the beneficial effects of GTP-EGCG against MetS through direct enhancement of glycogen synthesis in the liver and decreased hepatic lipogenesis.

GTP improved insulin resistance through improved serum parameters, insulin sensitivity and glucose intolerance in HFD ZF rats.

The findings of this chapter showed that GTP significantly improved serum parameters such as glucose, insulin, TG, NEFA and cholesterol, and improved insulin sensitivity and glucose intolerance in HFD ZF rats. The likely molecular mechanisms involved in the regulatory processes are insulin-signalling targets such as PKB/Akt, GLUT4 translocation and regulation of PKC translocation in skeletal muscle of HFD ZF rats. It is postulated that the decreased insulin-signalling in skeletal muscle of rats may be, in part, caused by serine/threonine phosphorylation by PKC. Therefore, the results of the study strongly support that GTP may be a beneficial natural and multi-targeted therapy for treatment and prevention of MetS and reduction of cardiovascular risk.

GTP improved NAFLD induced by HFD in ZF rats through decreased hepatic enzymes, TG content and lipids.

The results of this chapter support the beneficial effects of GTP in reducing hepatic fat accumulation and insulin resistance. On the basis of these results, GTP seems to be a promising candidate for both the treatment and prevention of NAFLD and other related hepatic disorders. This is evidenced by significant reduction of serum ALT and AST levels and hepatic TG content in GTP-treated HFD ZF rats. Mechanisms are likely to include upregulation of GSK3 β and GS expressions thereby enhancing glycogen synthesis and down-regulating *de novo* lipogenesis, through enhancing AMPK α and ACC mediated fatty-acid oxidation and glucose uptake in the liver and reducing PEPCK to inhibit hepatic glucose production.

7.2.Future Directions

It has been outlined in this thesis that the pathogenesis of MetS is very complex and is only partially understood at present. Although insulin resistance and obesity have been regarded as key components of MetS, insufficient knowledge on the mechanisms and a lack of effective treatment options for MetS are reasons for more extensive research and development in this area. Given the limited efficacy of, and long-term safety concerns with, the drugs currently used for the treatment of MetS, the discovery of novel therapeutic options is crucial for the forthcoming years. It is crucial that the targets of future investigation involve both clarification of the pathogenesis of MetS and the establishment of effective treatment and prevention of MetS and related diseases. Recently natural agents have been reported to hold great potential for the treatment of T2D, obesity, NAFLD and other metabolic disorders. In particular, teas have gained research interest for their effectiveness in regulating metabolism.

The results presented in this thesis have provided insights into the action of GTP in enhancing insulin-mediated glucose and lipid metabolism by regulating enzymes involved in glycogen synthesis and lipogenesis in liver and glucose uptake and lipolysis in adipose and hepatic cells. GTP also significantly improved serum parameters, such as glucose, insulin, TG, NEFA and cholesterol, ALT, AST and improved glucose intolerance in HFD ZF rats. The results of this thesis show that GTP may be a potential therapeutic agent for MetS and related disorders such as T2D, obesity and NAFLD.

The results of this thesis showed that GTP has beneficial effects on obesity and systemic metabolic dysfunction and moderately improved insulin resistance. Current pharmaceutical insulin sensitisers, such as metformin or thiazolidinediones (rosiglitazone and pioglitazone)

have potent effects on insulin resistance but they also exert significant side effects that reduce tolerability of long term of administration. It would be worthwhile to investigate into the synergistic effects of GTP and these oral medications in rodent models of MetS and T2D in the future study.

It may also be worthwhile to study other components of GTP, such as caffeine, that may give rise to metabolic effects. Studies have shown that habitual tea consumption reduces the risk of T2D and that caffeine may stimulate resting energy expenditure and increase oxidative lipid disposal. Therefore, it would be interesting and of value to study whether caffeine has an effect on glucose and lipid metabolism in models of insulin resistance, T2D and obesity.

Randomised, double-blinded clinical trials need to be carried out to study the effects of GTP on key clinical parameters, such as HbA1c levels, in subjects. It is important that either an optimal GTP dosage or a range of safe and effective GTP dosages are established and that high-quality GTP is administered to patients with MetS or T2D. The areas outlined above are currently being investigated or will be considered for future experimentation.

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