

Flavonol kaempferol in the regulation of glucose homeostasis in diabetes

By

Hana Alkhalidy

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Advisory Committee:

Dongmin Liu, Chair

Deborah Good

Honglin Jiang

Matthew Hulver

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ABSTRACT

Diabetes mellitus is a major public health concern. Although the accessible novel drugs, techniques, and surgical intervention has improved the survival rate of individuals with diabetes, the prevalence of diabetes is still rising. Type 2 diabetes (T2D) is a result of chronic insulin resistance (IR) and loss of β -cell mass and function. Therefore, the search for naturally occurring, low-cost, and safe compounds that could enhance insulin sensitivity and protect functional β -cell mass can be an effective strategy to prevent this disease. Kaempferol, a flavonol present in various medicinal herbs and edible plants, has been shown to elicit various pharmacological activities in preclinical studies. However, studies investigating the effect of kaempferol on diabetes are limited. In this dissertation, I explored the anti-diabetic potential of dietary intake of kaempferol in diet-induced obese mice and insulin-deficient diabetic mice. First, kaempferol was supplemented in the diet to determine whether it can prevent IR and hyperglycemia in high fat (HF) diet-induced obese mice or STZ-induced obese diabetic mice. To evaluate its efficacy for treating diabetes, kaempferol was administrated once daily via oral gavage to diet-induced obese and insulin-resistant mice or lean STZ-induced diabetic mice. The results demonstrated that long-term oral administration of kaempferol prevents HFD-induced metabolic disorders in middle-aged obese mice. Oral administration of kaempferol improved glucose intolerance and insulin sensitivity, and this effect was associated with increased Glut4 and AMPK α expression in muscle and adipose tissues. Consistent with our findings from the *in*

vitro study in C2C12 muscle cell line, these findings suggest that kaempferol may reduce IR at the molecular level by improving glucose metabolism in peripheral tissues. In the second study, dietary kaempferol supplementation prevented hyperglycemia and glucose intolerance by protecting β -cell against the induced damage in obese STZ-induced diabetic mice. In the third study, the administration of kaempferol by oral gavage significantly ameliorated hyperglycemia and glucose intolerance and reduced the incidence of diabetes from 100 % to 77.8% in lean STZ-induced diabetic mice. This kaempferol effect was associated with reduced hepatic glucose production, the primary contributor to hyperglycemia, and increased glucose oxidation in the muscle of diabetic mice. Kaempferol treatment restored hexokinase activity in the liver and skeletal muscle and reduced pyruvate carboxylase (PC) activity and glycogenolysis in the liver. Unlike its effect on T2D mice, kaempferol effect in lean STZ-induced diabetic mice was not associated with changes in plasma insulin levels. In the last study, we found that administration of kaempferol by oral gavage significantly improved blood glucose control by suppressing hepatic glucose production and improving glucose intolerance in obese insulin-resistant mice. Similar to its effect in old obese mice, kaempferol enhanced whole-body insulin sensitivity. Kaempferol increased Akt and hexokinase activity and decreased PC activity in the liver. However, kaempferol did not exert any changes in glucose metabolism or insulin sensitivity when administered to healthy lean mice. Overall, findings from these studies provide new insight into the role of kaempferol in the regulation of glucose homeostasis and suggest that kaempferol may be a naturally occurring anti-diabetic compound by improving insulin sensitivity, improving glucose regulation and metabolism, and preserving functional β -cell mass.

Keywords: Kaempferol, diabetes, glucose control, β -cells, insulin resistance, glucose production.

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DEDICATION

Thanks be to Allah for all His blessings.

I dedicate my dissertation work to my family, friends, supervisor, and lab-mates. Thank you for all your guidance, and continuous support. I could not have made it without you. Also, I would like to thank my committee for offering their time and providing helpful feedback. Lastly, I would like to extend my thanks to the faculty and staff at the Department of Human, Nutrition, Foods and Exercise/Virginia Tech University, and the Department of Nutrition and Food Technology/Jordan University of Science and Technology for their generous assistance that enabled me to accomplish this work.

LIST OF ABBREVIATIONS

A

AMPK: 5' AMP-activated protein kinase

ANOVA: Analysis of variance

AUC: Area under the curve

B

BW: Body weight

D

DAG: Diacylglycerol

DMEM: Dulbecco's modified eagle medium

DMSO: Dimethyl sulfoxide

E

ELISA: enzyme-linked immunosorbent assay

F

FA: Fatty acid

FAA: Free fatty acid

FBS: Fetal bovine serum

FoXO: Forkhead box O

G

G6Pase: Glucose-6-phosphatase

GCK: Glucokinase

GCKRP: Glucokinase regulatory protein

GLUT: Glucose transporter

GTT: Glucose tolerance test

H

HDL: High-density lipoprotein

HF: High fat

HK: hexokinase

I

IL-6: Interleukin 6

IMCL: Intramyocellular lipid

Ip: intraperitoneal

IR: Insulin resistance

IRS: insulin receptor substrate

ITT: Insulin tolerance test

L

LDL: Low-density lipoprotein

M

MetS: Metabolic syndrome

N

NO: Nitric oxide

P

PC: Pyruvate carboxylase

PI: Phosphatidylinositol

PKC: Protein kinase C

PTT: Pyruvate tolerance test

R

ROS: reactive oxygen species

S

SD: Standard diet

SGLT: Sodium-glucose linked transporter

STZ: Streptozotocin

T

T1D: Type 1 diabetes

T2D: Type 2 diabetes

TNF- α : tumor necrosis factor- α

W

WAT: White adipose tissue

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CHAPTER ONE

INTRODUCTION

Background

Diabetes is a progressive metabolic disease which is characterized by hyperglycemia associated with abnormalities in carbohydrate [1], lipid [2], and protein metabolism [3]. Diabetes mellitus is considered one of the leading causes of mortality in the United States [4]. Type 2 diabetes (T2D) is the most common type of diabetes accounting for approximately 90-95% of all diabetes cases [5]. While the cascade of events that leads to the development of T2D has long been the subject of debate [6, 7], it is well recognized that chronic insulin resistance (IR) and β -cell dysfunction are key defects in the pathogenesis of this disease [8]. Obesity is a leading pathogenic factor for developing IR, which along with IR is considered a risk factor for T2D [9]. In subjects with IR, β -cell failure to secrete an adequate amount of insulin to compensate for the defects in its action can lead to the development of hyperglycemia and overt diabetes [10]. Type 1 diabetes (T1D) is least common accounting for 5-10% of the cases. However, T1D is much serious and always accompanied by severe complications [11]. Unlike T2D, T1D results from destruction of pancreatic β -cells due to autoimmune process [12]. Therefore, subjects with T1D require insulin administration for survival [13].

Despite the tremendous knowledge about the disease and the availability of health practices that increase the survival rate of diabetic subjects, the prevalence of diabetes is still rising afflicting 9.3% of the U.S. population in 2012 [14], and this number is expected to double in 2050 [15]. In addition, the cost of treating diabetes and its complications is an increasing burden [16, 17]. Therefore, the search for novel agents that may prevent or treat hyperglycemia

by stimulating insulin sensitivity, protecting β -cell function, and improving glucose control may be an effective therapeutic strategy to reduce the risk of diabetes and its complications. Naturally occurring polyphenolic compounds gained a major interest in medicinal research due to their potential beneficial effects on various end points of human health, including diabetes [18, 19]. Kaempferol belongs to the subclass flavonols, one of the most common polyphenolic subclasses [20]. Kaempferol is identified in many plants used in traditional medicine and edible plants [21, 22] and has been shown to exert many pharmacological activities such as anti-oxidative [23], anti-microbial [21], anti-inflammatory [24], lipolytic [25], and anti-cancer [26, 27] effects. In addition, epidemiological studies show that dietary intake of kaempferol-containing foods has been associated with a reduced risk of certain cancers and cardiovascular diseases [21]. However, the studies regarding its effect on the pathogenesis of diabetes are very limited. This study was aimed at investigating the anti-diabetic potential of kaempferol and further elucidating the underlying mechanism of this action.

Hypothesis

It is well recognized that obesity induces IR and therefore increases the risk of developing T2D [28]. Elevation in free fatty acids observed in obesity [29] may contribute to the defects in insulin action in peripheral tissues and the liver [30-33]. Obesity-induced IR impairs energy metabolism [30, 31, 34], leading to excessive accumulation of intracellular fat content in liver and peripheral tissues, such as skeletal muscle, fat, as well as pancreatic islets [35, 36]. Moreover, persistent hyperlipidemia in obese individuals plays an important role in causing β -cell apoptosis and dysfunction, thereby contributing to the deterioration of glycemic control and the overt development of T2D [37-39]. A previous study demonstrated that kaempferol prevented apoptosis and improved insulin biosynthesis and secretion in β -cells and human islets exposed to

chronic hyperlipidemia [19]. However, it is not clear whether kaempferol can preserve β -cell function in vivo or promote insulin sensitivity, thereby preventing the onset of T2D. In the first study, **we tested the hypothesis that dietary supplementation of kaempferol prevents insulin resistance and hyperglycemia in high fat (HF) diet-induced obese mice and protects pancreatic β -cell function in streptozotocin (STZ)-induced obese diabetic mice.**

Glucose homeostasis is firmly regulated by the coordination of several complex pathways. Due to insulin deficiency, glucose metabolism is altered in T1D leading to the development of hyperglycemia [40]. Studies have reported that T1D is associated with reduced expression and activity of proteins that regulate glucose uptake and utilization [41, 42], and an increase in the proteins that stimulates hepatic glucose production [43]. In particular, excessive glucose production, mainly via gluconeogenesis, is a primary contributor to hyperglycemia. The unrestrained release of glucagon, which counter-regulates insulin action, due to insulin deficiency is a major part of the problem. Indeed, past studies demonstrated that suppressing glucagon secretion [44] or its function can prevent hyperglycemia in insulin-deficient rodent models of diabetes [45]. Thus, modulating the key proteins that regulate glucose homeostasis can be an important approach to preventing hyperglycemia. In a previous study, kaempferol was shown to dose-dependently (10–100 μ M) improve basal glucose uptake in a human hepatocyte cell line (HepG2) [46]. Results from another study demonstrated that kaempferol (50 μ M) inhibited the activity of glucose-6-phosphatase (G6Pase) [47], one of the key glucogenic enzymes responsible for committing the final step of gluconeogenesis in the liver [48]. However, it is unknown whether kaempferol can regulate hepatic glucose control in vivo. Therefore, **in the second study, we tested the hypothesis that dietary intake of kaempferol can ameliorate**

hyperglycemia by modulating the expression and/or activity of key enzymes involved in the regulation of glucose homeostasis in insulin-deficient diabetic mice.

One of the most common ways to induce obesity in rodent models is through the HF diet which leads to the development of metabolic disorders including hyperlipidemia, impaired insulin sensitivity, and glucose tolerance, as well as elevated blood insulin and glucose [49-52]. Indeed, obesity-induced hepatic IR alters hepatic glucose regulation in T2D, leading to reduced glycogen synthesis but excessive glucose production due to increased gluconeogenesis and glycogenolysis in the liver [53], which primarily contributes to the fasting hyperglycemia [54]. Therefore, targeting these pathways could be valuable in reversing IR and preventing the onset of T2D. **In the third study, we tested the hypothesis that oral administration of kaempferol can ameliorate hyperglycemia in diet-induced obese, insulin-resistant mice by modulating the expression and activity of key enzymes involved in the regulation of glucose homeostasis.**

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CHAPTER TWO

Literature Review

Abstract

Type 2 diabetes (T2D) is a progressive metabolic disease, and its prevalence is increasing globally. It is well established that insulin resistance (IR) and loss of functional β -cells play a major role in the etiology of the disease. Various proposed mechanisms may lead to the development of IR and β -cell dysfunction. Chronically elevated plasma levels of free fatty acids (FFAs) can alter insulin signaling pathway which contributes to the development of IR in the skeletal muscle and liver. Other factors such as genetic abnormalities, abnormal cytokine and adipokine levels, and endothelial dysfunction are presumed to play a role in inducing IR. Some of these factors can also impair β -cell function leading to β -cell death and/or reduced insulin secretion. Subjects with IR will progress to overt diabetes once β -cells fail to secrete adequate amount insulin to compensate for IR. Considering the gap between the current knowledge about prevention and treatment of T2D and its application in public health, it is necessary to search for naturally occurring anti-diabetic compounds that are abundant in certain types of foods. Flavonoids are a group of polyphenols that are recognized for their pharmacological activities. Experimental studies indicated that dietary intake of flavonoids might prevent or attenuate T2D. Consistent with these findings, flavonoids were shown to attenuate IR and T2D in clinical trials. Furthermore, epidemiological observations suggested a possible protective effect of flavonoids against T2D. Thus, flavonoids display a great potential in preventing and treating T2D. However, further studies are needed to test flavonoid effects at different dose and duration of treatment, and to elucidate the mechanisms of these actions. This chapter reviews the current knowledge on the role of IR and pancreatic β -cell in the pathogenesis of T2D. In addition, this

review discusses the classes of flavonoids, including their absorption and metabolism. Finally, it explores the existing evidence from experimental studies, epidemiological observations, and clinical trials on the anti-diabetic effect of flavonoids and the mechanisms of their actions.

1. Introduction

The prevalence of diabetes is rising rapidly. In 2012, 9.3 % of the US population had diabetes [1], and this number is expected to double in 2050 [2]. In addition, the economic cost of treating diabetes and its complications is an increasing burden [3]. In the U.S, the estimated cost of diagnosed diabetes increased from \$174 billion to \$245 billion between the years of 2007 and 2012 [4]. Type 2 diabetes (T2D) is a progressive metabolic disorder with a characteristic of hyperglycemia accompanied by abnormalities in carbohydrates [5], lipid [6], and protein metabolism [7]. The cascade of events that lead to the development of T2D has long been the subject of debate [8, 9]. However, it is well recognized that insulin resistance (IR), defects in insulin action, and impaired β -cell function are key features in T2D [10]. Subjects with IR will progress to overt diabetes if β -cells fail to secrete adequate amount of insulin to compensate for the defects in its action [11]. Therefore, β -cell failure plays a central role in the development T2D.

There is strong evidence that hyperglycemia plays a major role in the pathogenesis of diabetic complications that affects various organs in the body [12, 13]. Hyperglycemia increases glucose metabolism can lead to excessive reactive oxygen species (ROS) production that will impair cell function and survival [14]. Moreover, hyperglycemia, in turn, aggravates IR, thereby forming a vicious circle [15]. As hyperglycemia causes diabetic complications, improved

glucose control was shown to reduce the risk of diabetic complications such as microvascular complications [16-18]. The type and starting time of treatment upon diagnosis was associated with the risk of developing the complications [17, 19]. Diabetes treatments include inhibition of oligo- and disaccharides degradation, reduction of insulin demand, stimulation of endogenous insulin secretion, and enhancement of insulin action at target tissues [20, 21].

There is a considerable amount of knowledge about the means to prevent and treat T2D. However, this knowledge is not fully applied or practiced in public health [22]. Also, some diabetes therapies may have side effects [21] which necessitate the search for naturally occurring, cheaper, and safer compounds for preventing and treating diabetes. Flavonoids, polyphenolic compounds abundant in fruits, vegetables, and some medicinal herbs, are shown to exert many beneficial effects in various chronic diseases including diabetes [23]. This review will first summarize current knowledge of IR and pathogenesis of pancreatic β -cell in the context of the development of T2D. Further, it will briefly discuss various classes of flavonoids, including their absorption and metabolism. Primarily, this review will compile existing evidence from experimental studies, epidemiological observations, and clinical trials on the effects of flavonoids on T2D and the mechanisms of their actions.

2. Type 2 diabetes

Glucose homeostasis is tightly controlled by the harmonization of multiple pathways in the postprandial (fed) and post-absorptive (fasted) states [24]. After the ingestion of a meal, the majority of glucose is absorbed by the glucose transporters, SGLT1 and GLUT2, in the gut [25] to the portal vein. The rise in glucose levels in the portal vein induces glucose uptake followed

by phosphorylation of glucose to glucose-6-phosphate facilitated by glucokinase (GCK) in the liver [26], hence increasing glucose clearance and storage by the liver before it reaches the circulation [27]. The activation of GCK is accompanied by an inhibition of a key gluconeogenic enzyme, glucose-6-phosphatase (G6Pase) which is responsible for committing the last step in gluconeogenesis and glycogenolysis by dephosphorylating glucose to its free form [28]. Consequently, the rate of glucose production is suppressed [29]. Further, in response to increased glucose, insulin is secreted from pancreatic β -cells into the portal vein, which subsequently increases glycogen synthesis, reduces glycogenolysis while simultaneously inhibits gluconeogenesis [30, 31]. Moreover, muscle tissue also makes a major contribution to glucose uptake, accounting for about 30% of total glucose clearance as compared with about 39% by splanchnic tissues, primarily by the liver [32]. Glucose uptake is mediated by the combined influence of glucose and insulin [33]. Insulin stimulates the uptake of glucose. Intracellular glucose is phosphorylated by hexokinase in the muscle tissue [34], and then it is routed to different destinies; oxidation, glycogen synthesis, or glycolytic pathway [35, 36]. In the fasting state however, glycogen in the liver is hydrolyzed to produce glucose in addition to gluconeogenesis to maintain glucose homeostasis [37]. When glucose levels are low, glucagon levels increases and counteracts insulin action by increasing glucose production [38]. The pancreatic hormone glucagon rises the rate of glycogen breakdown and gluconeogenesis in the liver through modulating the transcription and activity of key glucogenic enzymes such as G6Pase and phosphoenolpyruvate carboxykinase (PEPCK) [39]. The liver is the primary supplier of glucose during fasting and responsible for about 90% of the overall produced glucose, whereas the kidneys produce the remainder percentage [40]. Although the muscle stores glycogen [41], it cannot be released to the circulation as free glucose due to the lack of the

enzyme G6Pase [42]. However, peripheral tissues including skeletal muscle and adipose tissues can supply the liver with glucogenic precursors such as amino acids and glycerol which are blocked in the presence of insulin [43].

Insulin is a peptide hormone synthesized and secreted by pancreatic β -cells into the circulation and acts on target tissues such as muscle, liver, and adipose tissues [9]. It binds to its plasma membrane receptor, a tyrosine kinase receptor, which then triggers a cascade of events that eventually affects many processes in the short and long term including glucose and lipid homeostasis [44]. However, IR in peripheral tissues such as skeletal muscle reduces glucose uptake, utilization, and storage [45]. Moreover, IR in the liver can result in excessive hepatic glucose production through stimulating gluconeogenesis and glycogenolysis, which make a significant contribution to fasting and postprandial hyperglycemia, the hallmarks in T2D [46-48]. Likewise, IR may impair the function of kidneys [49], thereby contributing to developing hyperglycemia in T2D, as kidney also plays a significant role in regulating glucose homeostasis that is usually not taken into consideration [50]. In normal subjects, kidneys act similarly to the liver in maintaining glucose homeostasis through glucose uptake and production in the fed [51] and fasting state [52]. There is a reciprocal relationship between liver and kidneys to maintain glucose homeostasis referred to as hepatorenal glucose reciprocity [53, 54]. In animal models of T2D, glucose transporters in kidneys are upregulated [55], which increases glucose reabsorption [56]. Similar findings were reported in T2D patients suggesting that the increased activity of glucose transporters in kidneys might contribute to hyperglycemia in T2D [57].

2.1 IR and T2D

The underlying mechanisms which lead to the development of IR are still an active area for investigation. Many genetic and environmental factors are involved in the development of IR [58]. One of the biggest difficulties investigating IR is the accompanying metabolic abnormalities referred to as IR syndrome (IRS), or more commonly metabolic syndrome (MetS) [59], which also increase the risk of developing T2D [60]. Elevated plasma free fatty acids (FFAs) associated with obesity [61] or independent of obesity, i.e. consumption of a large amount of dietary fat, may impair insulin signaling pathway leading to IR in the muscle and liver [62, 63]. Other factors considered having a role in inducing IR are genetic abnormalities, abnormal cytokine and adipokine levels, and endothelial dysfunction [61], which will be discussed in more details in this review.

2.1.1 IR in Muscle and development of T2D

There is strong evidence suggesting that IR in skeletal muscle is a primary risk factor for T2D [64, 65]. For glucose uptake, storage and utilization in the muscle, insulin activation of the insulin receptor substrate (IRS)-1/ phosphatidylinositol (PI)-3 kinase/ kinase B (or Akt) pathway is required [66]. However, this pathway was reported to be impaired in genetically predisposed subjects to developing T2D [67], and in T2D subjects [68]. It is proposed that due to this defect, IR develops primarily in the muscle leading to a decrease in glucose uptake and utilization, mostly glycogen synthesis [69].

Obesity is a major risk factor for developing T2D particularly once associated with IR [70]. In obesity, FFAs are constantly elevated [61], which in muscle, can directly inhibit insulin activation of the IRS-1/ PI-3 kinase/ Akt pathway leading to reduced glucose uptake and

phosphorylation, and decreased glycogen synthase activity in the muscle [62, 63]. One of the pathways connected to this alteration in insulin signaling is the diacylglycerol (DAG)/protein kinase C (PKC) pathway. Elevation of FFAs was associated with an increase in DAG level which in turn activates PKC- θ , - β 2 and - δ [71]. The activation of these isoforms phosphorylates IRS on Ser³⁰⁷ and then reduces insulin-stimulated tyrosine phosphorylation, thus inhibiting insulin signaling [72]. The excess deposit of intramyocellular lipid (IMCL) in the muscle, which is associated with IR in lean and obese subjects [73], may play a role in inducing IR by activating the DAG/PKC pathway similar to FFAs. However, IMCL activates another PKC isoform, PKC- ϵ which induces IR as well [74, 75]. Further, the increase in the activity of these PKC isoforms was also observed in animal models of obesity and T2D [76, 77].

Mitochondria are organelles that have major roles in cellular homeostasis through the generation of energy via oxidative phosphorylation of nutrients such as glucose and fatty acids and regulation of cell death [78]. Dysfunctional mitochondria has been implicated in the development of IR. Indeed, defects in glucose uptake in the muscle of T2D subjects was associated with decreased glucose oxidation [79] and impaired fatty acid metabolism [80], indicating mitochondrial dysfunction. The decrease in glucose uptake and utilization observed in the muscles of IR subjects may be the cause or the result of the mitochondrial dysfunction. For instance, impaired mitochondrial activity in muscles of IR subjects may lead to the increased IMCL deposition in the muscles which subsequently result in the development of IR [81]. Moreover, downregulation of genes encoding key enzymes for mitochondrial oxidative phosphorylation in the muscle can also be linked to IR and T2D [82]. However, the cause and effect relationship between IR and mitochondrial function is still elusive. For example, IR increases ROS production leading to impaired mitochondrial function [83]. For more information

about the role of dysfunctional mitochondria in developing IR and T2D, please refer to the comprehensive review by Szendroedi et al. [84].

The endothelium is essential for regulating vascular tone, and endothelial dysfunction impairs the release of nitric oxide (NO), thus affecting vascular homeostasis [85]. In particular, it has been found that dysfunction of peripheral vascular endothelium plays a role in the pathogenesis of IR [86]. This vascular dysfunction results in reduced expansion of the capillary network in the major target tissues of insulin such as the skeletal muscle, thereby reducing blood flow and supply of insulin to these tissues, which then subsequently impairs glucose and lipid metabolism [87].

2.1.2 IR in liver and development of T2D

The liver has a vital role in maintaining glucose homeostasis in both the fed and fasting states with a major contribution for the latter [24]. The increased hepatic glucose production is considered one of the early pathological changes in T2D subjects [46, 88, 89]. Evidence suggesting that such hepatic metabolic abnormality in T2D are caused primarily by IR [90]. Insulin is involved in the direct and indirect suppression of hepatic glucose production, which is impaired due to hepatic IR. In healthy subjects, insulin can suppress the flux of the glucogenic precursors from peripheral tissues such as nonesterified fatty acids to the liver by promoting lipogenesis and inhibiting lipolysis in the fat tissue [91]. Also, insulin inhibits glucagon production and subsequently reduces the expression and activity of the glucogenic enzymes such as PEPCCK and G6Pase [92]. However, in IR these indirect pathways are not blocked. Furthermore, insulin is unable to regulate the gene expression and enzymes' function that control

glucose production [93] such as PEPCK [94], and G6Pase [95]. Therefore, leading to excessive hepatic glucose production through gluconeogenesis and glycogenolysis, which primarily lead to fasting hyperglycemia [46-48]. Increased hepatic gluconeogenesis, in particular, is considered one of the early pathological changes in newly diagnosed T2D subjects [46]. The activation of Akt by insulin contributes to the control of hepatic glucose metabolism [96], and the reduction in hepatic glucose output by the stimulation of glycogen synthesis [97, 98], and possibly by downregulating PEPCK and G6Pase gene expression [99]. In T2D, a report demonstrated that the gene expression of PEPCK and G6Pase was not changed nor was associated with fasting hyperglycemia [100]. However, it was demonstrated that morbidly obese patients with T2D had an increase in hepatic glucose production rate which was associated with IR and an increase in liver G6Pase activity [101], suggesting that the increase in gluconeogenesis in IR and diabetic subjects might be controlled by these enzymes' activities rather than their expression.

The forkhead box O (FoxO) family of transcription factors plays important roles in a variety of physiological and pathological processes. Altered FOXO expression has been found to be associated with several metabolic diseases including diabetes. FoxO1 plays a role in mediating hormone-induced hepatic gluconeogenesis [102]. It promotes hepatic gluconeogenesis by transcriptionally activating gene expression of PEPCK and G6Pase [103]. Consistently, liver-specific FoxO1 knockout mice displayed fasting hypoglycemia associated with reduced expression of gluconeogenic genes [104]. In addition, FoxO3 and FoxO4, two other members of the FoxO family, were demonstrated to further enhance FoxO1-induced hepatic glucose production in mice [104], suggesting that these FoxO transcription factors contribute to the control of hepatic glucose production. Further, the action of FoxO1 in the liver is suppressed by the insulin-mediated pathways but is augmented by peroxisome proliferative activated receptor-

gamma co-activator 1alpha (PGC-1 α), which serves as a transcriptional co-activator for FoxO1, thereby augmenting the expression of gluconeogenic genes. [105]. Interestingly, PGC-1 α expression was upregulated in the liver of T2D subjects [106], which might partially explain the link between PGC-1 α , FoxO1 and glucose production in diabetes. Recently, another FoxO family member, FoxO6 was found to play a similar role as FoxO1 in regulating gluconeogenesis, as increased FoxO6 activity in the liver promoted gluconeogenesis and increased fasting blood glucose levels, whereas FoxO6 deletion in the liver reduced gluconeogenesis, resulting in fasting hypoglycemia. However, unlike FoxO1, the transcriptional activity of which is largely inhibited by insulin activation of the Akt-mediated pathway, which leads to their nuclear exclusion and degradation in hepatocytes. Thus, insulin signaling disables the transcriptional activity of FoxO6 without affecting its subcellular distribution [107].

It is suggested that IR in skeletal muscle can induce the development of IR in liver. In muscle tissues with IR, the failure of insulin to activate glycogen synthesis adequately diverts the energy substrates to de novo lipogenesis in the liver [108], which can lead to the accumulation of fat in hepatocytes [109]. Moreover, excess intrahepatocellular lipid (IHCL) accumulation and elevated FFAs may cause a wide spectrum of liver dysfunction, including IR. On the other hand, in some cases, hepatic IR may precede the development of IR in the muscle. For instance, high fat (HF) overfeeding induced hepatic IR in healthy subjects which were characterized by elevated fasting blood glucose levels and insulin secretion even before the development of IR in the muscle [110]. Consistently, short-term HF diet feeding in rats resulted in hepatic IR and hepatic steatosis, which was independent of IR in skeletal muscle. However, it was associated with activation of PKC- ϵ , attenuated insulin-stimulated signaling pathways, increased gluconeogenesis, and decreased insulin-dependent activation of glycogen synthase [111]. The

activation of PKC- ϵ by FFAs and IHCL in the liver was consistent with the activation of this isoform in the muscle. In this regard, DAG may play a role in the activation of PKC which then inhibits insulin activation of IRS and subsequently its initiated signaling, thereby leading to the development of hepatic IR and hyperglycemia [108, 112]. Furthermore, the accumulation of lipids in the liver and the promotion of fatty acid oxidation may increase ROS production which might impair the mitochondrial functions and induce abnormalities in liver functions [113].

2.1.3 Relationship between obesity, inflammation, and IR and development of T2D

Obesity, a condition of fat accumulation in the body and defined as having a BMI ≥ 30 , is a worldwide epidemic that is still increasing globally [114]. Obesity is strongly associated with the development of IR, dyslipidemia, and T2D [115]. Obesity can also induce endothelial dysfunction [116]. Abdominal obesity, in particular, is associated strongly with many chronic diseases where visceral fat is responsible for the abnormal production of many adipokines including tumor necrosis factor-alpha (TNF- α), interleukin 6 (IL-6), adiponectin, and leptin [117]. Indeed, obesity associated with IR is partly a chronic inflammatory disease. Moreover, studies have suggested a direct connection between obesity and systematic inflammation due to the upregulation of key genes of inflammation [118], and the increased secretion of inflammatory markers from white adipose tissue (WAT) [119]. Also, it was observed that obesity increases macrophage infiltration into WAT, which further increases secretion of inflammatory adipokines into circulation, leading to low-grade systematic inflammation and IR [120].

TNF- α secreted from adipose tissue [121] and from the infiltrated macrophages in adipose tissue [122] may play a role in IR accompanying obesity. It is documented that TNF- α gene expression and protein levels are elevated in obese and diabetic rodent models [123]. In addition, exposing adipocytes to TNF- α impaired insulin-stimulated glucose uptake via reducing insulin activation of IRS-1 [124], whereas neutralization of TNF- α improved insulin-mediated peripheral glucose uptake significantly [123] and its gene deletion in rodent models of obesity protected them from IR [125]. One of the proposed mechanism by which TNF- α induces IR is the activation of the c-Jun N-terminal kinase (JNK), and I kappa beta kinase (I κ K) by TNF- α , which subsequently phosphorylates IRS-1 on Ser³⁰⁷, thereby interfering with insulin action leading to IR [126]. In addition, TNF- α inhibits the activity of AMP-activated protein kinase (AMPK), which is consider a master regulator of energy homeostasis that is involved in regulating several energy metabolic pathways [127]. Indeed, it was demonstrated that the inhibition of AMPK in the muscle by TNF- α lead to the development of IR [128].

IL-6 is another cytokine that is secreted from adipose tissue and may be associated with IR. IL-6 plasma levels were elevated in obesity [129]. In T2D subjects, IL-6 was independently associated with IR and hyperglycemia [130]. Induction of the hepatic production of the inflammatory marker C-reactive protein (CRP) by IL-6 [131], suggests its role in IR [132]. However, it is still unclear by what means would IL-6 specifically affect glucose uptake and its metabolism. Findings in healthy mice and humans indicated that IL-6 may enhance fatty acid oxidation and insulin-stimulated glucose uptake [133, 134]. In T2D subjects, however, insulin-stimulated glucose uptake was not affected by administration of IL-6 [135].

Adiponectin is a plasma protein exclusively expressed and secreted from mature fat tissue [136]. It has been established that adiponectin is an important insulin-sensitizer. Unlike other

adipokines, adiponectin is inversely associated with markers of IR and inflammation [137]. Low levels of adiponectin found in obesity were associated with inflammation, whereas a loss in weight increased adiponectin [138]. Furthermore, adiponectin administration reversed IR in rodent models of obesity [139] [140]. Adiponectin binds to its receptors AdipoR1 and AdipoR2, which are expressed in the liver and skeletal muscle [141] and largely mediate its elicited various biological events, including activation of peroxisome proliferator-activated receptor (PPAR)- α [142] and AMPK [143]. Adiponectin may enhance insulin sensitivity via activating the AdipoR1/LKB1/AMPK pathway which suppresses the expression of sterol regulatory element-binding protein (SREBP)1c [144]. (SREBP)1c modulate fat metabolism by upregulating key enzymes of hepatic fatty acid synthesis pathway. Together, the suppression of SREBP1c and activation of AMPK improves fatty acid metabolism and prevent its accumulation in the liver thereby enhance insulin sensitivity [145]. Adiponectin may also have functions independent of its receptors. It is demonstrated that adiponectin enhanced insulin sensitivity in mice by inducing the production of IL-6 from macrophages which further upregulated hepatic IRS-2 expression independent of its known receptors [146]. Moreover, in a very recent study, it is reported that in addition to adiponectin effect on peripheral tissues, it could regulate glucose control through the neuroendocrine system as well. The intracerebroventricularly administration of adiponectin which target the central nervous system, improved glucose tolerance and reduced IR in rodent models of obesity [140].

The role of resistin, another adipocytokine, in IR is not clear. While some studies show that resistin is a risk factor for inflammation and IR independent of obesity [147], others found that resistin levels were not closely associated with markers of IR or obesity [148]. However,

resistin was found to be positively associated with markers of inflammation [137]. Also, it is strongly associated with obesity [149].

2.2 Impaired insulin secretion and development of T2D

It is well known that T2D is characterized by impaired insulin secretion [150]. Both impaired β -cell function and reduced mass contribute to insulin secretion abnormalities in T2D [151]. There seem common mechanisms that regulate both β -cell insulin secretory function and mass [152], suggesting that a combination of both β -cell dysfunction and loss of mass is the precipitating factor of the impaired insulin secretion in T2D [153].

Genetic [154] and environmental factors, such as diet and obesity, are well known for their effects on insulin secretion [155]. Obesity can increase the demands of insulin secretion [156]. As previously stated, obesity is associated with elevated FFA and adipokines levels, which could lead to β -cell apoptosis [157].

In T2D, there is a progressive reduction in both β -cell mass [158] and its insulin secretory function [159]. This progression of β -cell impairment may be at least partially due to increased production of ROS, which results from the abnormal antioxidant status in T2D [160, 161]. The increased ROS production in β -cells could be generated from excess amounts of saturated fatty acids (lipotoxicity) and glucose (glucotoxicity) that gradually cause β -cell apoptosis and impairs its function, thereby contributing to the pathogenesis of T2D [162-165].

One of the proposed scenarios of the leading cause of T2D is that β -cell dysfunction is the earliest and main genetic factor predisposing to the development of T2D [166]. In this scenario, it is suggested that IR is developed secondary to the defects in insulin secretion [167].

While the role of β -cell dysfunction is important in the development of T2D, it does not lessen the importance of IR in the pathogenesis of T2D. In IR, the adequacy of the compensatory insulin secretion depends on the genetic background [168]. Hence, it is of great importance taking both IR and insulin secretion into consideration when evaluating people at risk of developing T2D [169].

3. Flavonoids

3.1 Discovery and classifications

Flavonoids are largely present in the plant kingdom [170]. They are synthesized in plants as secondary metabolites from phenylalanine [171]. Many flavonoids have antioxidant capabilities [172] that protect plant membrane from desiccation, oxidation [173], and UV damage [174]. Flavonoids are important for the proper development of plants [175]. They can improve plant growth [176], and support plant defense system against microbial invasion [177].

Plants and food products containing flavonoids have been used to treat various human diseases since ancient times [178]. However, flavonoids were not discovered and characterized until the twentieth century. In the 1930s, Rusznyak and Szent-Gyorgyi extracted a substance containing a mixture of flavonoids from Hungarian peppers that had an action on vascular permeability and named it vitamin P [179]. The advances of flavonoids research in the 1970s led to the discovery of many other flavonoids, clearing the path for characterization of their structures and biological activities [180]. Although flavonoids were classified as semi-essential food components [181] and suggested as a new class of drugs due to their potential in treating many human diseases [170], the extensive research on their effects on disease prevention was not

started until the middle of the 1990s [182]. Recently, flavonoids are often referred to as nutraceuticals [183], a hybrid term that pointed to a product combined nutrients and pharmaceuticals [184] and defined as “any non-toxic food extract supplement that has scientifically proven health benefits for both the treatment and prevention of disease” [185].

Over 9,000 flavonoid compounds have been identified from plant sources [186] sharing the basic chemical structure which is a common three-ring structure (A-, C- and B-rings) with 15 carbon atoms (C₆–C₃–C₆) (Figure 1). The substitution of a functional group of the heterocycle ring (C-ring) with a methyl, hydroxyl, glycan, acetyl or other groups, along with the C-ring oxidation state determine various subclasses of flavonoids [187]. Flavonoids in each subclass are further structurally diversified due to different patterns of hydroxylation of the phenolic rings.

Flavonoids are divided into two main groups based on the structure; 2-phenylchromans (flavonoids) and 3-phenylchromans (isoflavonoids). The 2-phenylchromans group includes the subclasses of flavanones, flavones, flavonols, flavan-3-ols, and anthocyanidins; whereas the 3-phenylchromans includes the subclasses of isoflavones, isoflavans, and pterocarpan [188].

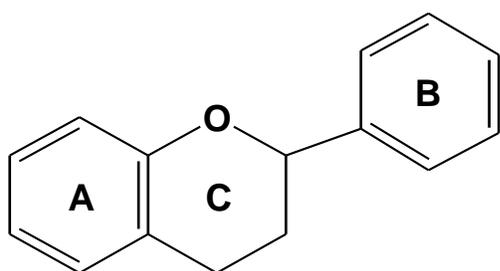


Figure 1 General structure of flavonoids.

The content of flavonoids in plants varies depending on many factors such as plant species, organ, stage of development, and environmental conditions [189]. In addition, food preparation and processing methods can significantly affect flavonoid contents in foods [190, 191]. Available food databases, including USDA's, provide information about food content of some flavonoids from six subclasses of flavonoids only and with isoflavonoids separated from other subclasses [192]. According to USDA database for the flavonoid content of selected foods (Table 1), the flavanones hesperetin, naringenin, and eriodictyol are primarily found in citrus fruits and their juices. Flavones luteolin and apigenin are predominant in aromatic herbs, parsley, celery, and peppers. Flavonols quercetin, kaempferol, myricetin, and isorhamnetin are found in many fruits and vegetables like apples, cranberries, onions, beans, and fennel. The flavan-3-ols (+)-catechin, (-)-epicatechin, (-)-epigallocatechin, theaflavin and their gallate esters are found in large amounts in tea, wine, and cocoa. Anthocyanidins cyanidin, delphinidin, malvidin, pelargonidin, peonidin, and petunidin are present in different varieties of berries, grapes, nuts and some vegetables. The USDA database for the isoflavone content of selected foods shows that the isoflavones daidzein, genistein, and glycitein are found in considerable quantities primarily in soybeans and soy products [193, 194].

Table 1 Major subclasses of flavonoids with examples and some of their major dietary resources.

Flavonoid subclasses	Examples	Dietary resources
Flavanones	Hesperetin, Naringenin, Eriodictyol	Spice (dried oregano), grapefruit, lemon, orange, grapefruit juice, lemon juice, orange juice.
Flavones	Luteolin, Apigenin	Spice (dried oregano, celery seed, dried parsley, thyme), celery, parsley, peppers.
Flavonols	Quercetin, Kaempferol, Myricetin, Isorhamnetin	Capers, spice (saffron), apples, cranberries, arugula, asparagus, broccoli, cabbage, chives, coriander, endive, fennel, ginger, mustard greens, okra, onions, peppers, radish (raw, seeds, leaves), beans, buckwheat.
Flavan-3-ols	(+)-Catechin, (+)-Gallocatechin, (-)-Epicatechin, (-)-Epigallocatechin, (-)-Epicatechin 3-gallate, (-)-Epigallocatechin 3-gallate, Theaflavin, Theaflavin 3-gallate, Theaflavin 3'-gallate, Theaflavin 3,3' digallate, Thearubigins	Apples, broad beans, pecans, pistachio, wine, cocoa, tea (green, black), soybeans.
Anthocyanidins	Cyanidin, Delphinidin, Malvidin, Pelargonidin, Peonidin, Petunidin	Berries (bilberry, blackberries, black, choke, crane, elderberries, raspberries), currants, grapes, plum, red cabbage, eggplant, pecans, pistachio, wine, black beans.
Isoflavones	Daidzein, Genistein, Glycitein	Red clover, soybeans and soybean products (milk, flour, yogurt and others).

3.2 Dietary intake

Several studies attempted to estimate the dietary intake of flavonoids in the U.S. [195-197] [198]. It was estimated in one study that total dietary intake of flavonoids by U.S adults was 190 mg/d [195] while this number was 345 mg/d in another study [198]. These two studies analyzed 24-h dietary recall data from the National Health and Nutrition Examination Survey (NHANES) and used the USDA databases to assess the intake of flavonoids. However the data used in these studies were from two different time periods, and the databases were from different releases and updates which may explain the difference in the estimated daily intake. Recently, USDA expanded flavonoids databases to include 2,900 foods with analyzed contents of the five subclasses of flavonoids and the major isoflavones instead of the roughly 500 foods in the original one [199]. This expansion is expected to provide researchers with a better tool to estimate dietary intake of flavonoids and study their effects on populations following exposure to these phytochemicals. Thus re-analysis of the data from NHANES using the most recent USDA databases is recommended to achieve a more accurate assessment of the intake. Moreover, the highest intake of all flavonoids documented in these studies was from the subclass flavan-3-ols corresponding to a higher intake of tea [195, 198].

3.3 Absorption, metabolism, and bioavailability

How well flavonoids are absorbed and to what degree they are metabolized in the human body are of importance to understand their potential efficacy for the treatment and prevention of diseases. Starting in the mouth, flavonoids are first released from the plant matrix and some flavonoid glycosides (with sugar moiety) are hydrolyzed to aglycones (without sugar moiety) by

saliva [200]. While some flavonoids are absorbed in the stomach [201], most of them undergo enzymatic hydrolysis and further metabolism in the small intestine [202, 203]. The hydrolysis involves the deglycosylation of flavonoids, removal of glycosides naturally-bound to flavonoids by beta-glucosidases [204]. The hydrolyzed flavonoids are further metabolized by the conjugation with glucuronic acid in the small intestine. The conjugation depends on the flavonoid structure with less predisposition to glucuronidation to flavonoids with a hydroxyl group on B-ring [205], but more extensive metabolism and/or conjugation to flavan-3-ols [206]. The conjugation pattern is also affected by nutritional status. For example, administration of isoflavone in the fasting state results in more conjugation with sulfates and less glucuronidation than in the non-fasting state in humans [201]. Once absorbed, flavonoids from the small intestine reach the liver, they can be further conjugated with sulfate and/or methyl groups or excreted back with bile components from the liver [207-209].

The majority of the ingested flavonoids may not go under hydrolysis or conjugation in the small intestine [210], and they are neither absorbed nor excreted from the bile [209]. Instead, these flavonoids pass to the colon where they are degraded by colonic flora into smaller molecules and phenolic acids that can then be absorbed [211, 212].

The absorption of flavonoids depends on many factors such as the configuration of their structures and glycosylation [213]. In foods, flavonoids mostly exist in the glycosylated forms [214]. Some flavonoids can be absorbed more readily when attached to glycosides in the small intestine [215], while other flavonoids are absorbed more efficiently as aglycones [216, 217]. In addition, the type of sugar moiety (galactose, rhamnose, arabinopyranose) of the glycoside [218], and the plant matrix can affect the absorption of flavonoids as well [219]. For instance, conjugation of the flavonol quercetin with glucose has a high absorption rate as compared with

that of quercetin rutinoside [220], which is irrespective of the glucose position on quercetin molecule [221].

In the past few decades research has been primarily focused on exploring the potential biological and pharmacological activities of flavonoids such as the antioxidant [222], anti-inflammatory [223], and anticancer activities [224]. In this context, many factors that may affect their bioavailability should be taken into consideration to validate their health-promoting effects. Flavonoid bioavailability is influenced by many factors such as their absorption rate, metabolism, and conjugation. As discussed previously, the absorption of flavonoids may vary depending on their structure and food sources which further determine their glycosylation type and availability from the plant matrix. Structure and molecular weight of flavonoids may affect their bioavailability [209, 225]. In general, the bioavailability of flavonoids is low, and the majority of flavonoids are detected in the conjugated form in the plasma [226, 227]. Among various classes of flavonoids, isoflavones have the highest bioavailability, whereas anthocyanins have the lowest bioavailability [228]. Ingestion of 50 mg anthocyanins aglycone equivalent only resulted in a maximal plasma concentration of about 30 nmol/L, while the plasma concentration could reach 3 μ mol/L with intake of the same amount of isoflavones [229]. Flavonoids can be detected in plasma after 30 minutes and last several hours after ingestion [230, 231], but the half-life for conjugated flavonoids could reach 28 hours [232]. Other factors that may affect the bioavailability of flavonoids are variation in absorption and metabolism of flavonoids between individuals [233], flavonoid dose [234], and duration of consumption [235].

While conjugation of flavonoids is reported to alter their structure and changes or even reduces their biological activities [222, 236], the microbial metabolism of flavonoids in the gut can generate a variety of metabolites, with different biological activities as well [237].

Flavonoids are exposed to drastic changes while being metabolized in the body, which affects their bioavailability and biological activity. Results from in vitro studies that explore biological roles of flavonoids using unconjugated forms of flavonoids or their metabolites that may not be detected in the plasma should be analyzed with caution. More focus should be given to investigating flavonoid metabolites rather than flavonoids themselves which are less detected in the plasma [233].

3.4 Potential adverse effects and toxicity

Our daily diet contains considerable amounts of flavonoids [181, 196]. Most of the flavonoids are considered safe. However, studies about flavonoid toxicities show controversial results with some studies raising safety concerns for some flavonoids. Galati and others reviewed the potential toxicity of flavonoids [238]. Although one of the proposed activities of many flavonoids is their antioxidant activities, in the presence of copper they can become pro-oxidants [222]. Another proposed health effect of some flavonoids is flavonoid ability to reduce tumor cell proliferation [239]. A study found that long-term intake of quercetin may cause mutagenicity [240]. Also, flavonoids may induce DNA damage that is further enhanced in the presence of copper, suggesting that mutagenesis caused by some flavonoids may be due to their pro-oxidant activity [241, 242]. The use of supplements, including non-nutritive supplements, has been on the rise [243]. However, the possible benefits from flavonoid supplementation have not been justified by results from scientific research [244]. Therefore the use of flavonoids as dietary supplements in large quantities should not be encouraged until more research is done to elucidate their biological effects [245]. In addition, more research should be conducted to

address the potential adverse effects of flavonoids, which may depend on their structure, dose, and duration of treatment [222, 246].

4. Flavonoids and T2D

As discussed above, T2D is a result of chronic IR and loss of β -cell mass and function [247, 248]. Thus, the search for agents that may promote insulin sensitivity and β -cell survival may provide a more effective strategy to prevent the onset of diabetes [249-252].

4.1 Flavonoids and the prevention and treatment of T2D

The use of animal models and various cell lines contributed to our understanding of IR, obesity, and T2D in humans [253-256]. For example, diet-induced T2D in C57BL/6J mice resembles metabolic phenotypes characteristics of human with T2D [257]. Interestingly, high fructose or sucrose diets can induce different degrees of IR, hypertriglyceridemia, and obesity that are similar to that in humans when there is an increased consumption of refined carbohydrates [258, 259]. Fructose-fed animals can further develop MetS [260]. A high-fat diet, particularly saturated fat, induces IR in rodents as well [261]. Various animal models have been also used to study the effects of flavonoids on T2D.

4.1.1 Antioxidant activity of flavonoids and T2D

One of the suggested triggers causing β -cell dysfunction and IR that ultimately lead to T2D is a high level of ROS production [262], which may be due to the activation of many stress signaling

pathways [263]. Flavonoids have been shown to reduce ROS levels due to their well-known antioxidant activities [264]. Results from studies using cell cultures and animal models show that flavonoids have a high scavenging activity towards ROS [265, 266]. In addition, flavonoids can protect and restore the antioxidant defense enzymes such as superoxide dismutase, catalase, and glutathione peroxidase [267, 268], and inhibit ROS-producing enzymes such as xanthine oxidase [269]. Subsequently, flavonoids can inhibit several ROS-stimulated biological events such as inhibiting oxidized LDL (oxLDL)-induced cell apoptosis, NF- κ B-mediated transcriptional activity and subsequent inflammation [270]. However, in humans, the plasma concentrations of flavonoids are typically far less than those used in vitro for achieving strong scavenging capabilities. However, there is possibility that some flavonoids may exert antioxidant activities in vivo through modulating protein kinases mediating ROS-induced signaling pathways [271]. It is also proposed that flavonoids themselves may only have minimal contribution to the antioxidant capacity in humans where the greatest contribution is from other components in flavonoid-rich foods [272]. Another possibility that flavonoids might exert antioxidant effects and ROS scavenging capabilities in the digestive tract [273].

4.1.2 Effects of flavonoids on postprandial blood glucose

The first point of carbohydrate metabolism is their breakdown into monosaccharides to make them available for absorption via their transporters in the small intestine. Carbohydrates are digested by enzymes such as α -amylase and α -glucosidase, and after that by other enzymes such as maltase, sucrase, and lactase [274]. The inhibition of these enzymes reduces glucose absorption, thereby lowering postprandial blood glucose levels. Indeed, those enzymes have been pharmacologically targeted for reducing postprandial hyperglycemia in T2D [275, 276].

4.1.2.1 In vitro and in vivo studies

Flavonoid-rich extracts, in particular, proanthocyanidins, from raspberry and rowanberry, inhibited α -amylase activity and acted synergistically with the drug acarbose to inhibit α -amylase activity in vitro [277]. Flavonoids such as luteolin and quercetin [278, 279], and flavonoid-rich extracts from Helichrysum and grapefruit suppressed the enzymatic activity of α -amylase and α -glycosidase [280]. While the inhibitory action of flavonoids on α -amylase activity was suggested to be structure-related [281], the flavones from bamboo leaf extract, specifically vitexin, orientin, isovitexin, and isoorientin, inhibited starch digestion by interacting with α -amylase enzyme and with starch molecule itself [282]. Moreover, some flavonoids have the ability to inhibit the activity of other digestive enzymes. For instance, rutin and kaempferol, two flavonols, reportedly inhibited maltase activity in vitro, whereas quercetin inhibited the activities of both maltase and sucrase in vitro and in vivo [283]. However, it is largely unclear how these flavonoids exert such actions.

In addition to inhibiting carbohydrate digestion, glucose absorption may be modulated by some flavonoids in the small intestine in vitro. Flavonoids such as quercetin [284], tiliroside [285], and tea flavonoids such as catechin, epicatechin gallate and epigallocatechin gallate (EGCG) [286, 287] reduced glucose absorption by competitive inhibition of the intestinal glucose transporter SGLT1. Other flavonoids such as anthocyanins mediated their action on glucose absorption by inhibiting SGLT1 and GLUT2 and their expression in human intestinal Caco-2 cells [288].

Findings from these *in vitro* studies as described above may have physiological relevance. Indeed, some flavonoids have been shown to modulate glycemic response after carbohydrate ingestion in a variety of animal models. Flavonoids such as quercetin and rutin reduced postprandial glucose levels in diabetic animals after ingestion of sucrose and/or glucose [283, 284, 289]. Also, tiliroside inhibited the elevation of blood glucose after an oral glucose load in IR animal model [285]. Consistently, the administration of a flavonoid-rich extract of serviceberry in diet-induced obese and hyperglycemic mice delayed carbohydrate absorption and subsequently ameliorated postprandial hyperglycemia [290]. Flavonoids are potential inhibitors of glucose/sugars digestion and absorption. While some studies demonstrated the specific biological action by which flavonoids exert this effect, the dose and long-term effects of flavonoids *in vitro* and *in vivo* are still largely unknown. Much effort should be directed to study the structure-based function to assist in predicting the efficacy of flavonoid in inhibiting enzymes involved in glucose digestion and absorption.

4.1.2.2 Clinical trials

Apple juice containing flavonoids such as phloridzin, catechin and epicatechin showed some ability to delay intestinal absorption of glucose in healthy subjects [291]. Additionally, flavonoids extract from sugarcane reduced the glycemic response to a high glycemic meal in healthy subjects [292]. Consumption of a variety of berries had different outcomes on the glycemic response in healthy and T2D subjects. Consumption of a berry meal (containing bilberries, blackcurrants, cranberries, and strawberries) sweetened with sucrose, delayed glucose appearance in blood of healthy subjects [293]. Similarly, in another study, sweetened blackcurrant juice fortified with crowberry powder improved postprandial glycemic control in

healthy subjects [294]. On the contrary, the addition of raspberries and blueberries to starch-based food was not able to reduce blood glucose in healthy subjects [295]. Also, the consumption of a sweetened cranberry juice did not improve the glycemic responses in healthy subjects, but some improvement was noted with the unsweetened cranberry juice [296]. The latter finding was consistent with the results from another study in which a cranberry product showed improvement in the glycemic response in T2D subjects when compared with cranberry products with higher content of sugar [297]. While these studies show promising results, more clinical studies are necessary to investigate flavonoid effect on carbohydrate digestion and absorption in diabetic subjects where the data are limited. Long- and short-term studies to test different doses of flavonoids on glycemic response of healthy and T2D subjects are also needed.

4.1.3 Effects of flavonoids on glucose disposal

Another approach to preventing IR, hyperglycemia, T2D and subsequent diabetic complications are through enhancing glucose uptake by peripheral tissues. Various isoforms of glucose transporters (GLUT) are primarily responsible for glucose flux into cells [298]. GLUT4, the most abundant glucose transporter in both skeletal muscle and adipose tissue, is primarily regulated by insulin [299]. Insulin stimulation of glucose entry into cells is executed via inducing the translocation of GLUT4 to the membrane of muscle and fat cells [300], which is promoted by a cascade of events including insulin signaling-triggered activation of PI3K/Akt pathway [301]. In liver cells, glucose can be transported into and out of liver cells by GLUT2 independent of insulin [302]. Additionally, activation of AMPK is considered one of the important insulin-independent targets for improving glucose uptake in both muscle and adipose tissue [303, 304].

4.1.3.1 In vitro and in vivo studies

Some flavonoids were shown to increase glucose uptake in different tissues by enhancing insulin-stimulated glucose uptake, mimicking insulin action, activating insulin-independent pathways, or regulating glucose transporters. The flavonoid eriodictyol increased glucose uptake in hepatocytes and adipocytes under high-glucose conditions via activating the PI3K/Akt pathway [305]. Procyanidins, which are polymers of the flavan-3-ols catechin and epicatechin, dose-dependently stimulated glucose uptake in L6E9 myotubes and 3T3-L1 adipocytes by activating Akt and ERK, another target of insulin signaling [306]. The long-term provision of procyanidin extracted from grape seed improved glucose control in diet-induced hyperinsulinemic rats, an effect that might be due to stimulation of glucose uptake by adipocytes [307]. Grape-seed procyanidin extract activated IRS/Akt, and MAPK in 3T3L1 adipocytes [308].

Another flavonoid 7-O-methylaromadendrin stimulated glucose uptake in adipocytes by increasing the gene expression and activity of the transcriptional factor PPAR γ 2, and improved high glucose-induced IR in human hepatocellular liver carcinoma (HepG2) through activation of the PI3K/Akt and AMPK dependent pathways [309]. In addition, kaempferol and quercetin also have been shown to activate PPAR γ and subsequently improved insulin-stimulated glucose uptake in 3T3-L1 adipocytes [310].

EGCG was demonstrated to enhance insulin-stimulated glucose uptake by increasing GLUT4 membrane translocation in L6 myotubes. In addition, it also inhibited dexamethasone-induced IR via activating AMPK and Akt [311]. In IR rats, treatment with green tea extract, which largely consists of epicatechin, epigallocatechin, and their gallates, increased the

expression of genes critical for glucose uptake and utilization such as Gsk3b, and Irs2 in the liver and Glut4 in the muscle [312]. Similarly, isoflavone genistein and its derivatives stimulated glucose uptake in L6 myotubes through activating AMPK and increasing the gene expression of GLUT4 and GLUT1 [313].

It is worth mentioning that at higher doses, which cannot be achieved by dietary intake of these compounds, some flavonoids may inhibit glucose uptake. For example, genistein inhibited insulin-stimulated glucose uptake in 3T3-L1 adipocytes and affected the function of GLUT4 [314]. Likewise, higher concentrations of silybin and dehydrosilybin inhibited glucose uptake by directly interacting with GLUT transporters [315]. However, there is no evidence that flavonoids at physiologically relevant concentrations inhibit insulin-stimulated glucose uptake. Prasad et al. found that kaempferitrin did not have any effect on glucose uptake or GLUT4 while it inhibited insulin-stimulated glucose uptake in 3T3-L1 adipocytes by inhibiting Akt activation and through a direct binding to GLUT4 [316]. However, Tzeng et al. found that kaempferitrin activated insulin signaling pathway, IRS1/PI3K/Akt, and increased GLUT4 translocation in 3T3-L1 adipocytes [317]. Although both studies used similar concentrations and treatment duration, it is unclear what caused the difference in results. However, data from an in vivo study demonstrated that kaempferitrin stimulated glucose uptake in muscles and lowered blood glucose in diabetic rats [318].

4.1.4 Effects of flavonoids on obesity, and inflammation

4.1.4.1 In vitro and in vivo studies

Some flavonoids have been shown to improve dyslipidemia, modulate adipokine secretion, and inhibit adipogenesis, which could thereby ameliorate IR and T2D. Studies showed that treatment with citrus flavonoids nobiletin and tangeretin increased adiponectin secretion and decreased the secretion of monocyte chemoattractant protein-1 (MCP-1), which regulates migration and infiltration of monocytes and macrophages, in 3T3-L1 adipocytes [319]. Naringin, found in grapefruit, ameliorated dyslipidemia, hepatic steatosis, and IR in T2D rats [320]. Treatment with cyanidin-3-glucoside increased serum adiponectin levels, attenuated dyslipidemia, IR, and reduced body weight gain in HF diet-fed rats [321].

HF-fed rats were treated with flavonoid extract of *Litsea coreana* to test their effect on IR. The results showed that *Litsea coreana* flavonoids reduced serum lipids such as TG, total cholesterol, and LDL-C, and improved insulin sensitivity in the insulin-resistant hyperlipidemia rats [322]. In another study, flavonoid extract of *Litsea coreana* improved insulin sensitivity and hyperlipidemia in HF diet-fed diabetic rats [323]. Similar results were found in obese and diabetic mice when treated with either silibinin or tiliroside [324, 325].

4.1.6 Effect of flavonoids on β -cells function

In IR, β -cells compensate for the defects in insulin action by releasing more insulin. T2D only develops when these cells are unable to secrete adequate amount for compensating for the decreased insulin sensitivity. The decrease in insulin secretion is largely due to insulin secretory dysfunction and significant loss of functional β -cells [326-330]. Indeed, individuals with T2D always manifest increased β -cell apoptosis and reduced β -cell mass [328, 329, 331]. There are several proposed mechanisms underlying the β -cell dysfunction including increased generation

of ROS, alterations in metabolic pathways, activation of endoplasmic reticulum stress, increases in intracellular calcium, among others [332].

4.1.6.1 In vitro and in vivo studies

Alloxan and streptozotocin (STZ) have been widely used to induce insulin-deficient diabetic animal models by selectively destroying β -cells [333]. Under this condition, flavonoids were shown to have positive effects in protecting and/or regenerating β -cells in vitro and in vivo. In STZ-induced diabetic rats, intraperitoneal (ip) injection of quercetin improved glucose tolerance and dyslipidemia [334]. This effect may be due to its protective effect on β -cell mass via a reduction in oxidative stress [335]. Similar results were observed with ip injection of naringenin 7-O- β -D-glucoside in STZ-induced diabetic rats [336]. Interestingly, treatment with epicatechin was shown to improve blood glucose levels by regeneration of functional pancreatic β -cells in alloxan-induced diabetic rats [337]. In addition, It is reported that epicatechin may also preserve β -cell mass and function through protection against oxidative stress [338]. Likewise, other flavonoids such as rutin and apigenin protected the islets against STZ-induced damage, probably due to their antioxidant activity [339].

The effects of soy isoflavones on diabetes were recently reviewed by Kwon et al. [340], which demonstrated that that dietary intake of isoflavones or soy products such as fermented soybean that enriched with isoflavones have beneficial effects on glucose homeostasis in animal models. The effects of soy flavonoids, especially the isoflavonoids genistein and daidzein, on pancreatic β -cell function, were extensively studied [341]. Dietary supplementation of genistein (250 mg/kg) improved hyperglycemia, glucose tolerance, and blood insulin levels in T2D mouse

model generated by HD-feeding and low doses of STZ injection [342]. The effect of genistein on β -cell may be mediated through enhancing proliferation at the gene level [343]. For more detailed information on the effects of on β -cell function, please refer to this recent review article [344].

4.2 Flavonoid intake and risk of T2D

Data from epidemiological studies shows that dietary intake of flavonoids may reduce the risk of T2D. A very recent meta-analysis consisting of 6 cohort studies indicated that total flavonoids intake was associated with a reduced risk of T2D [345]. However, in one observational study, no association was found between dietary intake of flavonoids and the risk of T2D in postmenopausal women [346]. However, the association between the intakes of flavonoids with the incident of T2D may be dependent of subclasses of flavonoids. However, an inverse relationship with intakes from flavanols and flavonols was observed [347]. These results are consistent with findings by Knekt et al. that a trend toward a reduction in the risk of T2D was associated with higher intakes of the flavonols quercetin and myricetin primarily due to higher intakes of apples and berries [348]. Data from several cohort studies demonstrated that tea, coffee, and their products which are rich in flavanols (Table 1), were associated with reduced risk of T2D [349-352]. Consistent with these results, a meta-analysis study concluded that consumption of 4 or more cups of different varieties of tea derived from *Camellia sinensis* per day may lower the risk of T2D [353]. Indeed, in a human trial, it was found that long-term effect of tea intake was shown to be associated with reduced levels of fasting blood glucose and lower risk of T2D in humans [354]. Similarly, higher intakes of anthocyanins or anthocyanin-rich plants like blueberries and grapes were also associated with a lower risk of T2D [355, 356].

While these studies show an association between dietary intake of flavonoids and the reduced incidence of T2D. It is noteworthy that most of these studies used flavonoid-containing foods which may consist of other components such as other classes of polyphenols and therefore possibly contributed to this association [357]. Moreover, food components and polyphenols rather than their constituents may have a synergistic effect on diseases [358-360]. Thus, it would be of importance to study the beneficial implication of the plant extract as well as its individual components.

4.3 Effects of flavonoids on T2D in clinical interventions

Results from clinical trials show different outcomes based on flavonoid subclasses. Some flavonoids may improve dyslipidemia and regulate glycemic control in diabetic subjects. Supplementation with flavonoids such as silymarin [361] and silybin-beta-cyclodextrin [362] improved glycemic and lipidemic profiles in T2D subjects. Similarly, cranberry juice consumption in addition to the dietary control for 3 months improved the glycemic control in T2D subjects [363], where consumption of chokeberry juice for 3 months improved both the glycemic and lipidemic profiles in T2D subjects [364].

Some flavonoids improved inflammatory biomarkers in obese and T2D subjects. Supplementation with grape seed extract improved markers of inflammation and glycemic control in obese T2D subjects [365]. Further, consumption of grape for 3 weeks lowered plasma levels of LDL-cholesterol and cholesterol in obese subjects [366]. Anthocyanin supplementation improved LDL- and HDL-cholesterol concentrations in dyslipidemic subjects [367] and reduced the inflammatory response in hypercholesterolemic subjects [368].

Epidemiological studies show that flavanols containing foods such as tea, coffee, and their products are associated with reduced risk of T2D [349-352]. However, clinical trials investigating tea catechins show controversial results. The consumption of green tea (456mg catechins for 2 months and 9 g of green tea for 1 month) did not exert any beneficial effect in T2D subjects [369, 370]. These outcomes were consistent with the results from other studies on green and black tea extracts effect (150 mg of green tea catechins and 75 mg of black tea theaflavin for 3 months) in T2D subjects [371] and green tea extract (500 mg tea catechins for 4 months) in obese subjects with T2D [372]. However, consumption of catechins (582.8 mg of catechins for 3 months) was found to reduce the body weight of obese subjects with T2D with some improvements in glucose control [373]. This finding was consistent with effects of the consumption of catechin-rich green tea (615 mg green tea catechins for 1 month) where it improved postprandial glucose in T2D subjects [374]. Collectively, It is still inconclusive that tea catechins may exert a significant beneficial in the treatment of T2D. It is unclear whether the individual catechins such as EGCG, rather than a mixture might be more effective in preventing or treating T2D in humans.

Different subclasses of flavonoids may have different effects depending on the metabolic state of subjects and duration of treatment. In a 3-month, randomized trial, a mixture of isoflavonoids composed of glycitein (58%), daidzein (36%) and genistein (6%) did not affect lipid profile or insulin sensitivity in postmenopausal women [375], whereas, supplementation with isoflavones (aglycone equivalent) in combination with flavan-3-ols (epicatechin) for one year was found to improve lipid profile and IR in postmenopausal women with T2D [376].

5. Summary

Type 2 diabetes (T2D) is a progressive metabolic disorder. T2D prevalence is increasing globally, and its cost of treatment is well recognized. Although the specific causes of T2D still need to be elucidated, there is a considerable body of evidence indicating that IR and loss of functional β -cell mass play a major role in the etiology of the disease. Various proposed mechanisms may lead to the development of IR and β -cell impairment. These mechanisms are mediated by many genetic and environmental factors. IR impairs glucose metabolism and storage in the muscle which might be due to elevated plasma FFA, accumulation of lipids in the muscle, endothelial dysfunction, and/or mitochondrial dysfunction. Moreover, muscle IR can contribute to the development of IR in the liver by diverting energy substrate to de novo lipogenesis in the liver leading to the accumulation of fat in the liver. This may alter insulin-signaling pathways and induce gluconeogenesis. In addition, the increased secretion of inflammatory markers in obesity may interfere with insulin signaling pathways that can contribute to IR. Many of these factors may also contribute to β -cell dysfunction and apoptosis leading to reduced insulin secretion. In the search of naturally occurring compounds to prevent and treat IR and T2D, flavonoids have drawn considerable attention for their potential anti-diabetic activities. Epidemiological studies indicated that higher intakes of flavonoids were associated with reduced risk of T2D. Experimental studies have shown that flavonoids may reduce postprandial glucose by inhibiting glucose digestion and transport in the small intestine. In addition, flavonoids were found to prevent or attenuate T2D by increasing glucose disposal in tissues, protecting and regenerating impaired β -cells, and/or enhancing pancreatic insulin secretion. Furthermore, flavonoids were shown to reduce IR, improve lipid metabolism, and modulate inflammation, which collectively or partially contribute to the beneficial effects of these compounds on T2D. Consistent with these findings, clinical trials have shown that

flavonoids may attenuate IR and T2D in humans. Further investigation is needed to establish the optimal anti-diabetic doses of flavonoid, and duration of treatment as well as to uncover the mechanisms of their actions.

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CHAPTER THREE

Small Molecule Kaempferol Promotes Insulin Sensitivity and Preserved Pancreatic β -Cell Mass in Middle-Aged Obese Diabetic Mice

Abstract

Insulin resistance and a progressive decline in functional β -cell mass are hallmarks of developing type 2 diabetes (T2D). Thus, searching for natural, low-cost compounds to target these two defects could be a promising strategy to prevent the pathogenesis of T2D. In the present study, we show that dietary intake of kaempferol (0.05% in the diet), a plant-derived flavonol, significantly ameliorated hyperglycemia, hyperinsulinemia, and circulating lipid profile, which were associated with the improved peripheral insulin sensitivity in middle-aged obese mice fed a high-fat (HF) diet. Kaempferol treatment reversed HF diet impaired glucose transport-4 (Glut4) and AMP-dependent protein kinase (AMPK) expression in both muscle and adipose tissues from obese mice. Further, kaempferol treatment increased lipolysis and prevented high fatty acid-impaired glucose uptake, glycogen synthesis, AMPK activity, and Glut4 expression in skeletal muscle cells. Using another mouse model of T2D generated by HF diet feeding and low doses of streptozotocin injection, we found that kaempferol treatment significantly improved hyperglycemia, glucose tolerance, and blood insulin levels in these middle-aged obese diabetic mice, which are associated with the improved functional islet β -cell mass. These results, along with our previous findings that kaempferol promoted viability and preserved the function of insulin-secreting cells and human islets chronically exposed to glucotoxicity or hyperlipidemic condition, demonstrate that kaempferol may be a naturally occurring anti-diabetic agent by improving peripheral insulin sensitivity and protecting against pancreatic β -cell dysfunction.

Keyword: kaempferol, diabetes, insulin resistance, blood glucose, islet, mice, high-fat diet

1. Introduction

Diabetes mellitus is a growing public health concern, presently affecting 25.8 million or 8.3% of the American population [1] and nearly 387 million people worldwide [2]. While the availability of novel drugs, techniques, and surgical intervention has improved the survival rate of individuals with diabetes, the prevalence of diabetes is still rising in Americans, with the number of people with diabetes projected to double by 2025 [3]. T2D is a result of chronic insulin resistance and loss of β -cell mass and function [4]. Both in experimental animals and people, obesity is a leading pathogenic factor for developing insulin resistance, which is always associated with the impairment in energy metabolism, causing increased intracellular fat content in skeletal muscle, liver, fat, as well as pancreatic islets. Constant insulin resistance will progress to T2D when β -cells are unable to secrete adequate amount of insulin to compensate for decreased insulin sensitivity, which is largely due to insulin secretory dysfunction and significant loss of functional β -cells [4-8]. Indeed, those individuals with T2D always manifest increased β -cell apoptosis and reduced β -cell mass [6, 7, 9]. As such, the search for novel agents that simultaneously promotes insulin sensitivity and β -cell survival may provide a more effective strategy to prevent the onset of diabetes [10].

Recently, naturally occurring polyphenolic compounds has been the focal point of medicinal research interest due to their pharmacological implications associated with human disease with considerable attention devoted to managing diabetes [11, 12]. Polyphenols exist naturally as secondary plant metabolites, and are the largest source of human dietary antioxidants, with a typical daily intake of roughly 1 g/day [13]. One of the most common polyphenolic subclasses is flavonols [14]. Kaempferol (3,5,7-trihydroxy-2-(4-hydroxyphenyl)-4H-1-benzopyran-4-one) is a flavonol, of low molecular weight (286.2 g/mol) that has been

identified in many plants used in traditional medicine, including *Equisetum spp*, *Sophora japonica*, and *Ginkgo biloba*, and edible plants, including beans, broccoli, cabbage, gooseberries, grapes, kale, strawberries, tea, and tomatoes [15, 16]. Dietary intake of kaempferol containing foods has been epidemiologically associated with a reduced risk of certain cancers and cardiovascular diseases [15]. It has been reported that kaempferol has anti-oxidative [17], anti-microbial [15], anti-inflammatory [18], lipolytic [19], and anti-cancer [20, 21] effects. However, to date, the studies regarding its effect on the pathogenesis of diabetes are very limited.

We recently demonstrated that kaempferol treatment prevented apoptosis and improved insulin biosynthesis and secretion in β -cells and human islets exposed to chronic hyperlipidemia [12]. Past studies have established that sustained hyperlipidemia in obese individuals plays an important role in causing β -cell apoptosis and dysfunction, thereby contributing to the deterioration of glycemic control and the overt development of T2D [22-24]. This study was conducted to investigate whether long-term dietary intake of kaempferol can promote metabolic homeostasis and thereby prevents diabetic pathogenesis. We show here that dietary intake of kaempferol for 5 months (mo) improved insulin sensitivity and glucose tolerances, which were associated with increased Glut4 and AMPK α expression in muscle and adipose tissues in middle-aged mice fed a high-fat (HF) diet. In vitro, kaempferol increased lipolysis and restored chronic high fatty acid-impaired glucose uptake and glycogen synthesis in skeletal muscle cells, which were associated with improved AMPK α activity and Glut4 expression. In addition, dietary kaempferol treatment preserved functional pancreatic β -cell mass and prevented hyperglycemia and glucose intolerance in STZ-induced diabetic mice. These results suggest that kaempferol may be a naturally occurring anti-diabetic agent.

2. Methods

2.1. *Animal Studies.* Male (10 mo old) C57BL/6J mice (NCI, NIH) were individually housed in an animal room maintained on a 12-h light/dark cycle under constant temperature (22–25 °C) with *ad libitum* access to food and water. After 1 wk of environment acclimation, we conducted the following two animal studies. The Institutional Animal Care and Use Committee at Virginia Tech approved the animal study protocols.

2.2. *High-Fat Diet-Induced Obese Mice.* Mice were divided into 4 groups (n=12 mice/group) with blood glucose and body weight balanced, and then fed a standard chow (SD) diet, with 10% of calories derived from fat, a HF diet (Research Diets Inc., New Brunswick, NJ) with 58% of calories from fat, or HF diet supplemented with kaempferol (0.01% or 0.05%) for 5 mo. Body weight and food intake were recorded weekly throughout the study. To assess fasting blood glucose, mice were fasted for 12 h, and blood glucose was measured in tail vein blood samples using a glucometer (Kroger, Cincinnati, OH). After 5 mo of dietary treatment, body composition was evaluated using an LF-90 instrument (Bruker Optics, Inc., Billerica MA). The LF-90 body composition instrument is based on Time Domain nuclear magnetic resonance (TD-NMR) technology, which provides an *in vivo* measurement of lean tissue, body fat, and body fluid in live mice without anesthesia. Following this procedure, glucose and insulin tolerance tests were performed. For the glucose tolerance tests (GTT), mice were fasted 12 h and injected intraperitoneally (ip) with a single bolus of glucose (2 g/kg BW). Glucose levels were measured at time points of 0, 15, 30, 60 and 120 min after glucose administration. For the insulin tolerance tests (ITT), mice were injected ip with insulin (0.75 units/kg BW), and blood glucose levels were measured at 0, 15, 30, 60, and 120 min after insulin administration. Area under the curve (AUC)

was calculated using the trapezoidal rule. At the end of the study, the mice were fasted overnight and euthanized, immediately followed by the collection of blood samples. Fasting plasma total cholesterol, HDL-cholesterol and triacylglycerol were measured by enzymatic methods using a Pointer 180 Analyzer (Pointe Scientific, Canton, MI) as described previously [25]. Plasma insulin levels were measured using a mouse insulin ELISA kit (Merckodia, Inc., Uppsala, Sweden). Blood HbA1c levels were determined using an assay kit (Henry Schein, Inc., Melville, NY). At the end of feeding experiment, mice were sacrificed and extensor digitorum longus muscle and abdominal adipose tissues were collected, snap-frozen in liquid nitrogen, and then stored at -80 °C for the Western blot analyses. In a separate experiment, mice were divided into 3 groups (n=8 mice/group) and fed a SD diet or SD diet containing kaempferol (0.01% or 0.05%) for 3 mo. Body weight and food intake were recorded weekly. Fasting and non-fasting blood glucose levels were measured biweekly. At the end of 3 mo, GTT and ITT were performed.

2.3. Streptozotocin (STZ)-Induced Diabetic Mice. For this study, 10 mo old male mice (NCI, NIH) were divided into 3 groups (n=10 mice/group) with initial fasting blood glucose and body weights balanced among groups. Mice were then fed a SD diet, a HF diet (58 kcal% fat), or HF diet containing 0.05% kaempferol. After 6 wk of dietary kaempferol supplementation, GTT, ITT, and body composition were evaluated as described above. After this procedure, mice received ip injections of STZ dissolved in 0.1 M cold sterile sodium citrate buffer (pH 4.5) at 45 mg/kg daily for 3 consecutive days. Control mice received ip injections of saline. Body weight, food intake, non-fasting and fasting blood glucose were measured biweekly throughout the study. Plasma insulin measurements were as stated above.

2.4. Immunohistochemistry. At the end of experiment, mice were euthanized, and the pancreata were dissected, weighed, and then fixed in 4% (vol/vol) formaldehyde buffer (pH 7.2). Pancreas samples were embedded in paraffin and sectioned by AML Laboratories Inc (Baltimore, MD). A series of tissue sections (5- μ m thickness at 200 μ m interval) were prepared, mounted on glass slides, and immunofluorescently stained with an insulin antibody and FITC-conjugated secondary antibody (Abcam, Cambridge, MA) for determining β -cell mass. Pancreatic β -cell area was measured using images acquired from insulin-stained pancreatic sections. Pancreatic β -cell mass was calculated by dividing the area of insulin-positive cells by the total area of pancreatic tissue and multiplied by the pancreas weight [25, 26]. Four pancreatic sections from 5 mice in each treatment group were evaluated.

2.5. Measurements of Pancreatic Insulin Content. Pancreata were rapidly excised and weighed. Insulin was then extracted from pancreas homogenates with acid-ethanol [75% ethanol, 25% acetic acid (25% vol/vol)] overnight at 4 °C. The homogenates were centrifuged (10 min, 2,000 g, 4°C) and the supernatants were neutralized with Tris buffer. Pancreatic insulin content was measured by ELISA and then normalized to the protein concentration in the same sample.

2.6. Cell Culture. The C2C12 mouse cells (American Type Culture Collection, Manassas, VA) were grown at 37°C and 5% CO₂ in Dulbecco's modified Eagle's medium (DMEM), supplemented with penicillin (50 I.U./mL), streptomycin (50 μ g/mL), and 10% fetal bovine serum (FBS). The cells were grown to 75% confluence and the growth medium was then switched to DMEM supplemented with 2% horse serum and penicillin (50 IU/mL), streptomycin (50 μ g/mL) for differentiation.

2.7. *Glucose Uptake Assay.* C2C12 myoblasts were grown to ~80% confluence in DMEM and then differentiated for 5 days in DMEM supplemented with 2% horse serum. On day 5, the myotubes were treated for 24 h with 10 μ M kaempferol or vehicle (DMSO) along with a mixture of fatty acids or vehicle (BSA). The fatty acid mixture contained a 2:1 ratio of palmitate to oleate for a final concentration of 0.4 mM complexed with 0.4% BSA in serum-free, low glucose DMEM. Following the fatty acid and kaempferol treatment, glucose uptake was assessed in Krebs-Ringer HEPES buffer (in mM: 136 NaCl, 4.7 KCl, 1.25 MgSO₄, 1.2 CaCl, and 20 HEPES, pH 7.4) with the addition of 10 μ M 2-deoxyglucose and 1.25 uCi/ml 2-deoxy-[3H] glucose. After 15 min of incubation, plates were placed on ice, washed three times with ice-cold PBS, and harvested in 400 μ l of 0.2 M NaOH for cell lysis. Glucose uptake was calculated based on specific activity and expressed relative to protein content.

2.8. *Glycogen Synthesis.* C2C12 myotubes were treated for 24 h with 10 μ M kaempferol or vehicle (DMSO) along with 0.4 mM fatty acid (FA) cocktail or BSA as state above. After 24 h with FA and kaempferol treatment, cells were further incubated with 1.25uCi/mL¹⁴C glucose (American Radiolabeled Chemicals, Saint Louis, MO) in the presence or absence of insulin (100 NM; Eli Lilly, Indianapolis, IN) for 3 h. Following this treatment, cells were rinsed twice with PBS at 4°C followed by solubilization by adding 250 μ L of 30% KOH. The samples were then mixed with 35 μ L of 60 mg/mL glycogen (Sigma-Aldrich, St. Louis, MO) in distilled water and heated at 80°C for 20 min. Glycogen in the samples was precipitated with ice-cold ethanol. Following centrifugation at 4°C (10,000 rpm) for 20 min, the pellet was collected and washed with 70% ethanol and then resuspended in 500 μ L distilled water. After 20 min of shaking, the

glycogen precipitate was counted for the presence of ^{14}C by liquid scintillation (LS 6500, Beckman Coulter, Brea, CA). Glycogen synthesis was calculated based on specific activity and was expressed relative to protein content.

2.9. Lipolysis Assay. C2C12 muscle cells were pre-incubated with kaempferol or vehicle (DMSO) for 30 min followed by addition of 20 mM glucose and 0.5 mM palmitate for 24 h. Glycerol released into the medium was then measured by using a free glycerol determination kit (Sigma-Aldrich, St. Louis, MO).

2.10. Western Blot Analysis. Animal tissues or cultured cells were homogenized in lysis buffer (50 mM HEPES, 0.1% (v/v) Triton X-100, 1 mM PMSF, 10 mM E-64, 10 mM pepstatin A, 10 mM TLCK, 100 mM leupeptin, pH 7.4). Supernatants of cultured muscle cell or mouse tissue lysates were collected, and protein content was measured using an assay kit. Equal amounts of protein extracts from mouse tissues or cells were subjected to Western blot analysis as described previously [27]. Nitrocellulose membranes were probed with antibody against Glut4, AMPK α , or phospho-AMPK α (Cell Signaling (Danvers, MA)). The immunoreactive proteins were detected by chemiluminescence (Thermo Fischer, Rockford, IL). Nitrocellulose membranes were then stripped and re-probed with β -actin (animal tissues) or AMPK α (culture cells). The protein bands were digitally imaged for densitometric quantitation with a software program (Image J, NIH). All proteins levels were normalized to those of β -actin or total AMPK α , where applicable, from the same samples.

2.11. Statistical Analysis. Data were analyzed with one-way ANOVA, using Sigmaplot software program, and are expressed as mean \pm standard error (SE) or mean \pm standard error of mean (SEM) [28], where applicable. Treatment differences were subjected to the Duncan's multiple comparison tests. Differences were considered significant at $p < 0.05$.

3. Results

3.1. Long-Term Dietary Intake of Kaempferol Reduced Body Weight Gain, Improved Body Composition and Plasma Lipid Profile in HF Diet-Induced Middle-Aged Obese Mice. In this study, we tested the metabolic effects of the long-term dietary supplementation of kaempferol (0.01% or 0.05% in the diet) in middle-aged mice fed a HF diet. The HF diet decreased the accumulative average food intake, but kaempferol supplementation for 5 mo did not alter the food consumption compared with HF diet-fed mice (Figure 1(a)). Five mo of consuming HF diet significantly increased body weight of mice. However, dietary intake of 0.05% kaempferol significantly ameliorated HF diet-induced body weight gain (Figure 1(b)). Consistently, mice fed the HF diet developed obesity as determined by measuring their relative percentage of fat (Figure 1(c)) and muscle mass (Figure 1(d)). However, kaempferol treatment had no significant effect on adiposity of obese mice. Fasting blood levels of cholesterol (Figure 1(e)), LDL-cholesterol (Figure 1(f)), and triglyceride (Figure 1(g)) were increased in HF diet-fed obese mice, which were significantly reduced by dietary intake of kaempferol. However, total cholesterol levels in kaempferol-fed mice were still significantly higher as compared to chow diet-fed mice. Fasting plasma HDL-cholesterol concentrations were increased by HF feeding, but were not further altered by kaempferol treatment (Figure 1(h)). In another study to determine whether kaempferol also improves metabolism and health of SD diet-fed older adult mice, we found that dietary intake of either 0.01% kaempferol (K1) or 0.05% kaempferol (K2) for 3 mo had no effects on body weight gain (5.9 ± 0.5 , 6.1 ± 0.4 , and 6.0 ± 0.6 g for control, K1, and K2 group, respectively), food intake (4.25, 4.27, and 4.23 g/d/mouse), fasting (136.4 ± 4.1 , 131.6 ± 3.4 , and 127.9 ± 5.8 mg/dl) and nonfasting (185.4 ± 11.1 , 180.5 ± 5.5 and 174.4 ± 12.2) blood glucose levels,

glucose tolerance (32672 ± 1653 , 31411 ± 1582 , and 31078 ± 1634 AUC), and insulin sensitivity (11073 ± 1065 , 10897 ± 9887 , and 10574 ± 1141 AUC). Given these results, these mice were not further studied and therefore euthanized.

3.2. Long-Term Provision of Kaempferol Maintained Blood Glucose Homeostasis and Insulin Sensitivity in Middle-Aged Obese Mice. After 2 mo of HF diet consumption, mice displayed hyperglycemia throughout this study, as shown by persistently over 50% higher circulating glucose levels as compared with mice that consumed the SD diet. Kaempferol supplementation at this point non-significantly reduced the HF diet-induced rise in blood glucose. After 3 mo however, mice fed HF diet supplemented with kaempferol exhibited significantly lower blood glucose levels than those in HF-fed mice; after 5 mo of treatment, mice fed kaempferol-supplemented diet still had significantly lower blood glucose levels (140 ± 10.5 mg/dl) as compared to HF diet-fed mice (197 ± 10.5 mg/dl), which were almost reduced to the levels shown in SD-fed mice (123.0 ± 4.8 mg/dl) (Figure 2(a)). We then performed a GTT. Data showed that kaempferol (0.05% in the diet) non-significantly improved glucose tolerance as demonstrated by lower blood glucose levels at 30 and 60 min (Figure 2(b)) as well as reduced AUC (Figure 2(c)) following ip glucose injection compared to HF-fed mice. Consistently, blood levels of HbA1c, which reflect an average of blood glucose over a period of two to three mo [29], were significantly lower in kaempferol-treated mice as compared to those in HF diet fed mice (Figure 2(d)). Insulin resistance is important to the etiology of T2D, and usually occurs in obesity. To determine if dietary intake of kaempferol improves insulin sensitivity in obese mice, we performed an ip ITT. As expected, HF diet treatment impaired whole body insulin sensitivity. However, dietary provision of kaempferol as low as 0.01 % significantly improved plasma

glucose levels (Figure 2(e)), and the AUC (Figure 2(f)) post-insulin injection in HF diet-fed mice, suggesting that kaempferol increases insulin sensitivity. We then measured plasma insulin levels after overnight fasting. We observed that fasting plasma insulin levels in HF mice were about 6-fold of those in mice that received the SD diet, suggesting that obese mice are insulin resistant (Figure 2(g)). However, plasma insulin levels in 0.05% kaempferol-treated mice were more than 50% lower as compared with HF-fed obese mice (Figure 2(g)). These data strongly suggest that long-term kaempferol supplementation maintained whole body insulin sensitivity in HF diet-induced obese mice.

3.3. Kaempferol Improves AMPK and Glut4 Expression in Skeletal Muscle and Adipose Tissues in Middle-Aged Obese Mice. Insulin resistance in adipose tissue and skeletal muscle, the primary site of glucose and fatty acid utilization, play a major role in the development of HF diet-induced T2D. To further determine the molecular events that are associated with improved insulin sensitivity by kaempferol treatment, we measured the expression of AMPK and Glut4 in skeletal muscle and adipose tissues, which are two critical molecules regulating glucose uptake [30, 31]. Consistent with impaired peripheral insulin sensitivity, the expression of AMPK α and Glut4 proteins in both skeletal muscle (Figure 3(a)-3(c)) and adipose tissue (Figure 3(d)-3(f)) from HF-diet fed mice were significantly attenuated as compared to the control mice. However, treatment with kaempferol completely reversed these detrimental effects caused by feeding the mice with HF diet (Figure 3 (a)-3(f)). These results further confirmed that long-term intake of kaempferol protects against developing insulin resistance in HF-diet induced obese mice.

3. 4. Kaempferol Treatment Prevented High Fatty Acid-Impaired Glucose Uptake and Glycogen Synthesis in Skeletal Muscle Cells. As skeletal muscle is the major site for fuel metabolism, and

obesity and insulin resistance are always accompanied with the impairment in energy metabolism, a leading pathogenic factor for T2D, we performed a series of in vitro assays to determine whether kaempferol has direct beneficial effects on glucose metabolism. In that regard, we cultured skeletal muscle cells with or without kaempferol in the presence of palmitate and oleic acid. We found that exposure of C2C12 myotubes to high fatty acids for 24 h impaired insulin-induced glucose uptake (Figure 4(a)) and glycogen synthesis (Figure 4(b)). However, these detrimental effects were partially reversed by treatment with 10 μ M kaempferol (Figure 4(a), 4(b)).

3.5. Kaempferol Promoted Lipolysis and Recovered High Glucose and Fatty Acid-Impaired AMPK Activity and Glut4 Expression in Skeletal Muscle Cells. To further characterize metabolic and insulin sensitizing effects of kaempferol, we cultured skeletal muscle cells with or without kaempferol in the presence of high glucose (20 mM) and saturated fatty acid palmitate (0.5 mM), an in-vitro environment that is frequently used to simulate diabetic condition in vivo. The results showed that kaempferol at physiologically relevant concentrations significantly improved lipolysis (Figure 5(a)) and reversed high fatty acid-impaired AMPK α activity (Figure 5(b)) and Glut4 protein expression (Figure 5(c)), a downstream target of activated AMPK α [31].

3.6. Dietary Intake of Kaempferol Ameliorated Hyperglycemia and Improved Insulin Levels in Middle-Aged Obese Diabetic Mice. While peripheral insulin resistance is common during obesity in rodents and people, its progression to T2D is largely due to insulin secretory dysfunction and significant apoptosis of functional β -cells [4-8], leading to an inability to compensate for insulin resistance. Past studies have established that sustained hyperlipidemia in obese individuals plays

an important role in causing β -cell apoptosis and dysfunction, thereby contributing to the deterioration of glycemic control and the overt development of T2D [22-24]. We recently found that kaempferol treatment prevented apoptosis of cultured pancreatic β -cells exposed to chronic hyperlipidemic condition [12]. We therefore further assessed whether kaempferol can also protect pancreatic β -cell function, thereby preventing diabetes by using a T2D mouse model that was generated through a combination of HF diet feeding and three consecutive injections of low-doses of STZ [32]. Regarding this, C57BL/6 mice (male, 10 mo old) were fed a SD diet, a HF diet, or HF diet containing 0.05 % kaempferol. Consistent with the observations from the animal study described above, treatment with kaempferol for 6 wk had no effect on body weight gain, food intake, fasting blood glucose levels, glucose intolerance, or insulin resistance of mice fed the HF diet (data not shown). After 6 wk of dietary treatment, STZ (40 mg/kg BW) was administered (ip) for 3 consecutive days to induce diabetes mediated by a destruction of islet β -cells in mice [33]. Our data showed that dietary ingestion of kaempferol significantly mitigated STZ-induced hyperglycemia in diabetic mice as determined by measuring fasting (Figure 6(a)) and non-fasting (Figure 6(b)) blood glucose levels. Consistently, kaempferol ameliorated the loss of body weight secondary to the development of diabetes [34] (data not shown). To determine if the improved glycemic control in mice fed the kaempferol-supplemented diet is the result of preserved islet function, we measured insulin levels in the plasma of the control and kaempferol-fed mice. As shown in Figure 6 (c), plasma insulin levels in mice fed diet containing kaempferol were significantly greater as compared to those in non-treated diabetic mice, suggesting that kaempferol may ameliorate hyperglycemia primarily via preserving islet β -cell function.

3.7. Dietary Intake of Kaempferol Improved Islet β -Cell Mass and Insulin Content in Obese Diabetic Mice. Since STZ causes diabetes by destroying islet β -cells [35], we then examined whether kaempferol treatment preserved β -cell mass in diabetic mice by using an immunohistochemical technique. We observed that HF diet-fed mice exhibited greater pancreas weight as compared with the control mice (Figure 7(a)), which was not modulated by kaempferol supplementation (Figure 7(a)). However, STZ administration caused severe destruction of pancreatic β -cells as determined by evaluating the area of insulin positive cells (Figure (b)) and islet mass (Figure 7(c)), which consequently led to the reduction of pancreatic insulin content by nearly 80% (Figure 7(d)). However, dietary provision of kaempferol partially preserved islet β -cell mass (Figure 7(b), 7(c)) and insulin content (Figure (7d)) in diabetic mice.

4. Discussion

Kaempferol is a flavonol that is relatively abundant in various natural plants [36]. It has been reported that kaempferol elicits a number of health benefits, including anti-oxidative [17, 37], anti-inflammatory [18, 38], anti-hypertensive [39, 40], lipolytic [19, 41], and anti-carcinogenic effects [20, 42, 43]. However, studies on whether this compound possesses anti-diabetic properties are very limited. In this study, we tested the anti-diabetic potential of this natural compound by using two mouse models and *in vitro* studies. It is worthy to note that, unlike many metabolic studies that used young adult mice, we used near middle-aged mice, which may be more clinically relevant, as T2D in humans often occurs during middle and late ages. We provide evidence that long-term dietary intake of kaempferol promoted metabolic homeostasis with the improved fasting blood glucose, HbA1c levels, insulin sensitivity and glucose tolerance in HF diet-induced obese mice, which was associated with increased fuel metabolism, AMPK activity, and Glut4 expression in skeletal muscle cells. Further, dietary kaempferol also ameliorates hyperglycemia in STZ-induced diabetic mice by preserving functional β -cell mass. Importantly, these beneficial metabolic effects, elicited by dietary intake of kaempferol, are not due to alteration in food intake. Given that both insulin resistance in peripheral tissues and progressive β -cell loss and dysfunction are key components in the pathogenesis of T2D [4-8], kaempferol could be a low-cost and safe natural compound to promote energy metabolism and maintain glucose homeostasis by targeting these two defects.

It is well established that obesity is an important risk factor for T2D [44, 45]. Therefore, HF diets are commonly used as a strategy to induce obesity in animal models, leading to the development of metabolic disorders including hyperlipidemia, impaired insulin sensitivity and glucose tolerance, as well as elevated blood insulin and glucose [46-49]. Consistently, our data

showed that consumption of the HF diet induced obesity and elevated plasma concentrations of triglycerides and cholesterol that are typical of obesity [46], which however were ameliorated in mice fed a HF diet containing kaempferol. Given that obesity is a leading pathogenic factor for developing insulin resistance and subsequent glucose intolerance [50], the improved insulin sensitivity and glucose homeostasis by kaempferol supplementation could be partially due to the secondary effects whereby long-term intake of kaempferol ameliorated obesity in mice fed a HF diet. While it is presently unclear how kaempferol affects lipid metabolism and body weight gain, previous studies demonstrated that several structurally similar flavonoids inhibit lipid absorption and lipogenesis [51-53]. Because food intake was not affected by long-term kaempferol supplementation, the decrease in fasting plasma cholesterol and triglycerides in the HF-fed mice could be caused by the regulation of the intestinal digestion and absorption of lipids and/or lipogenesis, which needs further investigation. As kaempferol treatment did not alter calorie intake in mice during the course of this study, it is also possible that the reduced body fat mass and body weight in kaempferol-fed mice is due to its effect on energy expenditure, given that obesity results from energy imbalance.

Several lines of evidence have shown that pharmacological activation of AMPK improves blood glucose homeostasis and lipid profile in insulin-resistant rodents [54]. AMPK is an energy sensing molecule highly conserved from yeast to all animals, is increasingly recognized as a master regulator of whole body energy homeostasis [55]. AMPK is a heterotrimeric protein kinase composed of a catalytic subunit (AMPK α) and two regulatory subunits (β and γ) that sense low cellular energy levels by monitoring changes in the AMP:ATP ratio. AMP binding to the γ subunit induces a conformational change that allows AMPK α to be phosphorylated at its threonine residue (Thr 172) by the AMPK-activating protein kinase

(LKB1). At the whole body level, AMPK integrates stress responses, nutrient and hormonal signals to the control of food intake, energy expenditure, and substrate utilization. At the cellular level, activated AMPK inhibits hepatic gluconeogenesis [56], promotes fatty acid oxidation [55], and regulates mitochondrial biogenesis [57]. In addition, activation of AMPK increases Glut4 expression and membrane translocation in skeletal muscle [31], thereby improving glucose uptake. Consistent with the impaired insulin sensitivity in HF-fed obese mice, we found that the protein levels of AMPK and Glut4 in skeletal muscle and adipose tissue from these animals were also considerably attenuated. However, these detrimental effects were completely reversed by supplementation of 0.05 % kaempferol in the HF diet. These results suggest that kaempferol may increase peripheral insulin sensitivity via the AMPK-mediated pathway.

Both in experimental animals and people, obesity-related insulin resistance is always associated with the dysfunctions of several metabolic pathways including reduced lipolysis, fatty acid oxidation, glucose uptake and glycogen synthesis coupled with increased glucose output [50]. Specifically, it is believed that elevated intramyocellular lipid accumulation coupled with diminished lipolysis and mitochondrial lipid oxidation play a role in the development of insulin resistance in skeletal muscle [58]. Consistently, it was found that inducing lipolysis and fatty acid oxidation in muscle cells protects against lipotoxicity-induced insulin resistance [59, 60]. In the present study, we further showed that kaempferol treatment as low as 1 μ M augmented lipolysis and reversed chronic hyperlipidemia-impaired glucose uptake, Glut4 expression, AMPK activity as well as glycogenesis in skeletal muscle cells, which provides further evidence at cellular levels that kaempferol might be an insulin sensitizing molecule by promoting energy metabolism. However, it is presently unclear how kaempferol exerts these beneficial effects in skeletal muscle cells. Kaempferol has been shown to possess antioxidant

property. While we found that pharmacological doses of kaempferol (10-50 μ M) showed significant free radical scavenging activity as evaluated by using an oxygen radical absorbance assay, it had no such an effect at 1 μ M. Therefore, kaempferol promotion of skeletal muscle cell function may not due to its potential antioxidant effect. We are presently investigating how kaempferol improve energy metabolism in skeletal muscle cells.

We further explored whether kaempferol directly protects pancreatic β -cell function in vivo by using a non-genetic mouse model of T2D, that was generated by employing a combination of feeding a HF diet and administering three mild doses (40 mg/kg) of STZ that does not cause diabetes in chow-fed mice, as demonstrated in our recent study [61]. This non-genetic diabetic mouse model manifests the metabolic characteristics of human T2D, including moderate levels of hyperglycemia, hyperlipidemia, insulin resistance, impaired insulin secretion, and reduced β -cell mass. The results in the present study show that kaempferol partially preserved β -cell mass in STZ-induced diabetic mice, which could be primarily attributable to its anti-diabetic action, given that diabetes was induced before kaempferol caused changes in body weight, adiposity, or other metabolic parameters. We speculate that kaempferol treatment may protect against β -cell apoptosis, thereby improving islet mass, given our recent observations that kaempferol promotes viability of isolated pancreatic islets exposed to chronic hyperglycemia [62] or hyperlipidemia [12].

In summary, we provide evidence that long-term dietary supplementation of kaempferol prevents HF diet-induced metabolic disorders in middle-aged obese mice. On cellular and molecular levels, kaempferol improves glycolysis, glucose uptake, glycogen synthesis, AMPK activity as well as Glut4 expression in skeletal muscle. In addition, dietary supplementation of kaempferol significantly ameliorated hyperglycemia and preserved functional islet mass in old

adult obese diabetic mice. These results indicate that phytonutrient kaempferol may be used as a dietary supplement to prevent metabolic disorders that are associated with obesity and aging.

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6. Figures

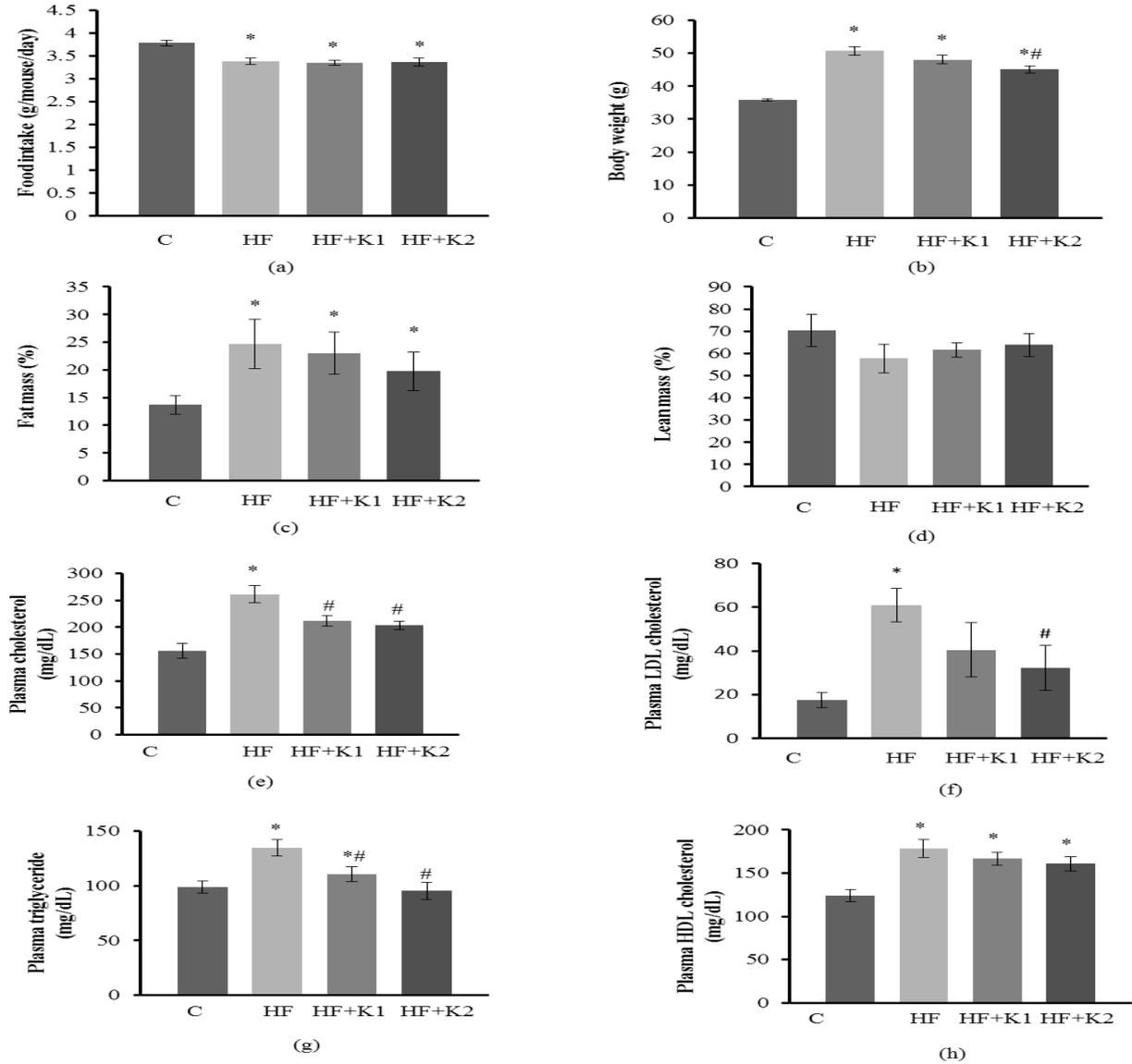


Figure 1. Kaempferol supplementation had no significant effects on food consumption, but reduced body weight gain and fat mass in HF diet-fed middle-aged mice. (a) Food intake was recorded twice a week and the average daily food intake was calculated. (b) Body weight of the individual mouse was measured each week. Data shown are the average body weight of last week of feeding experiment. Body composition including fat mass (c) and lean mass (d) was measured following 5 mo of standard diet or HF feeding. At the end of the experiment, fasting plasma total cholesterol (e), LDL-cholesterol (f), triglycerides (g), and HDL-cholesterol (h) were measured in duplicated samples by using mouse ELISA kits. Data are shown as means \pm SE (n=12). *, p<0.05 vs standard diet-fed mice (C); #, p<0.05 vs. HF-fed mice. C: standard diet; HF: high-fat diet; HF+K1: HF diet containing 0.01% kaempferol; HF+K2: HF diet supplemented with 0.05% kaempferol.

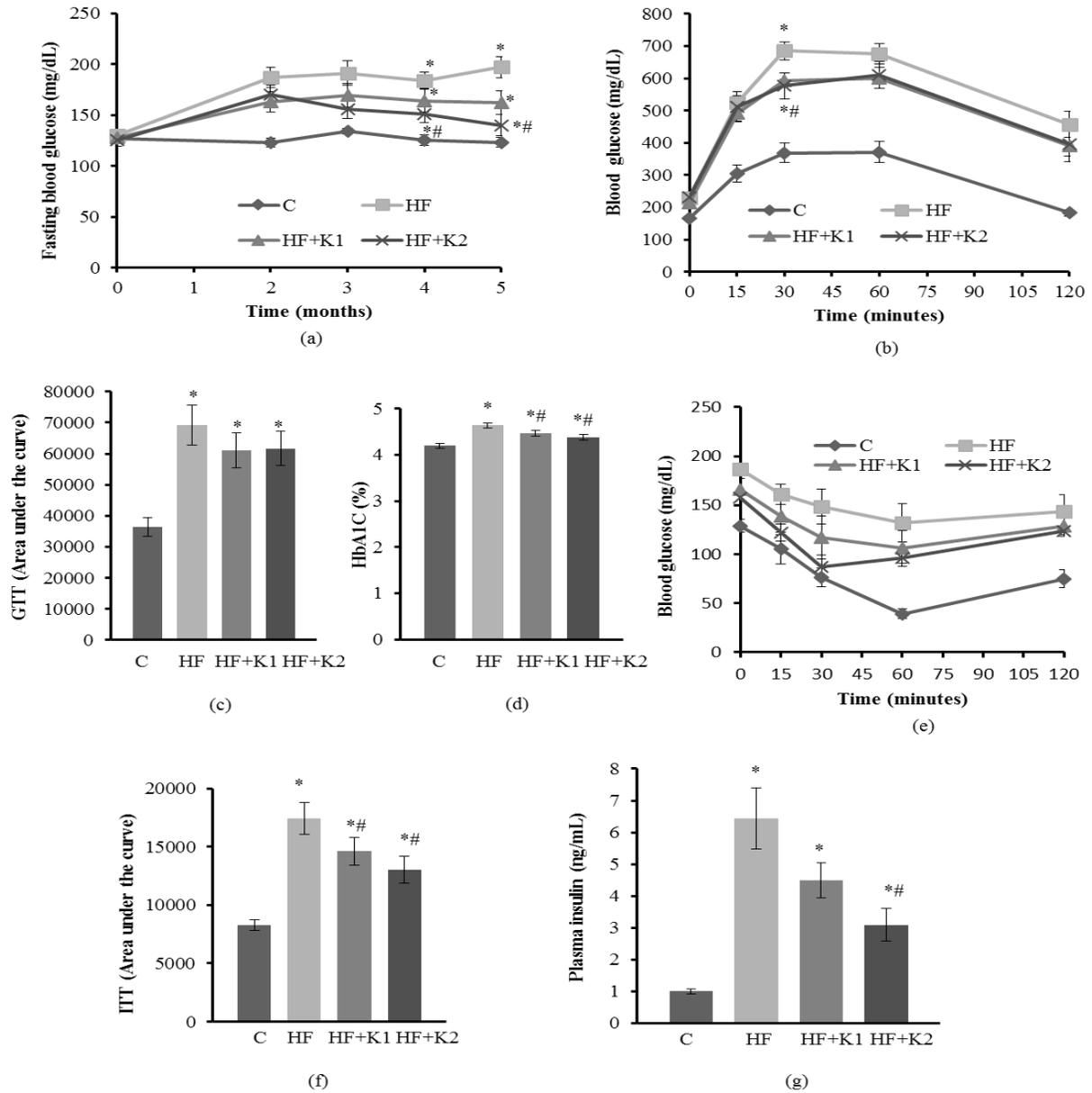


Figure 2. Kaempferol supplementation reduced blood glucose, HbA1c, and insulin concentrations, and improved insulin sensitivity in HF-diet fed mice. (a) Fasting blood glucose levels were measured at indicated time points of dietary treatment. GTT (b) and ITT (d) were performed as described in the Method section. The area under the curve (AUC) for GTT (c) and ITT (e) was calculated. (f) Plasma insulin levels were measured using an ELISA kit. Data are shown as means \pm SE (n=12). *, p<0.05 vs SD-fed mice (C); #, p<0.05 vs. HF-fed mice. C: standard diet; HF: high-fat diet; HF+K1: HF diet containing 0.01% kaempferol; HF+K2: HF diet supplemented with 0.05% kaempferol.

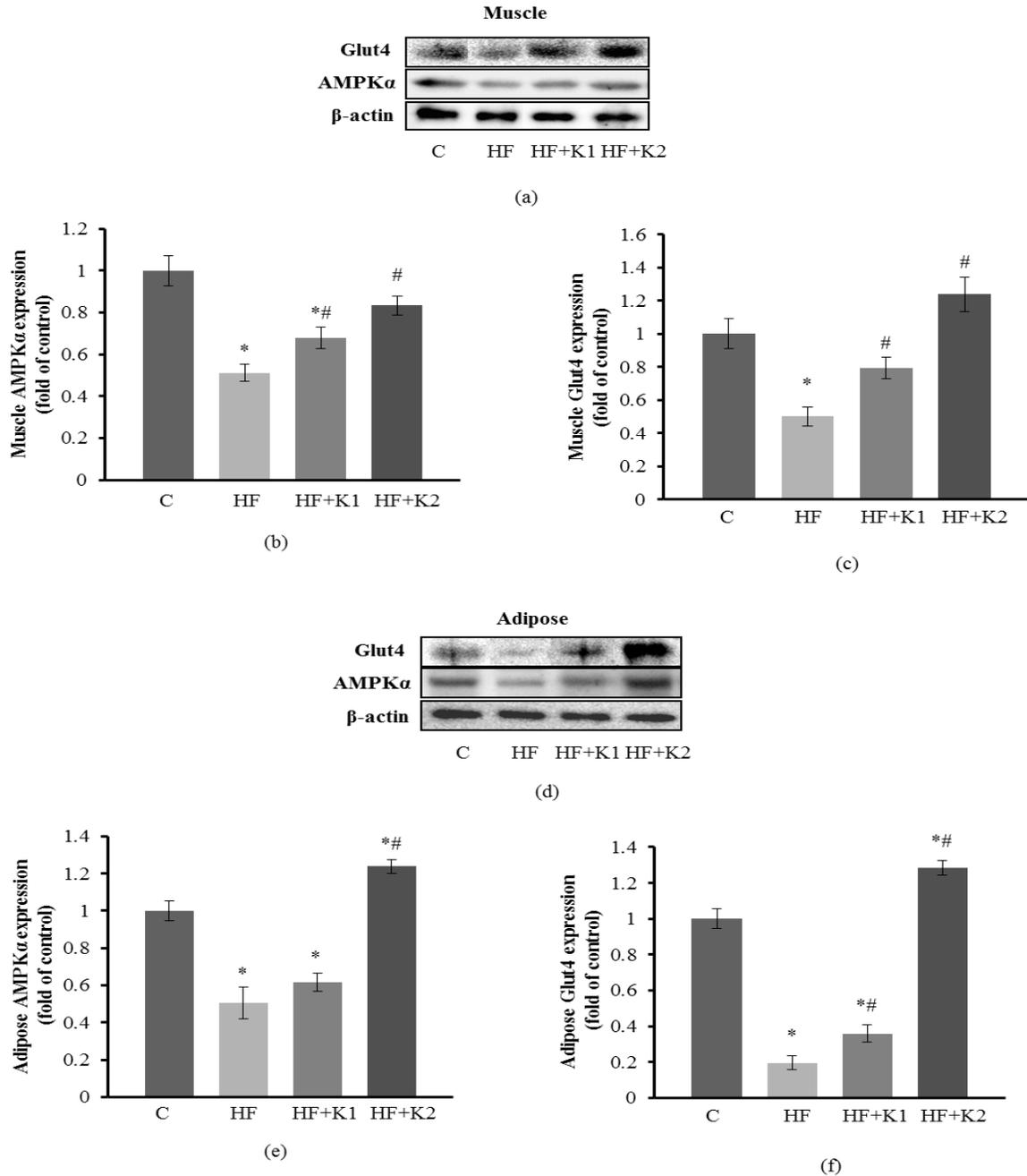


Figure 3. Kaempferol supplementation completely prevented the HF diet-induced decreases in AMPK α and Glut4 protein expression in skeletal muscle and adipose tissues of obese mice. At the end of feeding experiment, AMPK and Glut4 protein levels in whole cell lysates of skeletal muscle (a-c) and adipose tissue (d-f) of mice were measured by immunoblotting and normalized to β -actin content. Values are mean \pm SE from 4 mice per group. *, $p < 0.05$ vs standard diet-fed mice (C); #, $p < 0.05$ vs. HF-fed mice. C: standard diet; HF: high-fat diet; HF+K1: HF diet containing 0.01% kaempferol; HF+K2: HF diet supplemented with 0.05% kaempferol.

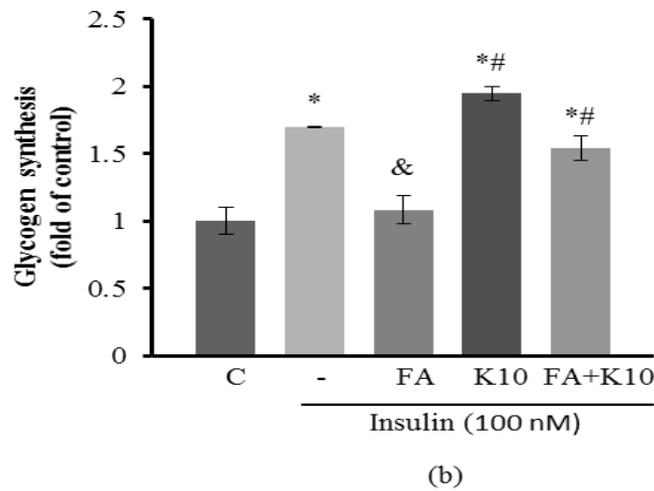
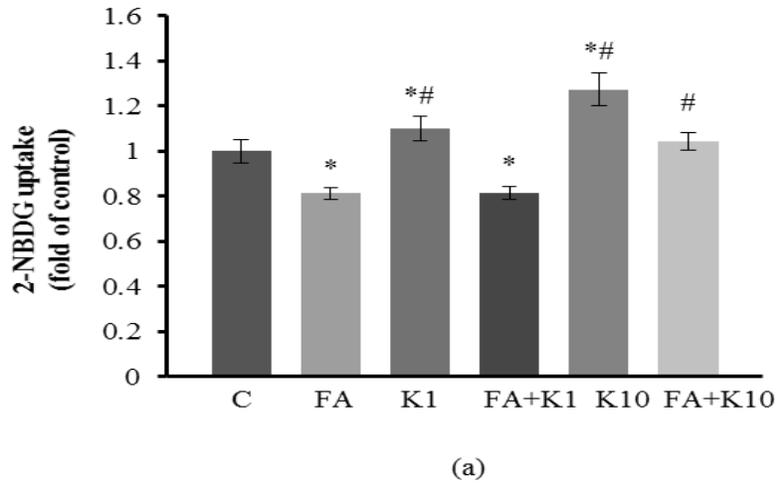
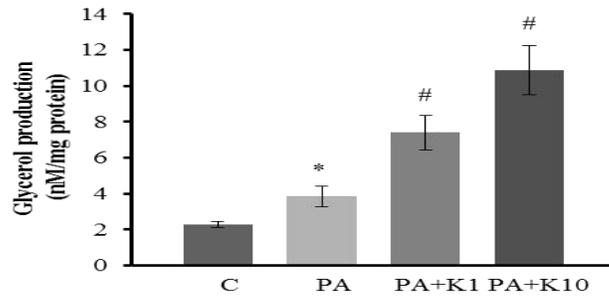
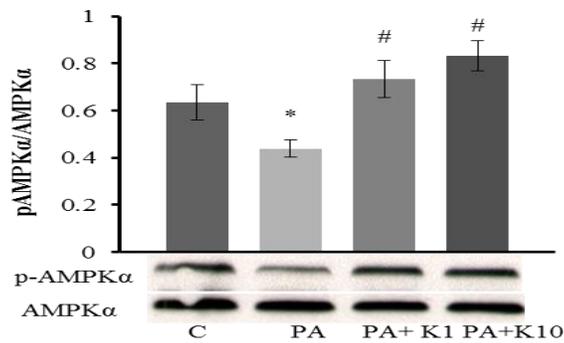


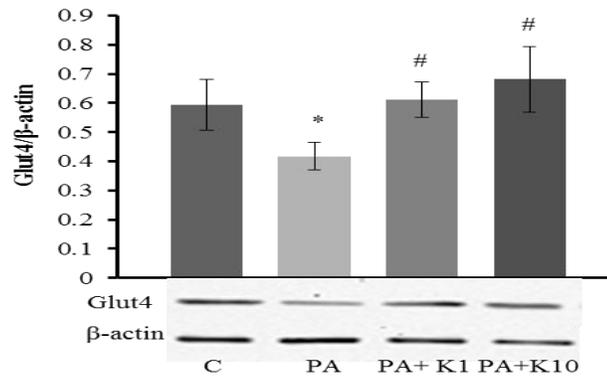
Figure 4. Kaempferol treatment reversed high fatty acid-impaired glucose uptake and glycogen synthesis in muscle cells. C2C12 myotubes were pre-treated for 30 min with kaempferol (K1: 1 μ M; K2: 10 μ M) or DMSO followed by addition of 0.4 mM fatty acid mixture (FA: 2:1 ratio of palmitate to oleate) or vehicle (C: BSA) as stated in the Method section. 24 h later, glucose uptake (a) and insulin-stimulated glycogen synthesis (b) were measured. Data are shown as mean \pm SEM (n=4) of duplicate or triplicated determinations each. *, P<0.05 vs. vehicle alone (C)-treated cells; &, p<0.05 vs. insulin alone-treated cells; #, p<0.05 vs. cells treated with FA and insulin.



(a)

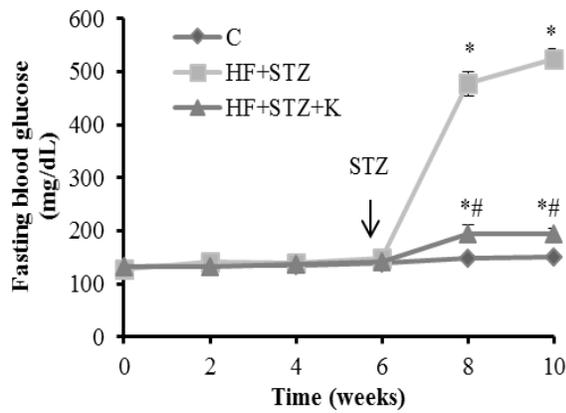


(b)

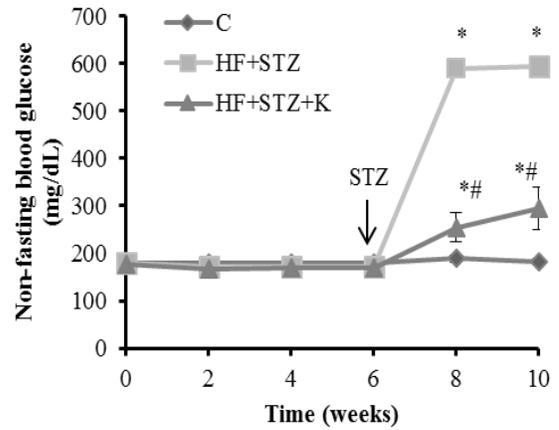


(c)

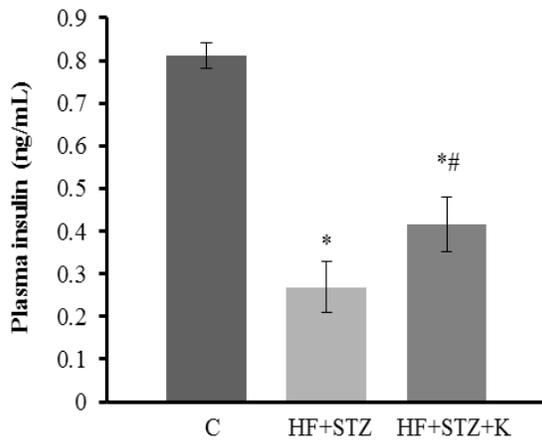
Figure 5. Kaempferol promoted lipolysis and reversed high glucose and fatty acid-impaired AMPK activity and Glut4 expression in muscle cells. C2C12 muscle cells were pre-treated with vehicle (C: DMSO) or kaempferol (K1: 1 μ M; K2: 10 μ M) for 24 h and then incubated in the presence or absence of 20 mM glucose and 0.5 mM palmitate (PA) for another 24 h. (a) Glycerol released into the medium was measured after 24h. (b) The phosphorylation of AMPK α (p-AMPK α) in whole cell lysates was measured by immunoblotting and normalized to total AMPK α . (c) Glut4 protein expression in the cell lysates was detected and normalized to β -actin content. Data are means \pm SE (n=3). *, p<0.05 vs. vehicle alone (C)-treated cells; #, p<0.05 vs. PA alone-treated cells.



(a)



(b)



(c)

Figure 6. Dietary intake of Kaempferol ameliorated hyperglycemia in STZ-induced obese diabetic mice. Male C57BL/6 mice (10 mo old) were fed a HF diet or HF diet containing 0.05% kaempferol (HF+K) in the diet for 6 wks prior to administration of STZ (40 mg/kg for 3 consecutive days) and continued on the same diet for 4 wks. Aged-matched mice were fed a SD diet (C). Fasting (a) and non-fasting blood glucose levels (b) were monitored biweekly throughout the study. (c) Plasma insulin levels in fasted mice were measured by ELISA. Data are means \pm SE (n=10-12 mice/group). *, $p < 0.05$ vs. healthy control (C); #, $p < 0.05$ vs. STZ alone-treated mice.

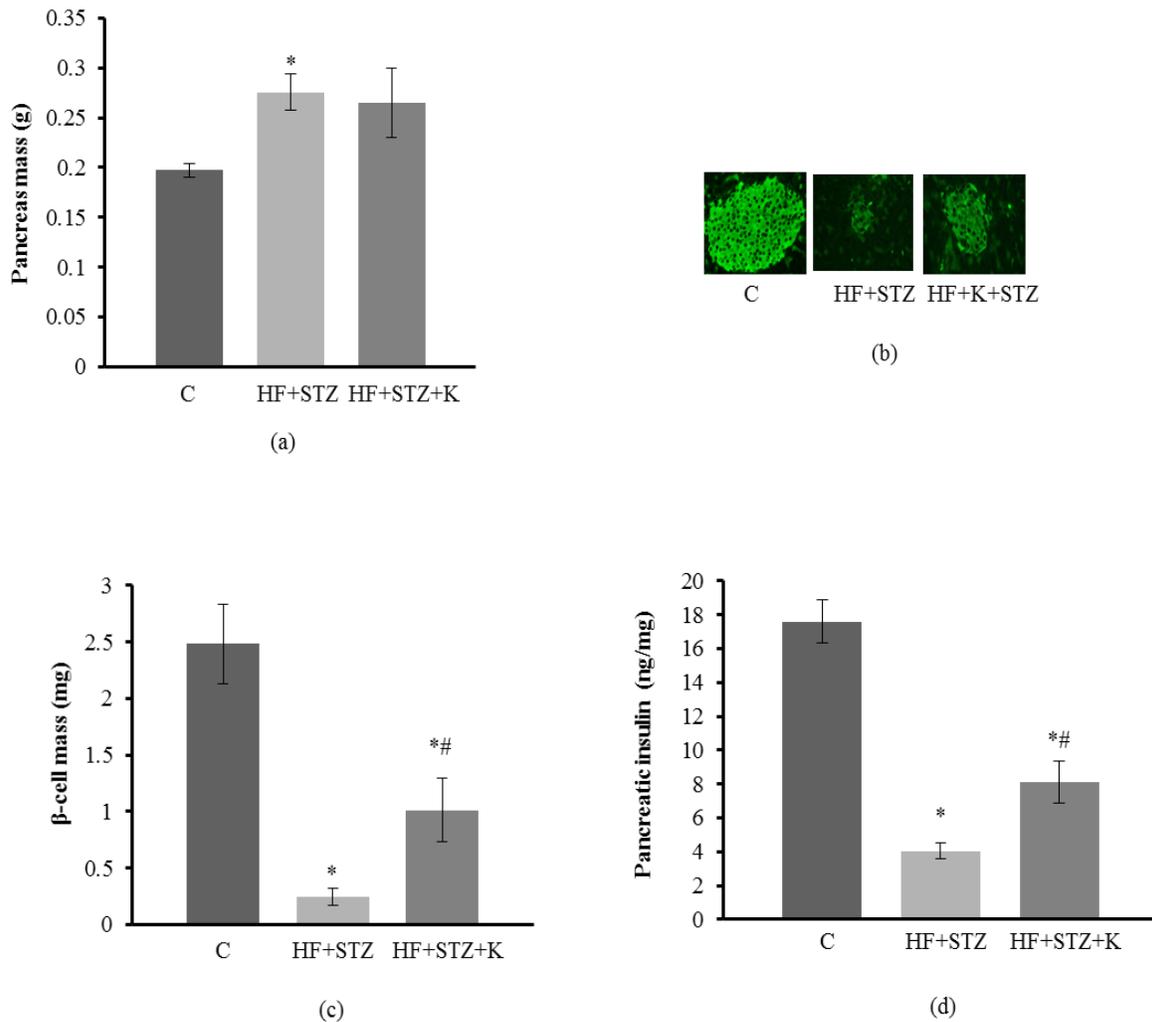


Figure 7. Kaempferol supplementation preserved pancreatic β -cell mass in STZ-induced diabetic mice. (a) Pancreas from mice given a SD diet (C), HF diet and STZ administration (HF+STZ), and HF+STZ supplemented with 0.05% kaempferol (HF+STZ+K) were isolated and weighed. (b) Fixed pancreas were then sectioned and fluorescently stained with insulin. (c) The β -cell mass was determined as described in the Method section. Data are shown as means \pm SE (n= 5 mice/group). (d) Insulin content in the pancreas was measured by ELISA and normalized to protein concentration in the same sample. Data are expressed as means \pm SEM (n=5-6 mice/group). *, p <0.05 vs. healthy control (C); #, p <0.05 vs. STZ alone-treated mice.

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CHAPTER FOUR

**Small molecule kaempferol protects against streptozotocin-induced diabetes through
suppressing hepatic glucose production**

Abstract

In both insulin-deficient type 1 diabetes and type 2 diabetes, the increase in the activity of the key enzymes that control glycogenolysis and primarily gluconeogenesis in the liver causes an increase in the rate of hepatic glucose production, which is the main contributor to the development of hyperglycemia, in particular, fasting hyperglycemia. In the present study, we investigated whether kaempferol, a naturally occurring flavonol present in some medicinal herbs and certain types of foods, can be used to ameliorate diabetes in an insulin-deficient mouse model and further explored the mechanism underlying the anti-diabetic action of this compound. We show that oral administration of kaempferol (50 mg/kg) to streptozotocin-induced diabetic mice significantly ameliorated hyperglycemia and glucose intolerance. After 12 wks of treatment, the incidence of overt diabetes was decreased from 100 % to 77.8%. This kaempferol effect was associated with reduced hepatic glucose production and increased glucose oxidation in the muscle of diabetic mice, whereas body weight (BW), food intake, body composition, or plasma insulin and glucagon levels were not affected. On the molecular level, kaempferol treatment restored hexokinase activity in the liver and skeletal muscle while reduced glycogenolysis and gluconeogenesis possibly via inhibiting pyruvate carboxylase in the liver. These findings suggest that kaempferol holds a great potential to treat insulin deficient diabetes by improving glucose metabolism in skeletal muscle and suppressing hepatic gluconeogenesis.

Keywords: insulin deficiency, type 1 diabetes, hepatic glucose production, kaempferol, hexokinase.

1. Introduction

Glucose homeostasis is regulated by the coordination of several complex pathways in the fed and fasted conditions. The rise in glucose levels in the portal vein post-absorption increases hepatic glucose uptake and subsequent metabolism, initiated by glucokinase (GCK) or hexokinase IV [1], and this hence increases glucose utilization and storage by the liver before it reaches the circulation [2]. Meanwhile, increased circulating glucose stimulates insulin secretion from pancreatic β -cells, which subsequently increases glycogen synthesis while inhibiting gluconeogenesis, thereby reducing hepatic glucose output [3, 4]. In addition, muscle tissue plays a significant role in disposing blood glucose, which accounts for over 30% of total disposed blood glucose as compared with about 39% by splanchnic tissues (mostly by the liver) [5]. On the other hand, low glucose levels trigger glucagon release which simultaneously increases glucose production through hydrolysis of glycogen as well as gluconeogenesis in the liver [6, 7]. Glucagon promotes the transcription of key glucogenic enzymes such as phosphoenolpyruvate carboxykinase (PEPCK), glucose-6-phosphatase (G6Pase) [8], and pyruvate carboxylase (PC) [9]. The liver is the primary supplier of glucose during fasting and responsible for about 90% of the overall glucose production with the kidneys generating about 10% of glucose output [10]. Peripheral tissues including the muscle and adipose tissues supply the liver with glucogenic precursors, which are blocked in the presence of insulin [11].

Type 1 diabetes (T1D) is an autoimmune disease characterized by immune cell-mediated destruction of pancreatic β -cells, leading to insulin deficiency and hyperglycemia. T1D accounts for 5-10 % of diabetes cases and accompanied by serious complications [12]. Like T2D, hepatic and peripheral glucose uptake and metabolism are also altered in T1D [13], which are associated with reduced GCK [14] and muscle hexokinase II (HK II) protein expression [15]. Additionally,

it is accompanied by excessive hepatic glucose production which primarily contributes to fasting hyperglycemia, the hallmark of diabetes [16]. Due to insulin deficiency, glucagon stimulation of the key glucogenic enzymes is not restrained [17]. Indeed, the use of agents that suppress glucagon [18] or its action can prevent hyperglycemia in insulin-deficient rodent models of diabetes [19]. Despite the tremendous knowledge about the T1D, there is no cure for it, and therefore patients require insulin administration for survival [20]. Continuous glucose monitoring and intensive treatment showed improvements in glycemic control of adult patients but weren't efficient in younger patients who are more commonly diagnosed with this disease [21]. Moreover, patients with T1D may have insulin resistance before diagnosis or develop it after diagnosis which makes the treatment even more complicated [22]. These observations suggest that, in addition to targeting the immune system and pancreatic β -cells for T1D treatment, it is also of importance to correct these dysregulated pathways due to insulin deficiency for more efficiently managing hyperglycemia in T1D.

Kaempferol (3,5,7-trihydroxy-2-(4-hydroxyphenyl)-4H-1-benzopyran-4-one), a flavonol found in many traditional and edible plants, exerted many pharmacological activities in preclinical studies, including anti-diabetic activities [23]. Kaempferol is a flavonol that is relatively abundant in various natural plants [24]. It has been reported that kaempferol elicits a number of health benefits, including anti-oxidative [25, 26], anti-inflammatory [27, 28], anti-hypertensive [29, 30], lipolytic [31, 32], and anti-carcinogenic effects [33-35]. However, studies on whether this compound possesses anti-diabetic properties are very limited. In a previous study, we observed that kaempferol prevented β -cell dysfunction and ameliorated hyperglycemia in mouse models for T2D, which was induced by high-fat (HF) diet feeding followed by exposure to low doses of STZ [36]. In this study, we examined whether kaempferol exhibits anti-

diabetic properties in a type 1-like diabetes, which was induced by intraperitoneal (ip) injection of multiple low doses of STZ to lean mice fed a standard chow (SD) diet [37]. Our data show that kaempferol treatment ameliorated hyperglycemia and improved glucose tolerance. After 12 wks of treatment, the incidence of overt diabetes was decreased from 100 % to 77.8%. Kaempferol treatment restored the activity of key enzymes which play crucial roles in glucose metabolism such as liver and muscle hexokinases. Kaempferol suppressed hepatic glucose production which was accompanied by a decrease in PC activity and glycogenolysis in the liver. These changes were independent of BW gain, body composition, or changes in plasma insulin levels of diabetic mice. These findings suggest that kaempferol could be effective for treating hyperglycemia and reversing diabetes caused by insulin deficiency.

2. Methods

2.1. Mice, STZ administration and kaempferol treatment

Male (5.5 mo old) C57BL/6 male mice (Envigo, Indianapolis, IN) were maintained on a 12-h light/dark cycle at constant temperature (22–25°C) in an animal room with *ad libitum* access to a standard chow (SD) diet and water. All experimental protocols were approved by the Institutional Animal Care and Use Committee at Virginia Tech. For the first study, mice were divided to receive either streptozotocin (STZ) (40mg/kg BW) (Sigma, St. Louis, MO) or vehicle (10 mM sodium citrate, pH 4.5) for 4 consecutive d to induce diabetes. Mice fasted for 4 h received ip injections of freshly prepared vehicle or STZ. Three wks after STZ injection, diabetic mice were divided into 2 groups ($n = 9$ mice/group), with non-fasting blood glucose levels, BW, and body composition balanced among groups and then received either kaempferol (50 mg/kg/d dissolved in 2% 2-methyl cellulose) or vehicle by oral gavage for 12 wks. Nondiabetic mice ($n = 9$ mice) served as a control group and received the vehicle. For second animal study, STZ-induced diabetic mice (C57BL/6 male) were divided into 2 groups ($n = 8-9$ mice/group). Six wks after STZ injection, mice received either kaempferol (50 mg/kg/d dissolved in 2% 2-methyl cellulose) or vehicle by oral gavage for 7 wks.

2.2. Metabolic studies

BW and food intake were recorded weekly. Non-fasting and fasting blood glucose levels were measured biweekly in tail vein blood samples using a glucometer (Kroger, Cincinnati, OH). Body composition of the mice was evaluated initially and 4 and 8 wks after treatment using an LF-90 instrument (Bruker Optics, Inc., Billerica, MA). Four and 6 wks after treatment with kaempferol, mice fasted for 15 h were ip injected with a single dose of pyruvate (1 g/kg BW), or

glucose (1 g/kg BW) for pyruvate and glucose tolerance tests, respectively. Blood glucose levels were then measured at 0, 30, 60, 90, and 180 min and 0, 15, 30, 60, and 120 min after administration of pyruvate or glucose. The area under the curve (AUC) for these tests was calculated using the trapezoidal rule [36]. At the end of the study, the mice were fasted for 15 h and were then euthanized between 9-11 am. Blood was collected immediately, and various organs were weighed and snap-frozen in liquid nitrogen and then stored at -80°C for further analyses. Plasma lipid profile was analyzed by enzymatic methods using assay kits (Teco Diagnostics, Anaheim, CA). Plasma insulin levels were measured using an ultrasensitive mouse insulin ELISA kit (Merckodia, Inc., Uppsala, Sweden). Plasma glucagon levels were measured using mouse glucagon ELISA kit (Crystal Chem, Downers Grove, IL).

2.3 Glucose oxidation

Fresh mouse liver and muscle (red) samples were used to analyze glucose oxidation as previously described [38, 39] with modifications. Briefly, liver or muscle tissues were homogenized in the buffer (0.250M Sucrose, 1mM EDTA, 0.01M Tris-HCl, and 2mM ATP, pH=7.4). The tissue homogenates were then incubated with ^{14}C -labeled glucose (American Radiolabeled Chemicals, St. Louis, MO) for glucose oxidation in a trapping device at 37°C for 1 h. The $^{14}\text{CO}_2$ produced was trapped with 70% perchloric acid and the resulting sodium hydroxide was collected to assess CO_2 production.

2. 4. Enzyme activity assays

Liver and muscle tissues were homogenized in ice-cold lysis solution and then centrifuged (13,000 rpm for 3 min at 4°C). Hexokinase activity in the cell lysates was measured using an

assay kit (Biomedical Research Services Center, Buffalo, NY) according to the manufacturer's instruction. The assay is based on the NADH-coupled reaction that leads to the reduction of tetrazolium salt INT (2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyl-2*H*-tetrazolium) to INT-formazan, which exhibits maximum absorbance at 492 nm. Briefly, the samples were treated with control solution and a reaction solution containing the substrate (20 mM glucose) for 30 min, and the reaction was stopped using 3% acetic acid. Absorbance at 492 nm was measured using a microplate reader. The activity of hexokinase in the cell lysates was calculated using this equation: IU/L unit= $\mu\text{M}/(\text{L}\cdot\text{min})=(\text{O.D.}\times 1000\times 110\ \mu\text{l})/(30\ \text{min}\times 0.6\text{cm}\times 18\times 10\ \mu\text{l})$. Pyruvate carboxylase activity was measured using a citrate synthase-coupled reaction as previously described [40].

2.5 Glycogen content

Mouse liver tissue was weighed, minced, and homogenized in phosphate buffer, pH 7.0. The homogenates were centrifuged (9,500 rpm for 10 min at 4°C) and the supernatant was used to determine glycogen concentration using a glycogen assay kit (Cayman, Ann Arbor, Michigan).

2.6. Western Blot Analysis

Tissues or cultured cells were homogenized in cell lysis buffer (Cell Signaling, Danvers, MA) containing protease and phosphatase inhibitor cocktail (ThermoFisher Scientific, Waltham, MA). After centrifugation, equal amounts of protein from supernatants were resolved by stain-free SDS-PAGE and transferred onto nitrocellulose membranes. The blots were blocked with 5% (w/v) BSA or milk protein in Tris-buffered saline containing 0.1% Tween-20 and then probed with antibody against PEPCCK (H-300), G6Pase (H-60), PC (H-300), GCK (H-88), or GCK

regulatory protein (N-19) (Santa Cruz Biotechnology, Inc., Dallas, Texas). The immune-reactive proteins were detected by chemiluminescence and quantified using ChemiDoc™ Touch Imaging System (Bio-Rad, Hercules, California). The protein levels were normalized to total protein per lane.

2.7. Statistical Analysis

The data were analyzed by one-way ANOVA using SigmaPlot® (Version 11 Systate Software Inc., San Jose, California). If significant differences ($P < 0.05$) were observed, Duncan's multiple range test was then performed for pairwise comparisons. Values are expressed as mean \pm standard error of mean (SEM).

3. Results

3.1 Oral administration of kaempferol improved hyperglycemia in diabetic mice.

To determine whether kaempferol is capable of reversing or ameliorating hyperglycemia following overt diabetes, we first induced diabetes in mice via injecting multiple low doses of STZ [41]. Three wks post STZ administration, mice developed moderate to severe hyperglycemia as determined by measuring their fasting (206.2 ± 11.2 and 191.1 ± 20.1 for STZ and STZ+Kaemp, respectively) and non-fasting blood glucose levels (537.4 ± 24.6 and 512.7 ± 32.0 for STZ and STZ+Kaemp, respectively), all of which were higher ($P < 0.05$) as compared with healthy control mice (120.8 ± 8.3 and 194.7 ± 5.8 for fasting and non-fasting blood glucose levels, respectively). Kaempferol treatment significantly reduced both fasting (Figure 1(a)) and non-fasting (Figure 1(b)) blood glucose levels of diabetic mice. Glucose clearance (Figure 1(c, d)) was reduced significantly in diabetic mice as compared those to non-diabetic mice [42]. Consistently, kaempferol treatment improved ($P < 0.05$) glucose tolerance (69314.2 ± 283.2 and 64277.5 ± 1852.7 AUC for STZ and STZ+Kaemp, respectively), but this change may largely be ascribed to the initial difference of blood glucose concentrations between the diabetic control and kaempferol group. While diabetic mice displayed significantly lower non-fasting plasma insulin levels than those in non-diabetic mice, kaempferol treatment did not affect circulating insulin levels in diabetic mice during the treatment period (Figure 1(e)), suggesting that better glycemic control elicited by kaempferol treatment was not due to the improved insulin secretory function of pancreatic islets. Mice were considered overtly diabetic when non-fasting blood glucose concentration is more than 300 mg/dl [43]. Based on this diagnostic threshold, we calculated the percentage of diabetes before and 11 wks after kaempferol treatment. We found that kaempferol reversed the incidence of diabetes from 100 % to 77.8%, whereas the percentage of diabetic

control mice remained at 100% (Figure 1(f)). However, oral provision of kaempferol had no effect on BW (Figure 2(a)), food intake (Figure 2(b)), fat mass (Figure 2(c)) or lean mass (Figure 2(d)). Consistently, the inguinal and visceral fat contents were significantly lower in diabetic mice as compared with those in nondiabetic control mice, and kaempferol treatment did not alter these parameters (Figure 2(e)). To assess the effect of kaempferol treatment on lipid profiles, we measured the concentrations of plasma lipids. We observed that diabetic mice in the control group had lower total cholesterol, and HDL-cholesterol ($P<0.05$), and LDL-cholesterol ($P<0.05$) levels when compared to those in non-diabetic mice. Kaempferol treatment reversed these changes to the levels similar as seen in non-diabetic mice, but triglycerides levels did not differ between all groups (Figure 2(f)).

To further confirm the anti-diabetic effect of kaempferol, we performed the second study and evaluated whether kaempferol treatment is still effective when mice are diabetic for a longer period. In that regard, mice became overt diabetic for 6 wks before initiating treatment with kaempferol. Consistently, we observed that kaempferol did not affect BW or food intake during the time course of the study (Figure 3(a, b)). Consistently, kaempferol reduced the extent of non-fasting hyperglycemia once the treatment started (Figure 3(c)), similar to the observation in the first animal study. However, it took longer time (4 wks of treatment vs. 2 wks in the first study) for kaempferol to ameliorate fasting blood glucose (Figure 3(d)). In addition, kaempferol reduced the incidence of overt diabetes from 100 % to 87.5 % 5 wks after treatment (Figure 3(e)). These findings confirmed that kaempferol is effective in mitigating hyperglycemia, and it is more effective in ameliorating hyperglycemia in mice if the treatment started at the earlier stage of diabetes.

3.3 Kaempferol suppressed hepatic gluconeogenesis.

To determine the anti-diabetic mechanism of kaempferol in STZ-induced diabetic mice, we evaluated gluconeogenesis by performing pyruvate tolerance test. Hepatic glucose production increased ($P<0.05$) in diabetic mice as compared to non-diabetic mice [42]. As shown in Figure 5(a,b), kaempferol treatment suppressed hepatic gluconeogenesis ($P<0.05$), thereby glucose output (78771.7 ± 3555.9 and 66826.9 ± 5029.9 AUC for STZ and STZ+Kaemp, respectively). Next, we measured fasting circulating glucagon and insulin levels and calculated the molar ratio of insulin to glucagon. There were no significant differences in glucagon or insulin levels between treated and control diabetic mice. Also, the ratio of these two hormones was not altered by kaempferol treatment (Figure 4(a-c)). Further, we examined the key enzymes that regulate gluconeogenesis in the liver including PEPCK, G6Pase, and PC. Kaempferol treatment had no effect on PEPCK (Figure 5(c, g)), PC (Figure 5(d, g)), or G6Pase (Figure 5(e, g)) protein levels in diabetic mice. Diabetic mice displayed significantly higher PC activity as compared with healthy mice, but kaempferol almost normalized PC activity ($P<0.05$) in diabetic mice (Figure 5(f)). These data demonstrate that kaempferol improves glycemic control in diabetic mice at least in part through suppressing gluconeogenesis in the liver, and further suggest that kaempferol may inhibit gluconeogenesis via regulating pyruvate carboxylation, the first and critical step in gluconeogenesis.

3.4 Kaempferol increased GCK activity and glycogen content in the liver without affecting glucose oxidation.

In insulin-deficient mice, GCK expression in the liver decreases which impair hepatic glucose uptake and utilization [14]. Here, we found that diabetic mice had lower GCK protein levels as compared with non-diabetic mice, whereas glucokinase regulatory protein (GCKRP) did not differ between two groups. Kaempferol treatment restored GCK activity ($P<0.05$), whereas it had no effect on either GCK or GCKRP protein expression in diabetic mice (Figure 6(a-c, e)). An increase in GCK activity can divert glucose into glycogen synthesis and indirectly suppresses the overall glucose output in the liver [1, 2]. To determine whether kaempferol suppresses glycogenolysis, thereby contributing to the reduced glucose production as observed in kaempferol-treated mice, we measured glycogen contents in the liver. As shown in Figures 6(d), diabetic mice had lower glycogen contents ($P<0.05$) in the liver as compared with the healthy mice. However, treatment with kaempferol partially restored glycogen contents ($P<0.05$) in diabetic mice (Figure 6(d)). To rule out that kaempferol may have any effect on channeling glucose to other destinations, we measured glucose oxidation in the liver. There was no effect of kaempferol treatment on glucose oxidation in the liver (Figure 6(f)).

3.5 Kaempferol increased hexokinase activity and glucose oxidation in red muscle.

Insulin stimulates the transport and uptake of glucose [44] and consequently increases hexokinase activity in muscle [45]. Activation of hexokinase induces glucose phosphorylation that subsequently triggers glycolysis and glycogen synthetic processes [46]. Therefore, insulin deficiency reduces muscle glucose uptake as well as glucose metabolism. To examine whether kaempferol affected glucose metabolism in skeletal muscle, we measured hexokinase protein level and activity as well as glucose oxidation. Diabetic mice had lower ($P<0.05$) hexokinase protein levels as compared with non-diabetic mice. Consistent with the observation in the liver,

kaempferol restored the reduced hexokinase activity to the level comparable to that in nondiabetic mice (Figure 7(a, b)). In addition, kaempferol increased glucose oxidation in skeletal muscle ($P<0.05$) (Figure 7(c)), which may be the secondary whereby kaempferol treatment improved hexokinase activity. These findings suggest that kaempferol enhanced glucose metabolism in skeletal muscle of diabetic mice.

4. Discussion

In the present study, we show that a single daily dose of kaempferol via gavage can significantly improve hyperglycemia and glucose tolerance in STZ-induced diabetic mice, which is associated with increased glucose uptake and oxidation in muscle and suppressed hepatic glucose production. These beneficial effects are mediated by normalizing the activity of the key enzymes involved in regulating glucose metabolism and homeostasis. These effects of kaempferol were independent of insulin or glucagon concentrations and weren't accompanied by changes in BW gain, food intake, or body composition. In both T1D and T2D, dysregulated glucose metabolism characterized by the combination of increased hepatic glucose production and reduced glucose utilization plays an important role in the deterioration of blood glucose control. Thus, kaempferol could be used as an adjuvant treatment to maintain glucose homeostasis by targeting the glucose production and metabolic pathways.

The reciprocal relationship between insulin and glucagon in the regulation of glucose homeostasis is disrupted in STZ-induced diabetic mice due to the destruction of pancreatic β -cells [47]. Therefore, the amount of insulin secreted from the residual β -cells is insufficient to oppose glucagon action on hepatic glucose production or to inhibit its secretion, which results in excessive glucose production [48, 49]. In the present study, it is evident that kaempferol can mitigate diabetes by decreasing fasting and non-fasting blood glucose levels without altering insulin or glucagon concentrations in diabetic mice. At 11 wks into the study and 1 wk before the end of the treatment, non-fasting blood glucose levels of kaempferol-treated mice were lower than the initial levels and the incidence of overt diabetes among the mice decreased from 100% to 77.8 %, indicating that diabetes could be completely reversed if kaempferol treatment was provided for a longer period. The improved glucose homeostasis in kaempferol-treated mice

could be partly explained by the increase in the activity of hexokinases in the liver and muscle tissues due to their significant role in glucose disposal and metabolism. While, insulin promotes the transcription of the major hexokinase in the liver, GCK, glucagon inhibits it [50]. Although our results show that kaempferol non-significantly increased GCK protein levels, which were still lower than those in non-diabetic mice, its activity was completely restored by kaempferol treatment. However, it is still unclear how kaempferol increased GCK activity. GCK activity is regulated by a particular protein, GCKRP, which sequester GCK in the nucleus in its inactive form during fasting while releasing it to the cytoplasm when glucose supply increases [51]. While we found that kaempferol did not alter GCKRP protein expression, it is presently unclear whether kaempferol directly activates GCK activity or affects its cytoplasmic translocation, which requires further investigation.

It is well established that glucose uptake is mediated by the combined influence of glucose and insulin [52]. In the muscle tissue, insulin was reported to stimulate glucose transportation and phosphorylation pathway [53]. Further, the phosphorylated glucose is routed to different destinies; oxidation, glycogen synthesis, or glycolysis pathways [54, 55]. Insulin can also increase the activity of muscle hexokinase through increasing its protein level and modifying the already existing kinase [45]. In the present study, hexokinase levels and activity, as well as glucose oxidation, were decreased in the muscle of insulin-deficient diabetic mice. However, kaempferol restored hexokinase function and increased the rate of glucose oxidation. The increase in glucose oxidation in skeletal muscle of kaempferol-treated diabetic mice might be secondary whereby kaempferol increased hexokinase activity, which in turn stimulates glycolysis [46]. Further, it has been shown that an increase in hexokinase activity is associated with increased membrane translocation of the primary glucose transporter (GLUT4) [56], which

subsequently increases glucose uptake [57], suggesting that kaempferol may increase glucose uptake and utilization via modulating this pathway. However, more studies are needed to determine whether these molecules are directly targeted by kaempferol.

T1D is associated with lipoprotein abnormalities [58]. Although the abnormalities may vary, it is documented that T1D subjects may experience normal to low levels of blood lipoprotein concentrations when compared to non-diabetic subjects [59]. Similar findings were observed in SD-fed STZ-induced diabetic mice [60]. Here we show that kaempferol reversed the abnormalities of LDL and HDL concentrations that might be due to improvements in glucose control [61]. Nevertheless, the defects in composition and size of lipoproteins, and in particular LDL [62] may lead to atherosclerosis in diabetic subjects [58, 59] and experimental animals [60]. Thus, experiments may be necessary to help understand how kaempferol is improving lipoprotein synthesis and metabolism.

Excessive glucose production is a major contributor to hyperglycemia in experimental diabetes [63] and T1D subjects [16]. PC, a mitochondrial enzyme, catalyzes the reaction that produces oxaloacetate from pyruvate, the initial and critical step in gluconeogenesis [64]. While the functions and regulation of PC are still under investigation [65], its crucial role in the control of gluconeogenesis is well-established [9, 66, 67]. Recently, it has been proposed that reducing PC activity in the liver is a potential target for reducing hepatic glucose production, for the most part, gluconeogenesis [68]. Therefore, the anti-diabetic action of kaempferol we observed in this study is at least partially ascribed to its suppressing action on hepatic glucose production, as examined by performing the pyruvate tolerance test. This effect of kaempferol might be mediated via inhibiting the activity of PC. Additionally, increased glycogen content in the liver observed in kaempferol-treated mice, which could be due to the reduced glycogenolysis may also

contribute to the overall decreased glucose output in kaempferol-treated diabetic mice. However, the mechanism by which kaempferol reduces PC activity and glycogenolysis in the liver is currently unknown. Future research is needed to study these pathways.

In summary, we provide evidence that oral provision of kaempferol ameliorated hyperglycemia in insulin-deficient diabetic mice. This effect of kaempferol is associated with reduced hepatic gluconeogenesis and improved metabolism in the liver and skeletal muscle. These results indicate that kaempferol may be a naturally occurring, low-cost anti-diabetic agent to be used as an adjuvant treatment for diabetes. More research is needed to elucidate the underlying mechanism by which kaempferol regulates gluconeogenesis and glucose metabolism.

5. Figures

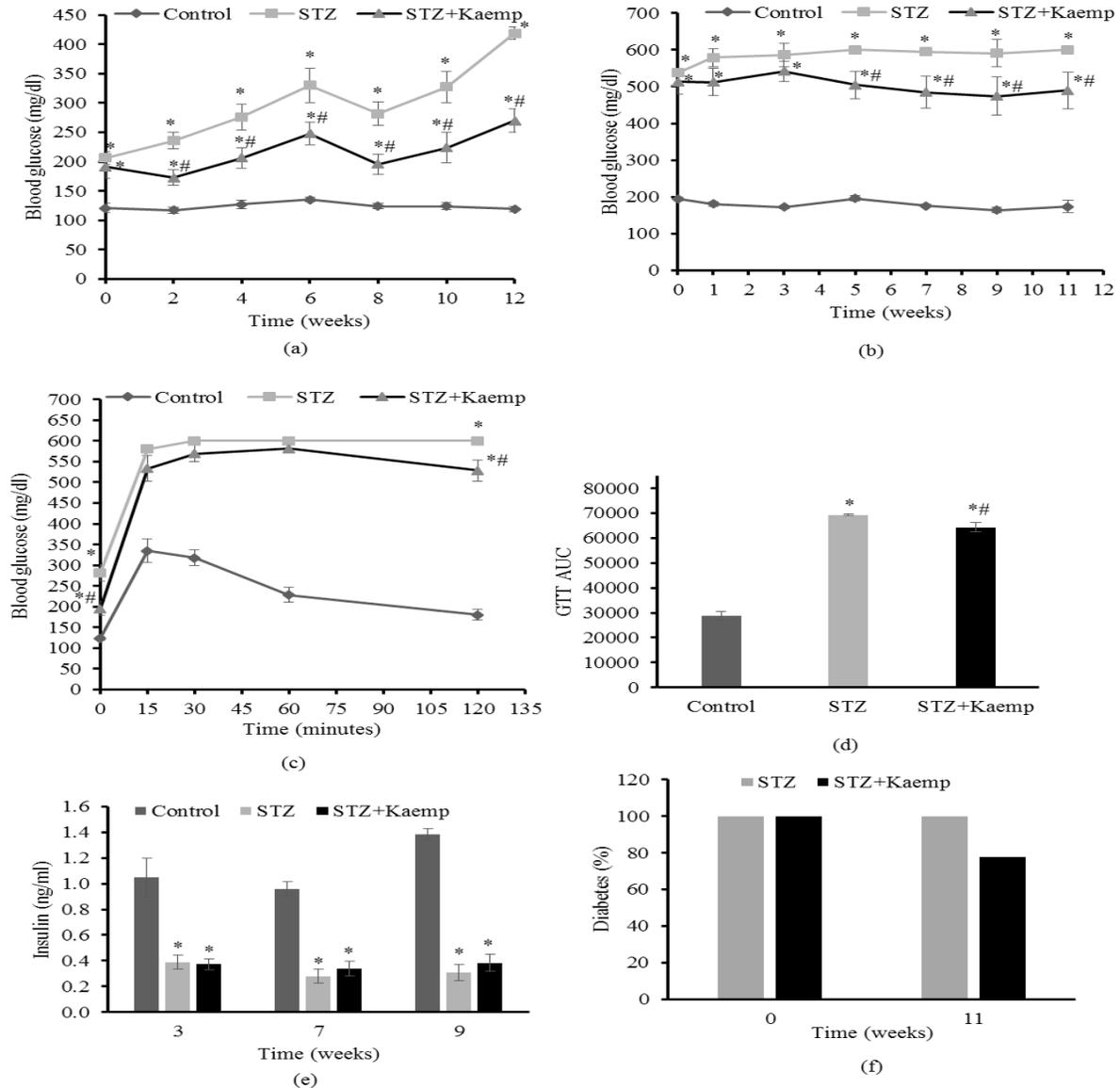


Figure 1. Kaempferol treatment reduced fasting and non-fasting blood glucose and improved glucose tolerance in STZ-induced diabetic mice. (a) Fasting and (b) non-fasting blood glucose levels were measured at indicated time points of dietary treatment. GTT (c) was performed as described in the Method section. The area under the curve (AUC) for GTT (d) was calculated. (e) Non-fasting blood glucose was withdrawn at week 3, 7, and 9 after treatment for measuring plasma insulin levels. (f) The incidence of diabetes was recorded with the percentage, and overt diabetes was calculated. Data are shown as Mean \pm SEM (n=9). *, $P < 0.05$ vs. non-diabetic mice; # vs. STZ-induced diabetic mice. Control: nondiabetic mice; STZ: STZ-induced diabetic mice; STZ+Kaemp: STZ-induced diabetic mice treated with kaempferol (50mg/kg BW).

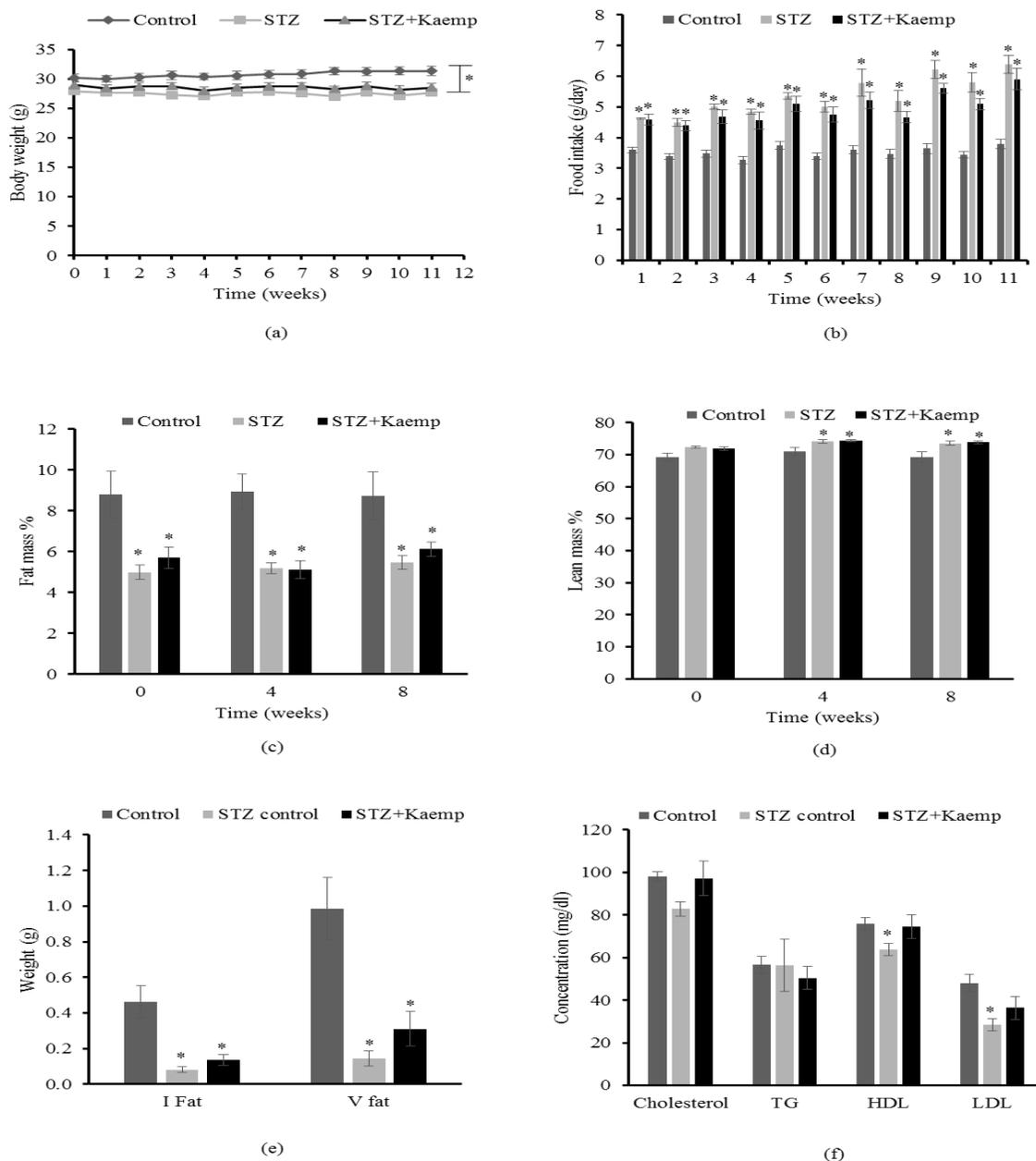


Figure 2. Kaempferol treatment had no significant effect on BW, food consumption, or body composition but restored lipid profiles in STZ-induced diabetic mice. (a) BW of the individual mouse was measured weekly. (b) Food intake was recorded each week, and the average daily food intake was calculated. Body composition including fat mass (c), and lean mass (d) were measured at 0, 4, and 8 wks of treatment. At the end of the study, (e) inguinal and visceral fat weight and (f) plasma lipid profile were measured. Data are shown as Mean \pm SEM (n=9). *, $P < 0.05$ vs. Control nondiabetic mice. Control: nondiabetic mice; STZ: STZ-induced diabetic mice; STZ+Kaemp: STZ-induced diabetic mice treated with kaempferol (50mg/kg BW).

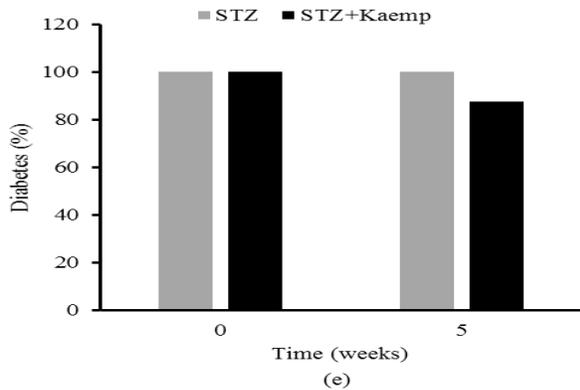
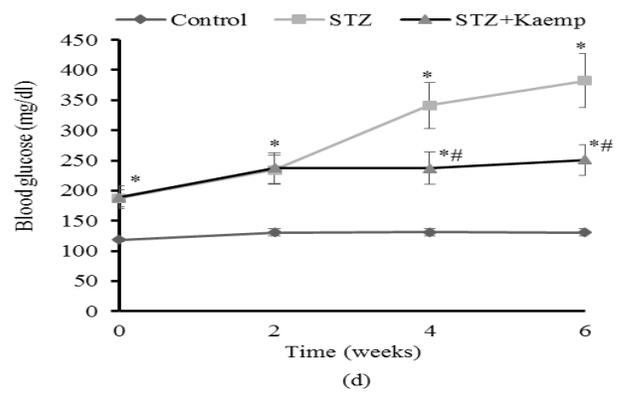
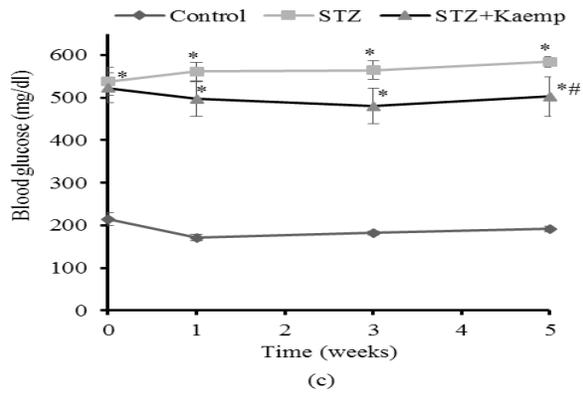
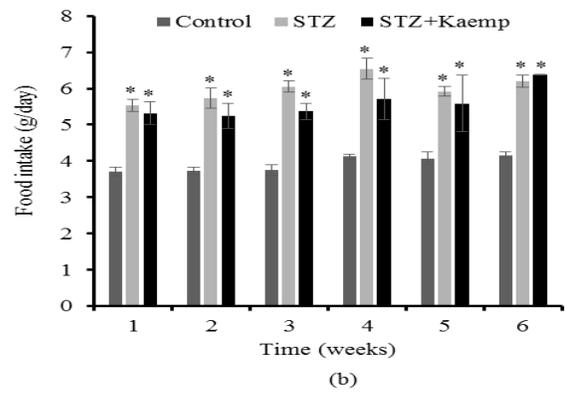
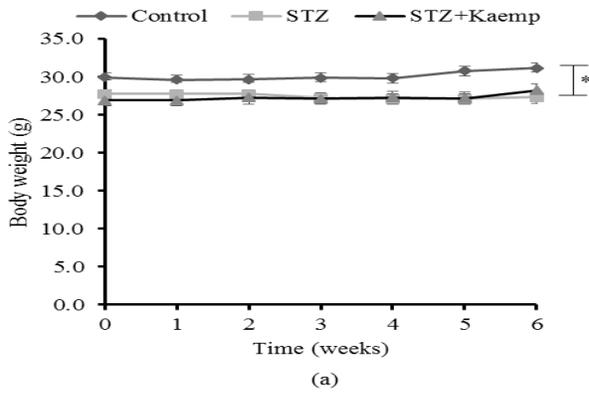


Figure 3. Kaempferol treatment 6 wks after STZ injections improved fasting and non-fasting blood glucose independent of BW gain, or food intake of STZ-induced diabetic mice. (a) BW of the individual mouse was measured weekly. (b) Food intake was recorded each week, and the average daily food intake was calculated. (c) Non-fasting and (d) fasting blood glucose levels were measured at indicated time points of dietary treatment. (e) Diabetes percentage was calculated. Data are shown as Mean \pm SEM (n=8-9). *, $P < 0.05$ vs. non-diabetic mice; # vs. STZ-induced diabetic mice. Control: nondiabetic mice; STZ: STZ-induced diabetic mice; STZ+Kaemp: STZ-induced diabetic mice treated with kaempferol (50mg/kg BW).

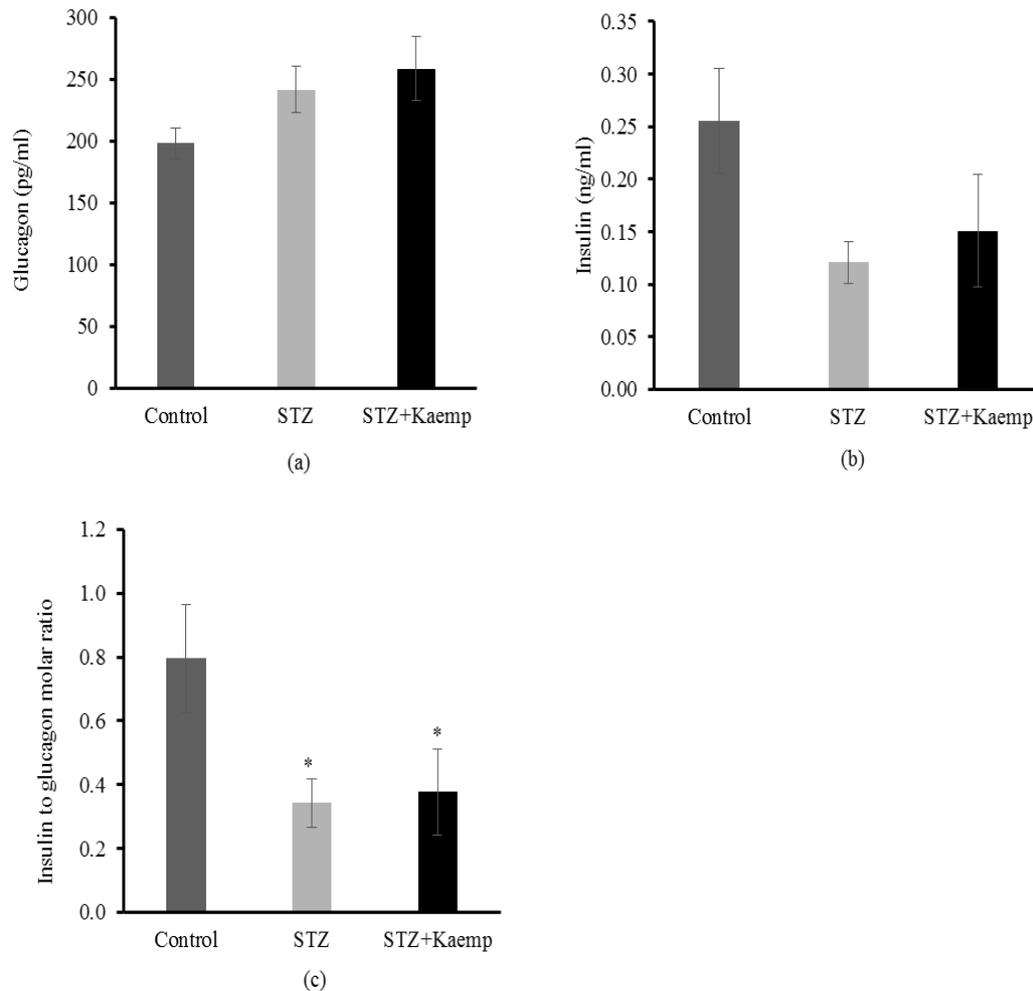


Figure 4. Kaempferol treatment had no effect on the concentrations of plasma insulin or glucagon. At the end of feeding experiment, (a) glucagon, and (b) insulin levels were measured using ELISA kits, and (c) their molar ratio was calculated. Data are shown as Mean \pm SEM (n=8-9). *, $P < 0.05$ vs. Control nondiabetic mice. Control: nondiabetic mice; STZ: STZ-induced diabetic mice; STZ+Kaemp: STZ-induced diabetic mice treated with kaempferol (50mg/kg BW).

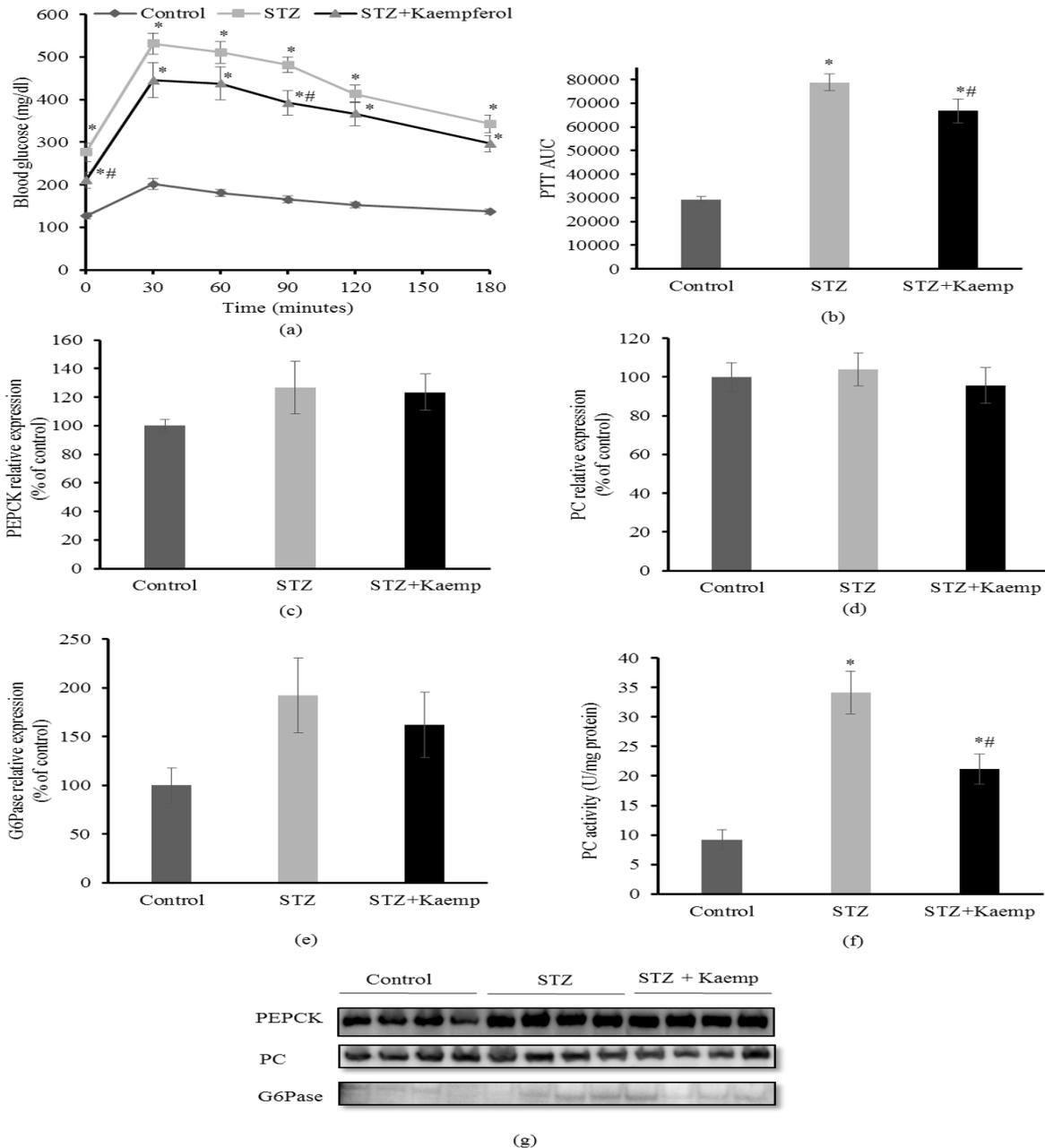


Figure 5. Kaempferol suppressed hepatic glucose production from pyruvate in diabetic mice and restored hepatic PC activity without altering protein expressions of PC, PEPCK, or G6Pase. PTT (a) was performed as described in the Method section. The area under the curve (AUC) for PTT (b) was calculated. At the end of feeding experiment, (c, g) PEPCK, (d, g) PC, and (e, g) G6Pase protein levels in whole cell lysates of liver tissue were measured by immunoblotting and normalized to total protein contents. (f) PC activity was measured as described in the Method section. Data are shown as Mean \pm SEM (n=8-9). *, $P < 0.05$ vs. non-diabetic mice; # vs. STZ-induced diabetic mice. Control: nondiabetic mice; STZ: STZ-induced diabetic mice; STZ+Kaemp: STZ-induced diabetic mice treated with kaempferol (50mg/kg BW).

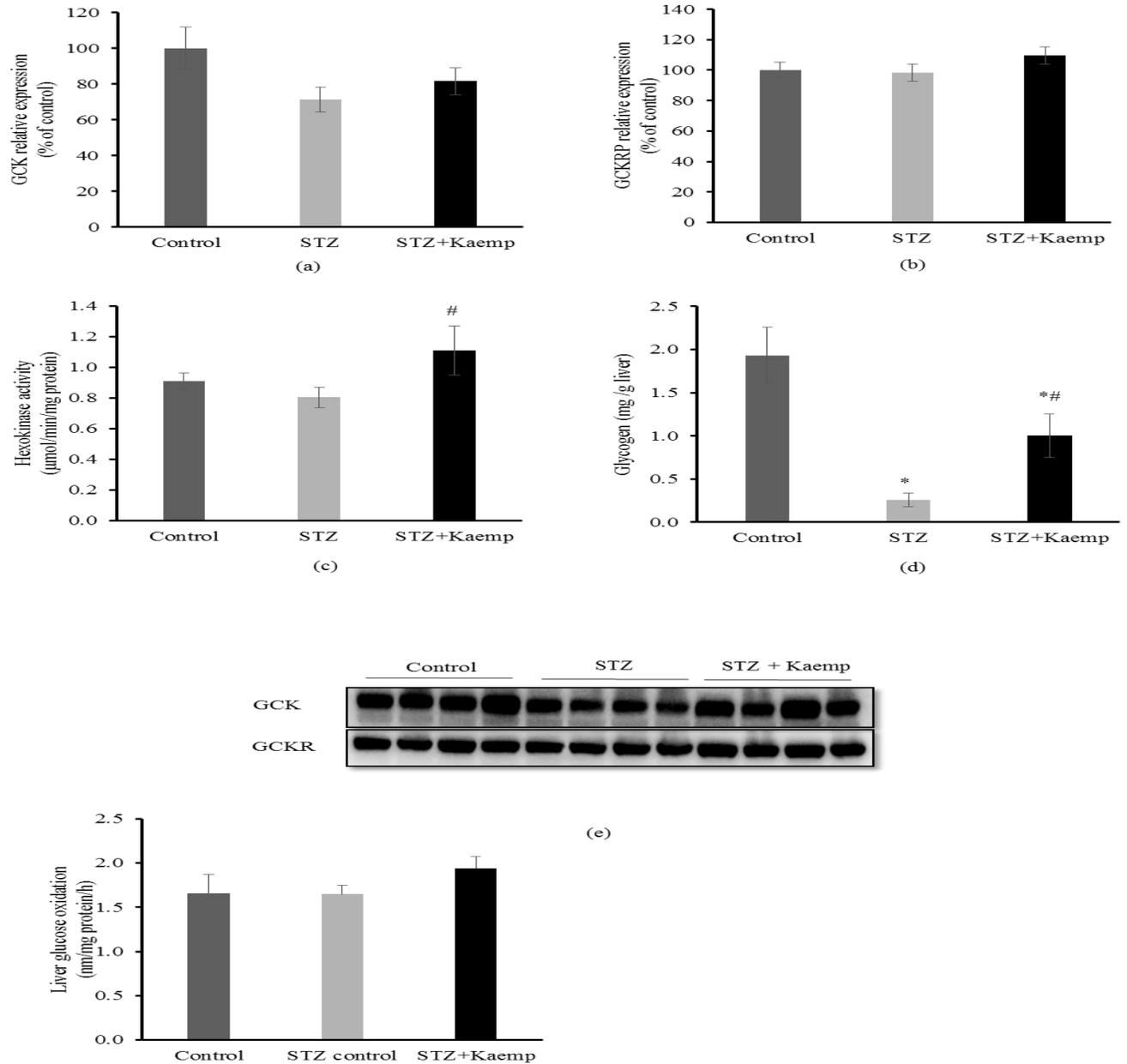


Figure 6. Kaempferol treatment increased GCK activity and glycogen contents in STZ-induced diabetic. (a, e) GCK, and (b, e) GCKRP protein levels in whole cell lysates of liver tissue of mice were measured by immunoblotting and normalized to total protein contents. Hexokinase activity (c) and glycogen content (d) were measured as described in the Method section. (f) Glucose oxidation was measured in fresh mouse liver homogenates using ^{14}C -labeled glucose as described in the Method section. Data are shown as Mean \pm SEM (n=8-9). *, $P < 0.05$ vs. non-diabetic mice; # vs. STZ-induced diabetic mice. Control: nondiabetic mice; STZ: STZ-induced diabetic mice; STZ+Kaemp: STZ-induced diabetic mice treated with kaempferol (50mg/kg BW).

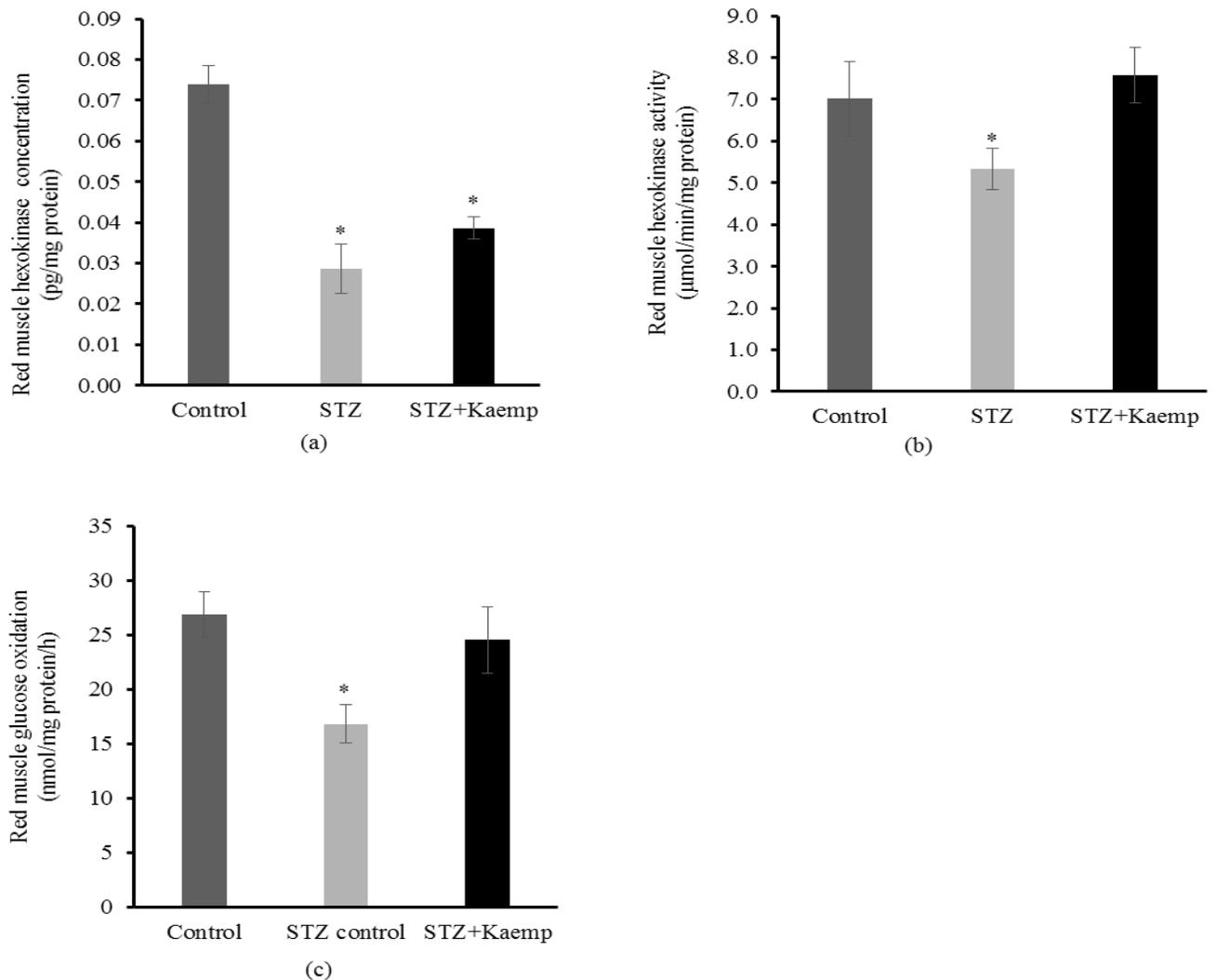


Figure 7. Kaempferol increased hexokinase activity and glucose oxidation in red skeletal muscle of STZ-induced diabetic mice. (a) Hexokinase concentration and (b) activity were measured in whole cell lysates of liver tissue of mice. (c) Glucose oxidation was measured in fresh mouse muscle homogenates using ^{14}C -labeled glucose as described in the Method section. Data are shown as Mean \pm SEM (n=8-9). *, $P < 0.05$ vs. Control nondiabetic mice. Control: nondiabetic mice; STZ: STZ-induced diabetic mice; STZ+Kaemp: STZ-induced diabetic mice treated with kaempferol (50mg/kg BW).

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CHAPTER FIVE

Kaempferol improves glucose control and suppresses glucose production by enhancing insulin sensitivity in diet-induced obesity and insulin resistant mice.

Abstract

Obesity and its associated insulin resistance (IR) are at the center of the pathophysiology of several metabolic diseases including type 2 diabetes (T2D). IR in the liver and muscle disturb glucose control that could lead to T2D. In particular, hepatic IR contributes to the increase in hepatic glucose production and the development of fasting hyperglycemia. In this study, we explore the role of kaempferol, a flavonoid isolated from Ginkgo biloba, on the regulation of blood glucose homeostasis in high-fat (HF) diet-fed obese mice. Oral administration of kaempferol (50 mg/kg) significantly improved blood glucose control through suppressing hepatic glucose production in obese mice. Kaempferol also enhanced whole body insulin sensitivity without altering body weight gain, food consumption, or adiposity of obese mice. In addition, kaempferol treatment increased Akt and hexokinase activity but decreased pyruvate carboxylase activity in the liver. Overall, these findings suggest that kaempferol may be a naturally occurring anti-diabetic compound by improving insulin sensitivity and suppressing glucose production.

Keywords: Insulin resistance, type 2 diabetes, hepatic glucose production, kaempferol.

1. Introduction

Obesity and insulin resistance (IR) are at the core of many prevalent diseases such as cardiovascular diseases [1], liver diseases, and diabetes [2, 3]. Diabetes is considered one of the major leading causes of mortality in the United States [4]. The prevalence of diabetes is still rising rapidly [5], reaching 9.3% of the U.S. population in 2012 [6], and this number is expected to double in 2050 [7]. The economic cost of treating diabetes and its complications is an increasing burden [8]. The estimated cost of diagnosed diabetes in the U.S. increased \$71 billion in 5 years [9]. While the cascade of events that leads to the development of T2D has long been the subject of debate [10, 11], it is well recognized that IR and β -cell dysfunction are key features in the pathogenesis of T2D [12].

Obesity is considered a major risk factor for developing IR and T2D [13]. Obesity-induced hepatic IR alters the hepatic regulation of glucose metabolism and production, through reducing glycogen synthesis and increasing gluconeogenesis and glycogenolysis [14], which significantly contributes to the fasting hyperglycemia [15]. Therefore, the liver is one of the primary targets for preventing and treating hyperglycemia and reducing the risk of diabetes. As both obesity and diabetes [16, 17] increasingly become a public health problem and economic burden [18], searching for naturally occurring, low-cost, and safe anti-diabetic compounds could be an effective strategy to prevent this disease. Flavonoids, polyphenolic compounds abundant in fruits, vegetables, and some medicinal herbs, are shown to exert some beneficial effects on diseases [19]. In the past few decades, research has been primarily focused on identifying the potential pharmacological activities of flavonoids including the antioxidant [20], anti-inflammatory [21], and anticancer activities [22]. From these flavonoids is kaempferol (3,5,7-trihydroxy-2-(4-hydroxyphenyl)-4H-1-benzopyran-4-one), a flavonol found in many traditional

and edible plants, which may possess anti-diabetic activities [23]. There are limited studies on the anti-diabetic action of kaempferol. We recently reported that long-term dietary intake of kaempferol supplemented in HF diet (0.05%) improved insulin sensitivity and hyperglycemia in middle-aged obese mice [24]. In addition, kaempferol was shown to improve basal glucose uptake and in a human hepatocyte cell line (Hep G2) in a dose-dependent manner (10–100 μ M) [25]. In a bioassay, a pharmacological dose of kaempferol (50 μ M) inhibited the activity of glucose-6-phosphatase (G6Pase) [26], which is one of the key gluconeogenic enzymes responsible for committing the final step of gluconeogenesis in the liver [27]. However, the physiological relevance of these in vitro findings are unclear, given the high doses of kaempferol are needed for the observed effects, which are likely unachievable via dietary intake of this compound. Moreover, how kaempferol exerts these effects is still unclear. In this study, we investigated the anti-diabetic effect of kaempferol in diet-induced obese insulin-resistant mice and further explored the mechanism underlying its effect. We show that oral administration of kaempferol (50 mg/kg BW) improved whole-body insulin sensitivity, and glucose tolerance and suppressed glucose production in HF diet-induced obese mice. Kaempferol treatment was associated with increased Akt and hexokinase activities but reduced pyruvate carboxylase (PC) activity in liver. These results suggest that kaempferol may ameliorate hyperglycemia and IR by restoring the role of the liver in maintaining glucose homeostasis that is disturbed in obesity.

2. Methods

2.1. Animals and experimental design

C57BL/6 male mice (4 mo old, Envigo, Indianapolis, IN) were maintained on a 12-h light/dark cycle at constant temperature (22–25°C) in an animal room with *ad libitum* access to a standard chow (SD) diet and water. All experimental protocols were approved by the Institutional Animal Care and Use Committee at Virginia Tech. Mice were divided into 2 groups ($n = 18$ mice/group) and fed either a SD diet, with 10% of calories derived from fat, or a high fat (HF) diet (Research Diets Inc., New Brunswick, NJ), with 58% of calories from fat for 8 wks to induce obesity and IR. Mice were then divided into 4 groups ($n = 9$ mice/group) with blood glucose, BW, and body composition balanced for the same dietary treatment groups, and then received either kaempferol (50 mg/kg/d dissolved in 2% 2-methyl cellulose) or vehicle (2% 2-methyl cellulose) by oral gavage for 6 wks.

2.2. Metabolic studies

BW and food intake were recorded weekly. Non-fasting and fasting blood glucose (15 h fasting) levels were measured weekly in tail vein blood samples using a glucometer (Kroger, Cincinnati, OH). Body composition was evaluated using an LF-90 instrument (Bruker Optics, Inc., Billerica, MA) at 0 and 4 wks after treatment. At 4 and 5 wks after kaempferol treatment, mice were fasted for 15 h and then injected intraperitoneally (IP) with a single dose of pyruvate (2 g/kg BW) or glucose (1.5 g/kg BW) for pyruvate and glucose tolerance tests, respectively. Blood glucose levels were then measured at 0, 30, 60, 90, and 180 min after administration of pyruvate and at 0, 15, 30, 60, and 120 min post-injection of glucose. For insulin tolerance test (ITT), mice were fasted for 4 h followed by ip injection of insulin (0.75 units/kg BW), and blood glucose levels

were then measured at 0, 15, 30, 60, and 120 min after insulin administration. The area under the curve (AUC) for all tests was calculated using the trapezoidal rule. At the end of the study, the mice were fasted for 15 h and then euthanized between 9-11 am. Blood was collected immediately, and multiple organs were weighed, snap-frozen in liquid nitrogen, and then stored at -80°C for further analyses. Plasma insulin levels were measured using an ultrasensitive mouse insulin ELISA kit (Merckodia, Inc., Uppsala, Sweden). Plasma glucagon levels were measured using a mouse glucagon ELISA kit (Crystal Chem, Downers Grove, IL).

2.3 Pyruvate and glucose oxidation

Fresh mouse liver samples were used to analyze pyruvate and glucose oxidation as previously described [28, 29] with modifications. Briefly, liver tissues were homogenized in the buffer (0.25 M Sucrose, 1 mM EDTA, 0.01 M Tris-HCl, and 2 mM ATP, pH=7.4). The tissue homogenates were then incubated with either ^{14}C -labeled pyruvate for pyruvate oxidation, or ^{14}C -labeled glucose (American Radiolabeled Chemicals, St. Louis, MO) for glucose oxidation in a trapping device at 37°C for 1 h. The $^{14}\text{CO}_2$ produced was trapped with 70% perchloric acid and the resulting sodium hydroxide was collected to assess CO_2 production.

2. 4. Enzyme activity analysis

Liver and muscle tissues were homogenized in ice-cold lysis solution and then centrifuged (13,000 rpm for 3 min at 4°C). Hexokinase activity in the cell lysates was measured using an assay kit (Biomedical Research Services Center, Buffalo, NY) according to the manufacturer's instruction. The test is based on the NADH-coupled reaction that leads to the reduction of tetrazolium salt INT to INT-formazan, which exhibits maximum absorbance at 492 nm. Briefly,

the samples were treated with control solution and a reaction solution containing the substrate (20 mM glucose) for 30 min, and the reaction was stopped using 3% acetic acid. Absorbance at 492 nm was measured using a microplate reader. The activity of hexokinase in the cell lysates was calculated using this equation: IU/L unit = $\mu\text{M}/(\text{L}\cdot\text{min}) = (\text{O.D.} \times 1000 \times 110 \mu\text{l}) / (30 \text{ min} \times 0.6 \text{ cm} \times 18 \times 10 \mu\text{l})$. Total hexokinase concentrations in the samples were determined using a mouse hexokinase ELISA kit (Elabscience, Beijing, China). PC activity was measured using a citrate synthase-coupled reaction as previously described [30].

2.5. Western Blot Analysis

Tissues or cultured cells were homogenized in cell lysis buffer (Cell Signaling, Danvers, MA) containing Halt Protease and phosphatase inhibitor cocktail (ThermoFisher Scientific, Waltham, MA). After centrifugation, equal amounts of protein from supernatants were resolved using stain-free SDS-PAGE and transferred onto nitrocellulose membranes. Blots were blocked with 5% (w/v) milk protein or BSA/Tris-buffered saline plus 0.1% Tween-20 and probed against phospho-Akt (Ser473) (#9271), Akt (#9272) (Cell Signaling Technology, Inc, Danvers, Massachusetts), PEPCCK (H-300), G6Pase (H-60), PC (H-300), glucokinase (H-88), and glucokinase regulatory protein (N-19) (Santa Cruz Biotechnology, Inc, Dallas, Texas). Proteins of interest were normalized to total protein per lane. The immunoreactive proteins were detected and imaged using ChemiDoc™ Touch Imaging System (Bio-Rad, Hercules, California).

2.6. Statistical Analysis

The data were analyzed by one-way ANOVA using SigmaPlot® (Version 11 Systate Software Inc., San Jose, California). If significant differences ($P < 0.05$) were observed, Duncan's multiple

range test was then performed for pairwise comparisons. Values are expressed as mean \pm standard error of mean (SEM).

3. Results

3.1. Kaempferol improved blood glucose control and insulin sensitivity in obese mice.

To determine whether kaempferol can reverse or ameliorate IR and further prevent the development of T2D, mice were first fed HF diet (containing 58% of energy from fat) for 8 wks to induce obesity and IR [31] before the treatment. Fasting and non-fasting blood glucose levels in mice were evaluated throughout the study (Figure 1 (a, b)). Obese mice had significantly higher fasting blood glucose levels than those of lean mice. However, oral treatment with kaempferol gradually reduced fasting blood glucose levels over time in obese mice. After 4 wks of treatment, kaempferol-treated obese mice displayed lower ($P<0.05$) fasting blood glucose levels as compared with the control obese mice (155.6 ± 7.5 mg/dl and 183.7 ± 5.6 mg/dl, respectively). After 6 wks of treatment, kaempferol treatment reversed fasting blood glucose levels comparable to those in SD-fed mice. However, kaempferol had no effect on fasting blood glucose of lean mice.

IR contributes to the development of impaired glucose tolerance (IGT) [32, 33]. Therefore we studied glucose tolerance of these mice. Obese mice had IGT when compared to lean mice. However, kaempferol treatment significantly improved glucose tolerance in obese mice but had no effect on glucose tolerance in lean mice (Figure 1 (c, d)). We further studied whether the improvement in glucose tolerance was associated with an increase in insulin levels at baseline or by IP glucose stimulation since there is an association between IGT and defects in insulin secretion [32]. To compensate for IR, β -cells increase insulin secretion to achieve normal glycemia [34]. Obese mice displayed hyperinsulinemia with higher ($P<0.05$) fasting plasma insulin levels than those in lean mice. Regarding their response to IP glucose injection, obese mice exhibited IR characteristics which were associated with an increase in insulin secretion and

rates [35]. Obese mice had significantly higher plasma insulin levels after 30 and 60 min of glucose injection than those detected in lean mice. Kaempferol did not modulate either basal insulin levels or glucose-stimulated insulin secretion (Figure 1 (e)). Next, we assessed whole body insulin sensitivity in the mice by performing ITT. As shown in (Figure 1 (f, g)), obese mice were insulin resistant as compared with lean mice, but kaempferol treatment normalized whole body insulin sensitivity in obese mice.

3.2. Kaempferol had no effect on BW, food intake, or body composition of lean and obese mice.

The average BW of experimental mice fed HF diet (45.4 ± 1.14 g) was higher ($P < 0.05$) than mice fed SD (32.0 ± 0.89 g) at the beginning of the study and remained higher throughout the study. Kaempferol had no effect on either BW (Figure 2 (a)) or food intake of obese and lean mice (Figure 2 (b)). Body fat percentages for obese mice (23.7 ± 0.95 %) were significantly higher than lean mice (6.6 ± 1.01 %) throughout the study (Figure 2 (c)). Conversely, lean mass % was lower (Figure 2 (d)). Kaempferol had no effect on the degree of adiposity in either obese or lean mice. At the end of the study, the inguinal and visceral fat were significantly heavier in the obese mice than lean mice (Figure 2 (e)). Kaempferol had no effect on either fat type.

For the pancreatic β -cells to compensate for IR before the development of overt hyperglycemia and T2D, their mass increase [36] and the pancreas weight is reported to become heavier [37]. Although obese insulin-resistant mice in the control group had heavier pancreas than lean mice (223.8 ± 18.7 and 180.1 ± 9.3 mg, respectively), the difference was not significant. Also, kaempferol treatment did not have a significant influence on the pancreas weight (Figure 2 (f)).

3.3. Kaempferol suppressed liver gluconeogenesis and PC activity in obese mice.

As the oral administration of kaempferol mitigated fasting hyperglycemia in obese mice, which is mainly caused by the increased hepatic glucose production in IR and T2D [38], we examined whether kaempferol affected hepatic gluconeogenesis, which appears to be predominantly responsible for the excessive hepatic glucose output in T2D [39]. We show that kaempferol significantly decreased hepatic glucose production in obese mice ($P<0.05$) as determined by a pyruvate tolerance test (Figure 3 (a, b)).

To further assess kaempferol effect on glucose production, we studied some key enzymes that play a vital role in hepatic gluconeogenesis. PC (Figure 3 (c, g)) and G6Pase (Figure 3 (d, g)) protein levels were significantly higher in obese mice as compared with lean mice, whereas PEPCCK protein expression did not differ between groups (Figure 3 (e, g)). Although kaempferol did not have a significant effect on protein expression of these enzymes in the liver, it almost restored the reduced PC activity in obese mice to the levels similar to that observed in the lean mice ($P<0.05$) (Figure 3 (d)).

3.4. Kaempferol increased Akt and hexokinase activity but had no effect on pyruvate and glucose oxidation in obese mice liver.

Glucagon regulates glucose homeostasis and counteracts insulin action by increasing hepatic glucose production by increasing glycogenolysis and gluconeogenesis [40]. To determine whether kaempferol regulation of fasting blood glucose is due to an effect on glucagon levels, we measured fasting plasma glucagon concentrations. As seen in Figure 4 (a), circulating glucagon

concentrations did not differ between groups. We then evaluated Akt phosphorylation in the liver, as the activation of Akt mediates insulin suppression of gluconeogenesis while increasing glycogen synthesis [41]. We observed that Akt protein levels in obese mice liver were higher ($P<0.05$) than those of lean mice (Figure 4 (b, h)). Kaempferol administration increased Akt phosphorylation in obese mice (Figure 4 (c, h)). In addition, the activity of hepatic hexokinase, which plays a primary role in glucose disposition in the liver by catalyzing the first step in the glycolytic process and inducing glycogen synthesis [42], was significantly increased ($P<0.05$) in kaempferol-treated obese mice (Figure 4 (d)). The increase in hepatic hexokinases activity wasn't associated with a change in protein expression of total hexokinases (Figure 4 (g)) or GCK (Figure 4 (e, h)), which accounts for 90 and 95% of the total activity of glucose phosphorylation in rat and human liver, respectively [43]. Moreover, GCK regulatory protein (GCKRP) contents were similar between all groups (Figure 4 (f, h)). Further, kaempferol treatment did not modulate glucose or pyruvate oxidation in hepatic tissues, suggesting that the reduction of hepatic glucose production observed in kaempferol-treated mice was not due to increased glucose glycolysis or oxidation (Figure 5 (a, b)).

3.5. Kaempferol had no effects on Akt and hexokinase in skeletal muscle.

As kaempferol increased Akt phosphorylation and GCK activity in the liver, we further assessed whether similar outcomes occurred in muscle tissue in the fasting state thus contributing to glucose control. We measured skeletal muscle Akt and hexokinase protein levels and activities. Unlike kaempferol effect on the liver, neither proteins nor their activities were changed in the muscle tissues (Figure 6 (a-d)).

4. Discussion

In this study, we demonstrate that daily oral administration of kaempferol (50 mg/kg) for 4 wks could restore glucose homeostasis and insulin sensitivity independent of BW or adiposity in HF diet-induced obese mice. These effects were associated with a robust suppression of hepatic glucose production. Impaired intraperitoneal IGT and fasting hyperglycemia may reflect the abnormal response of β -cells to circulating glucose and impaired insulin sensitivity. However, kaempferol had no effects on basal circulating insulin levels or glucose-stimulated insulin secretion, the reduction in IGT and fasting blood glucose observed in kaempferol-treated mice may be primarily due to the direct actions of kaempferol on hepatic gluconeogenesis and glucose metabolism. Indeed, we observed an increase in hepatic Akt and GCK activities in obese mice treated with kaempferol. Also, kaempferol decreased PC activity. Therefore, the improvement of glucose homeostasis appears to be the result of reduced hepatic glucose production. Importantly, kaempferol did not exert an effect on the metabolic phenotype of lean, healthy mice, suggesting that the potential side effect of nutritional supplementation of kaempferol is minimal and that the health benefits of some flavonoids may depend on the metabolic state [44]. These results, together with our recent findings that long-term dietary intake of kaempferol prevented diet-induced IGT and IR in middle-aged mice [24], suggest that kaempferol could potentially be a safe and inexpensive natural compound for preventing and treating T2D via modulating hepatic glucose production and insulin sensitivity.

It is well recognized that obesity increases the risk for developing IR and T2D. C57BL/6 mice were fed HF diet for 8 wks before kaempferol treatment to induce obesity, IR, and glucose intolerance to determine the potential therapeutic effect of kaempferol on obesity-related IR and glucose intolerance. The predisposition of these mice to developing T2D is initiated by the

development of obesity followed by IR, which will further become overt diabetes [45]. The diet-induced obese mouse models, therefore, has been widely used due to the phenotypic similarities induced by Western diet seen in humans [31], although IR can develop at any degree of adiposity in humans [46]. In the present study, we observed that kaempferol improved glucose tolerance and insulin sensitivity without altering insulin levels, BW gain or the degree of adiposity, suggesting that the anti-diabetic action of kaempferol is not a secondary effect whereby it modulated these metabolic parameters. Consistently, previous studies demonstrated that treatment with kaempferol and some of its glycosides prepared from unripe Jinadi soybean leaves (0.095% and 0.15%, respectively incorporated in SD) for 4 wks slightly improved glucose tolerance and lowered HbA_{1c} levels without affecting BW, food intake, or adiposity in T2D KKA^y-mice, [47]. However, these mice spontaneously develop diabetes at a young age even when fed a SD chow diet. In another study, kaempferol glycosides from unripe soybean leaves (0.15% supplemented in HF diet) improved insulin sensitivity, reduced BW and adiposity and decreased fasting blood glucose levels in HF diet-induced obese mice 13 wks after treatment [48]. These results from various mouse models and experiment design collectively provide evidence that kaempferol indeed exerts anti-diabetic action.

The liver plays an integral role in maintaining glucose homeostasis [49]. Hepatic IR is associated with impaired regulation of the expression and activity of enzymes that control glucose production [50] such as PC, PEPCK [51], and G6Pase [52], consequently leading to excessive hepatic glucose production through gluconeogenesis and glycogenolysis, which primarily contribute to fasting hyperglycemia [53-55]. Increased hepatic gluconeogenesis, in particular, is considered one of the early pathological changes in newly diagnosed T2D subjects [53]. The activation of Akt by insulin contributes to the control of hepatic glucose metabolism

[56]. In addition, induction of Akt signaling stimulates glycogen synthesis [57, 58] and downregulates PEPCK and G6Pase gene expression [59], thereby reducing hepatic glucose output. In this study, we found that kaempferol increased hepatic Akt activity of obese mice. However, PEPCK or G6Pase protein levels were not altered by kaempferol treatment. Our results show that G6Pase protein levels increased significantly in obese when compared to lean mice, whereas PEPCK protein levels were not altered. This observation was consistent with the results of another study [60]. In T2D, it was demonstrated that neither was the gene expression of PEPCK and G6Pase changed nor were their expression levels associated with fasting hyperglycemia [61]. Further, the elevated hepatic glucose production observed in morbidly obese diabetic patients was associated with an increase in liver G6Pase activity [62], suggesting that the increase in gluconeogenesis in IR and diabetic subjects might be primarily due to changes in the activities of one or more enzymes rather than increases in protein expression.

PEPCK and G6Pase play a vital role in gluconeogenesis. However, they display a relatively weak control over its flux [63]. Instead, PC, which is responsible for the first step in gluconeogenesis, was found to be strongly associated with glycemia in humans [64], as its inhibition greatly reduced gluconeogenesis in vitro and in vivo [65]. To our surprise, we observed that kaempferol treatment greatly inhibited PC activity in the liver. Therefore, it is conceivable that kaempferol may suppress gluconeogenesis and thus hepatic glucose output via inhibition of this glucogenic enzyme. Moreover, in our study, the inhibition of PC activity was not due to modifications at the protein level, and it is presently unclear whether kaempferol directly or indirectly modified PC activity in the livers. There is a possibility that the reduction in PC activity by kaempferol could partially explain the increase in Akt activity and the reduced hepatic IR in kaempferol-treated obese mice. An interesting finding from a study conducted by

Kumashiro et al. may clarify this relation. In their study, they specifically inhibited hepatic and adipose tissue PC expression through ip injection of a specific antisense oligonucleotide in HF-fed rats. The inhibition in PC in liver and adipose tissue improved hepatic insulin sensitivity reflected by suppression of hepatic glucose production and an increase in hepatic Akt activity [64].

Activation of GCK, the predominant hexokinase in the liver, is proposed to be a potential target for diabetes treatment due to its contribution to improved hepatic glucose regulation [42]. When activated, GCK phosphorylates glucose and increases its clearance by diverting glucose into glycolysis and inducing glycogen synthesis in the liver [66]. Although the factors that regulate GCK are not entirely studied, several studies demonstrated the involvement of insulin and GCKRP in GCK regulation [67]. In T2D, hepatic glucose uptake and transport are altered thus its flux into hepatocytes decreases. It is suggested that these alterations associated with IR and diabetes are mainly due to reduced GCK activity [68]. Therefore, targeting GCK to promote its activity could be substantial for glycemic control. Several studies demonstrated that the use of GCK activators improved glucose control through improving glucose tolerance and increasing hepatic glucose uptake in T2D rodent models [69], and enhanced insulin sensitivity in HF diet-fed mice [70]. Consistently, we found that kaempferol treatment increased GCK activity in HF diet-fed mice, which may contribute to the improved glucose control and enhanced insulin sensitivity as observed in these mice. While the increase in GCK activity might increase glycolysis and glycogen synthesis, in our study the increase in GCK was not accompanied by an increase in hepatic glycolysis. Glycogen synthesis is primarily controlled at the level of GCK and its product, glucose-6-phosphate, whereas glycolysis is controlled by other enzymes such as phosphofructokinase, and to a lesser extent by GCK [71, 72]. However, it is unclear whether this

activation was a direct effect of kaempferol or a result of the alteration in other metabolic pathways, which warrants further investigation.

In summary, we confirm in this study that the oral administration of kaempferol ameliorates IR in obese insulin-resistant mice. This effect of kaempferol is associated with improved glucose tolerance and suppressed hepatic glucose production as well as improved whole-body insulin sensitivity. Therefore, kaempferol may be a natural agent with a promising potential for the treatment of IR and prevention of T2D. To determine whether kaempferol activates these enzymes' directly or through inducing other pathways warrants further investigation.

5. Figures

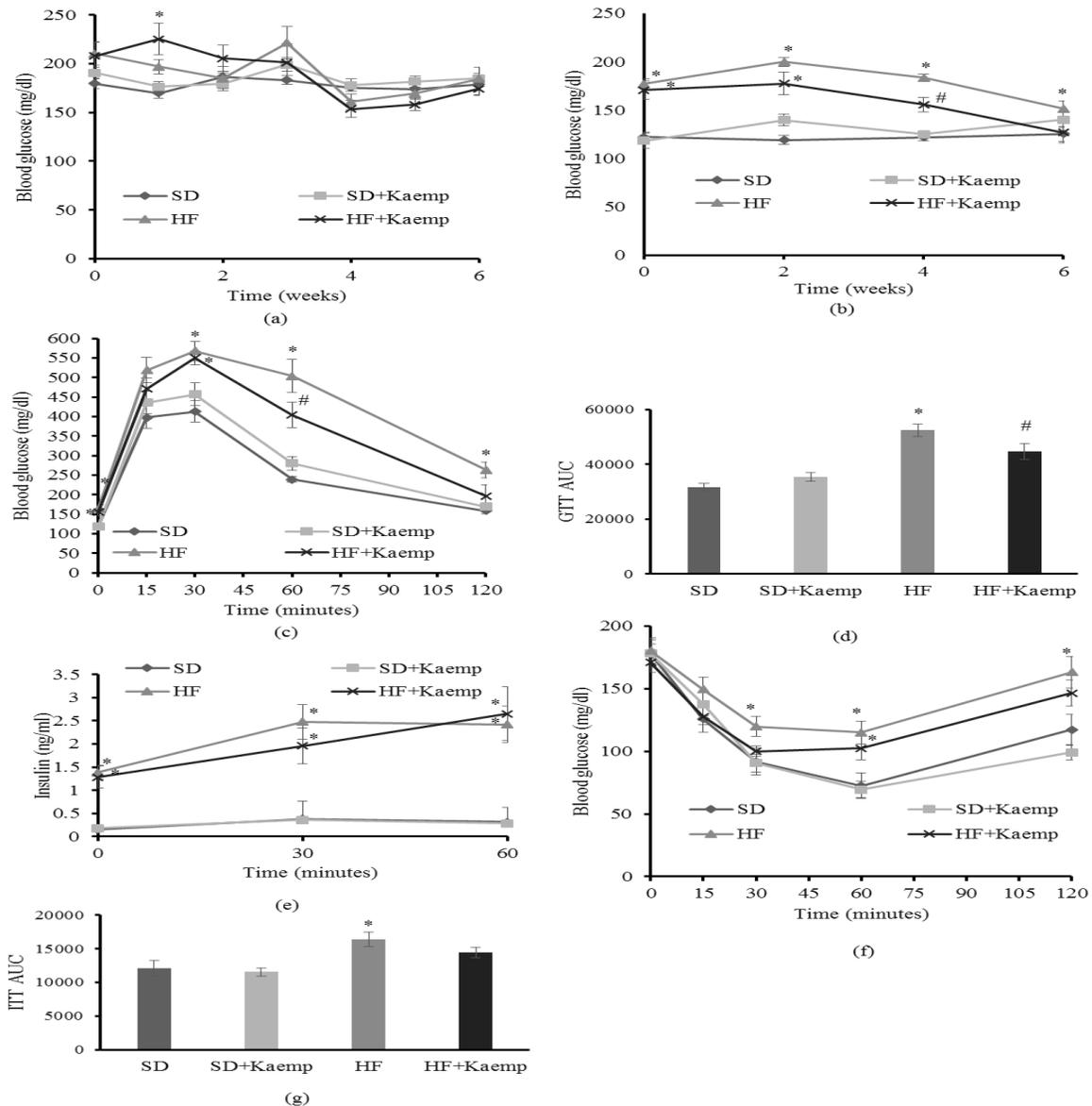


Figure 1. Kaempferol treatment reduced fasting blood glucose, improved glucose tolerance, and improved insulin sensitivity in HF diet-fed mice. (a) Non-Fasting and (b) fasting blood glucose levels were measured at indicated time points of dietary treatment. GTT (c) and (f) ITT were performed as described in the Method section. The area under the curve (AUC) for GTT (d), and ITT (g) was calculated. (e) Blood glucose was withdrawn at time 0, 30, and 60 min after IP glucose injection to measure plasma insulin levels. Data are shown as Mean \pm SEM (n=9). *, $P < 0.05$ vs. SD-fed mice (SD); #, $P < 0.05$ vs. HF-fed mice (HF). SD: SD diet; SD + Kaemp: SD diet with kaempferol treatment (50mg/kg BW); HF: HF diet; HF + Kaemp: HF diet with kaempferol treatment (50mg/kg BW).

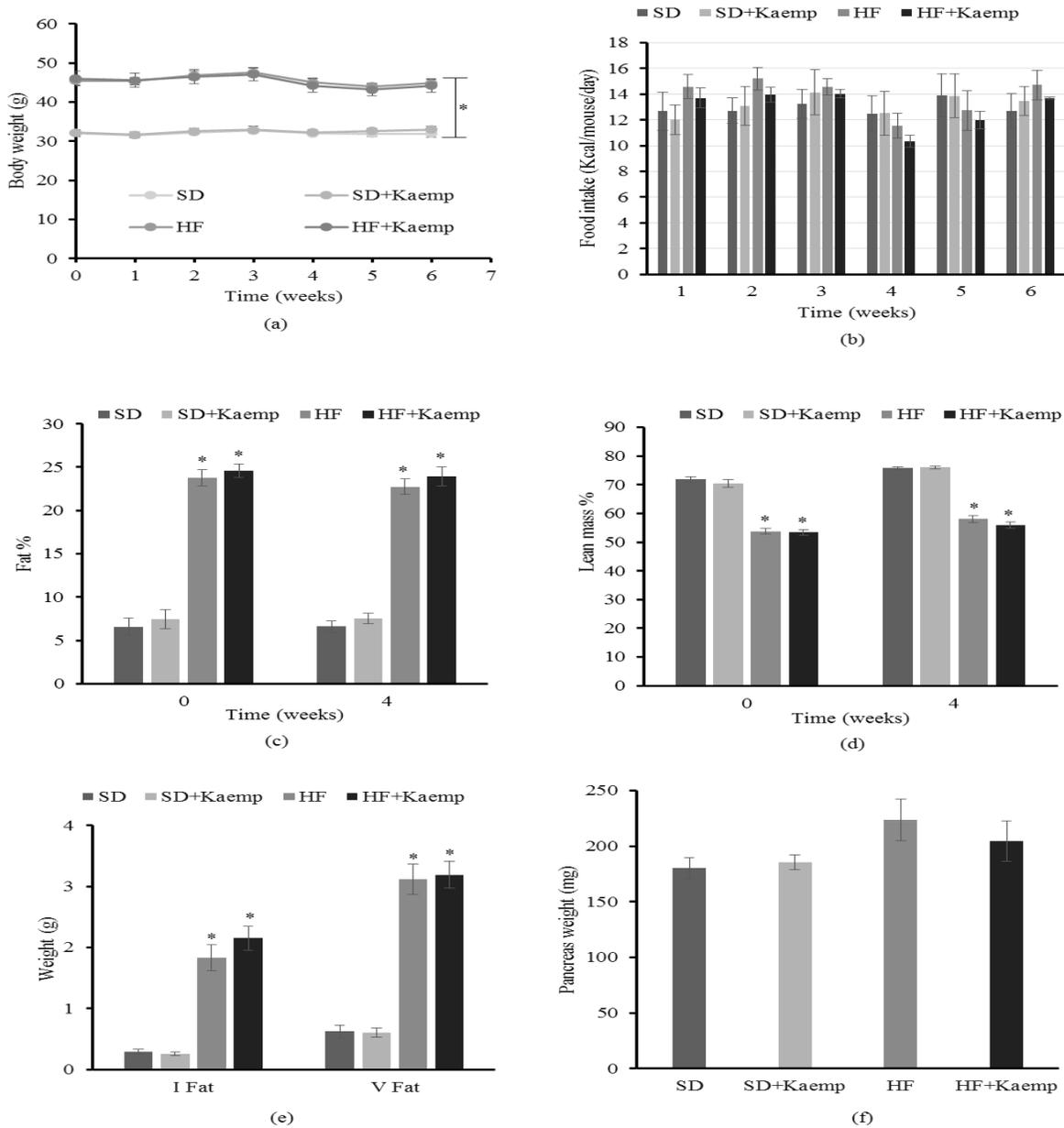


Figure 2. Kaempferol treatment had no significant effect on BW gain, food consumption, body composition, fat tissue or pancreas weight in HF diet-fed mice. (a) BW of the individual mouse was measured weekly. (b) Food intake was recorded each week, and the average daily food intake was calculated. Fat mass (c) and lean mass (d) at 0 and 4 wks after treatment were measured and expressed as percent of BW. At the end of the experiment, (e) inguinal (I fat) and visceral fat (V fat), and (f) pancreas were weighed. Data are shown as Mean \pm SEM (n=9). *, $P < 0.05$ vs. standard diet-fed mice (SD). SD: SD diet; SD + Kaemp: SD with kaempferol treatment (50mg/kg BW); HF: HF diet; HF + Kaemp: HF diet with kaempferol treatment (50mg/kg BW).

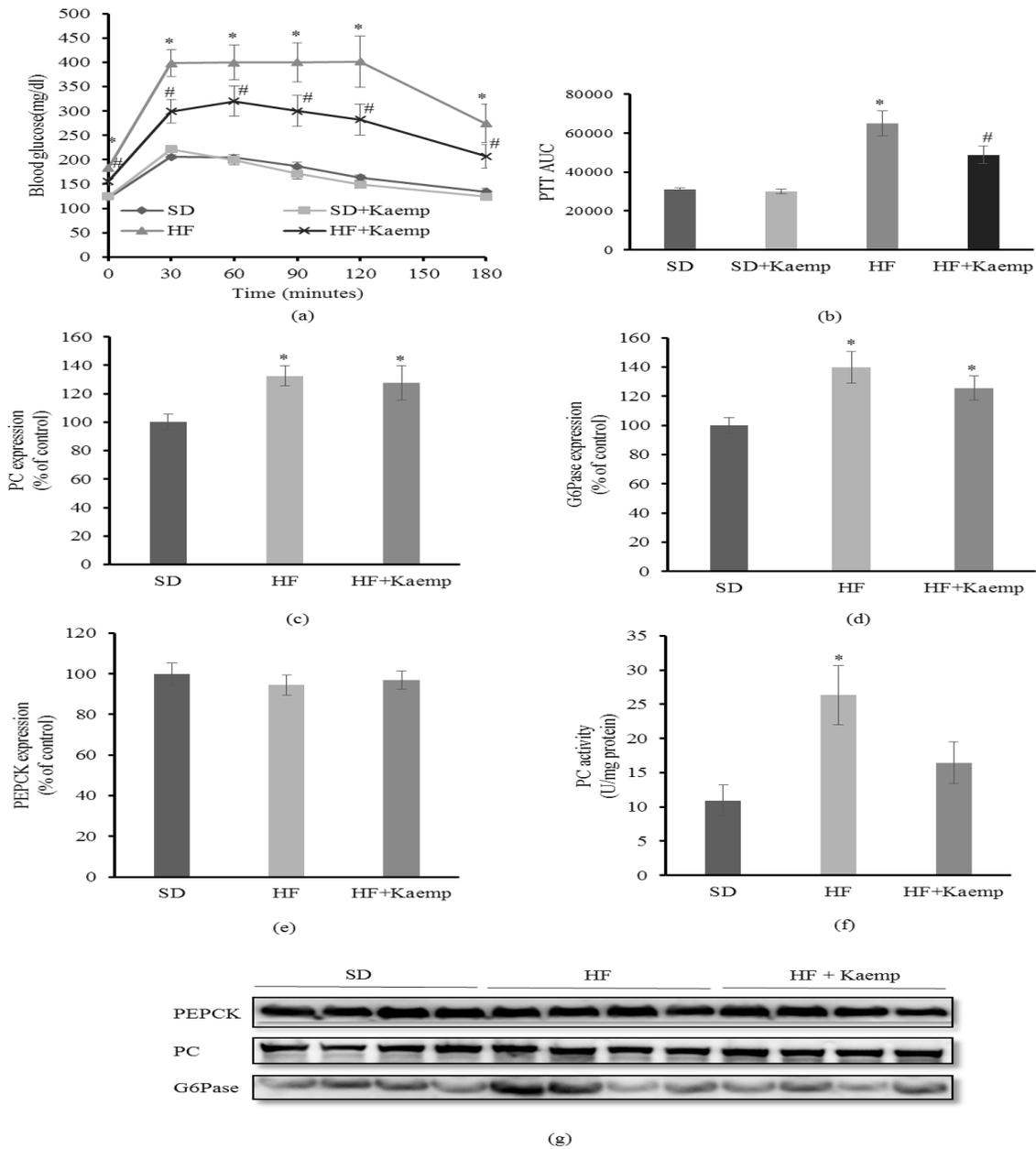


Figure 3. Kaempferol suppressed hepatic glucose production and restored the impaired hepatic PC activity without altering its protein expression in HF diet-fed mice. (a) PTT was performed in the Method section and the area under the curve (AUC) for PTT (b) was calculated. At the end of feeding experiment, (c,g) PC, (d,g) G6Pase, and (e,g) PEPCK protein levels in whole cell lysates of liver tissue of mice were measured by immunoblotting and normalized to total protein, which were not altered by kaempferol. (f) PC activity was measured as described in the Method section. Values are Mean \pm SEM from 8-9 mice per group. *, $P < 0.05$ vs. standard diet-fed mice (SD); #, $P < 0.05$ vs. HF-fed mice (HF). SD: SD diet; SD + Kaemp: SD with kaempferol treatment (50mg/kg BW); HF: HF diet; HF + Kaemp: HF diet with kaempferol treatment (50mg/kg BW).

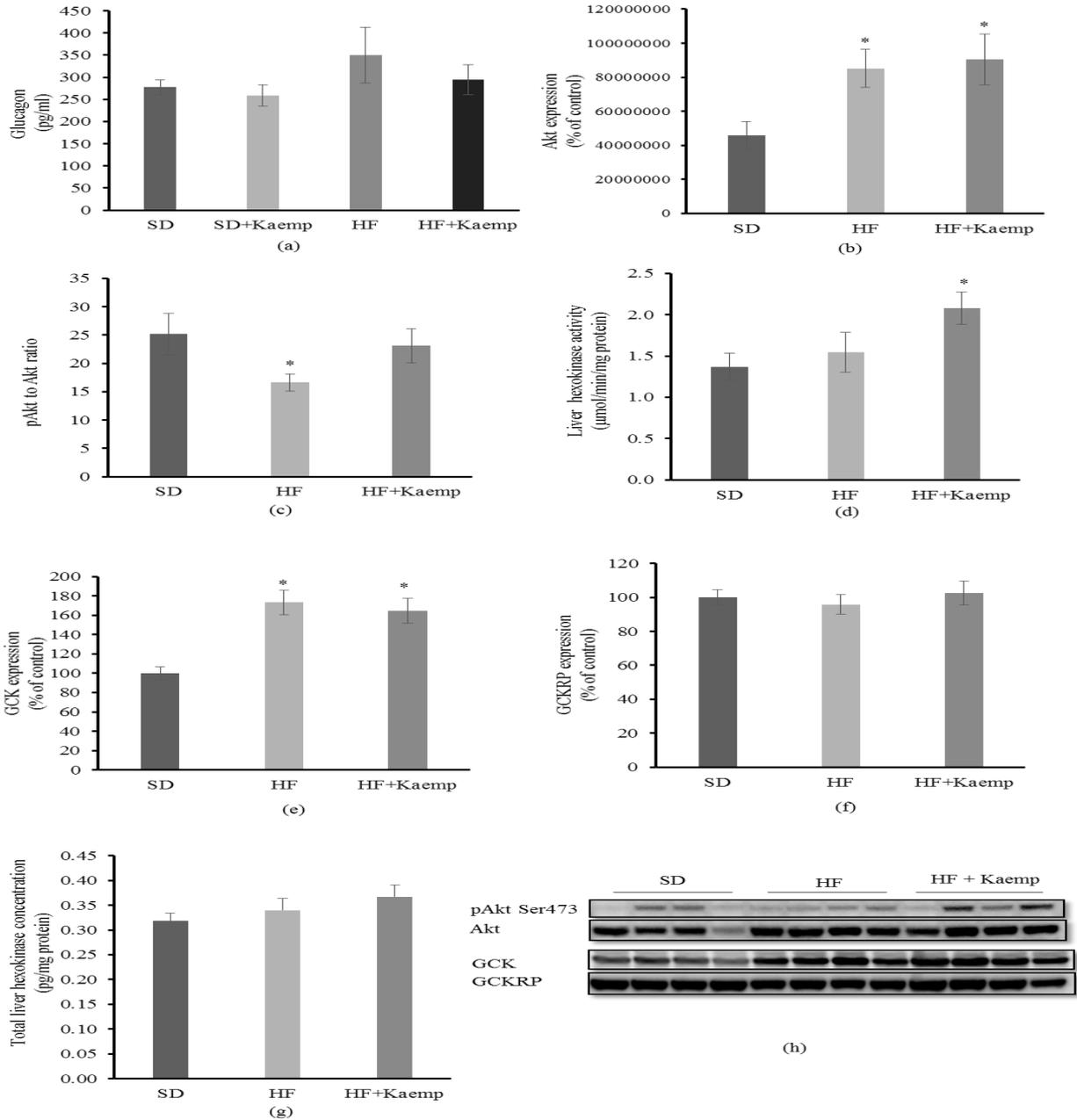


Figure 4. Kaempferol treatment increased liver Akt and GCK activity in HF-fed mice. At the end of the feeding experiment, (a) fasting plasma glucagon levels were measured using ELISA kit. (b, h) Akt, (c, h) pAkt, (e, h) GCK and (f, h) GCKRP protein levels in whole cell lysates of liver tissue of mice were measured by immunoblotting and normalized to total protein. (d) Hexokinase activity and (g) total hexokinase contents in the liver were measured as described in the Method section. Values are Mean \pm SEM from 8-9 mice per group. *, $P < 0.05$ vs. standard diet-fed mice (SD). SD: SD diet; HF: HF diet; HF + Kaemp: Hf diet with kaempferol treatment (50mg/kg BW).

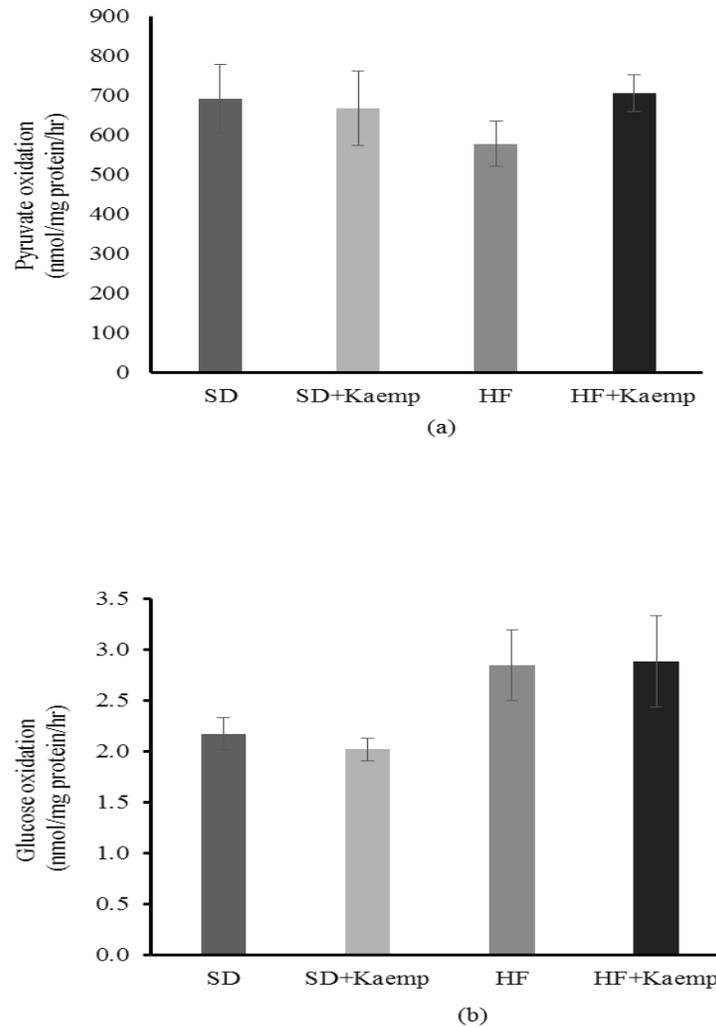


Figure 5. Kaempferol had no effect on glucose and pyruvate oxidation in the liver of HF diet-fed mice. (a) Pyruvate and (b) glucose oxidation were measured in fresh mouse liver homogenates using either ^{14}C -labeled pyruvate for pyruvate oxidation or ^{14}C -labeled glucose as described in the Method section. Values are Mean \pm SEM from 7 mice per group. SD: SD diet; SD + Kaemp: SD diet with kaempferol treatment (50mg/kg BW); HF: HF diet; HF + Kaemp: HF diet with kaempferol treatment (50mg/kg BW).

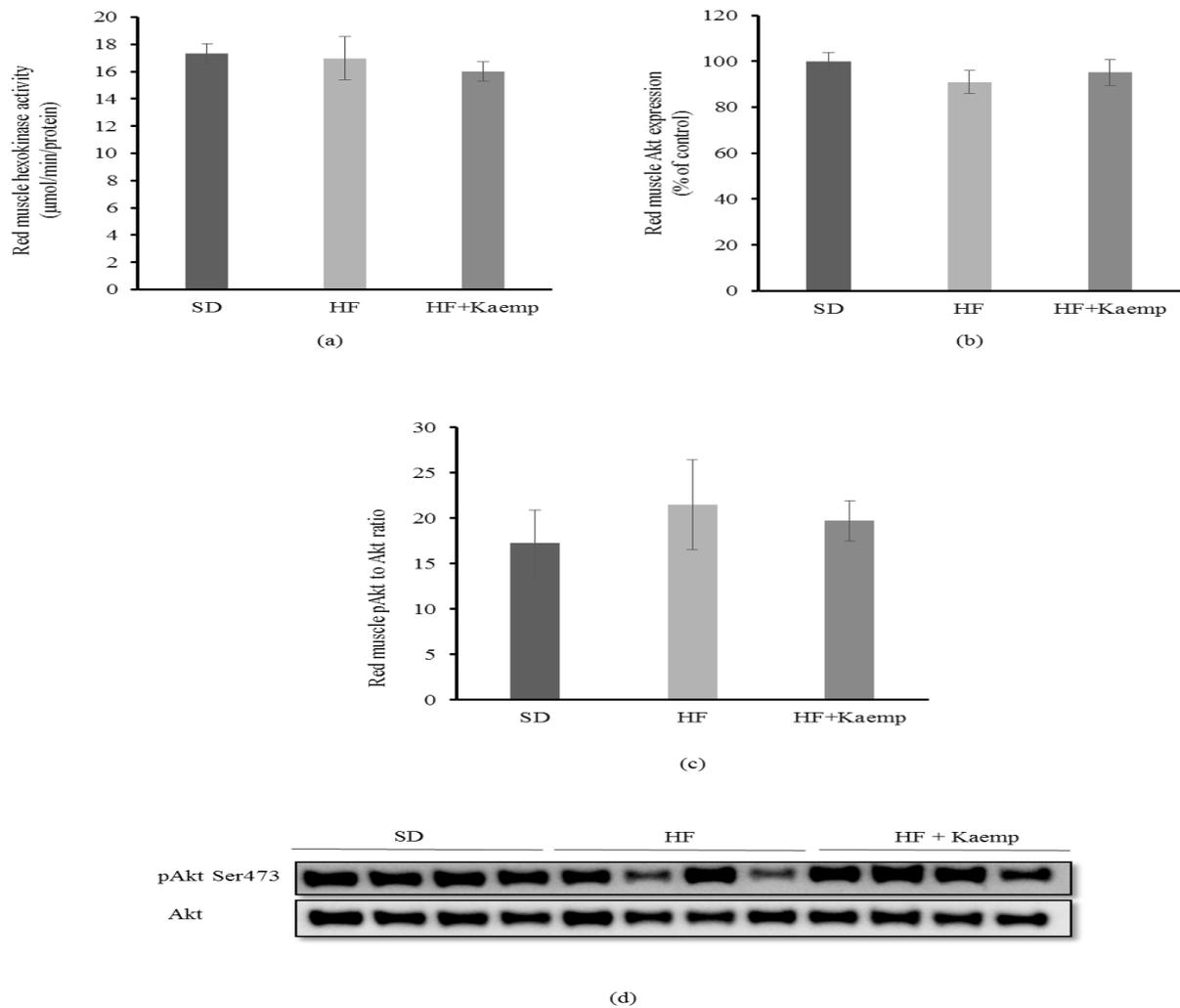


Figure 6. Kaempferol had no significant effect on Akt or hexokinase activities in red muscle of HF diet-fed mice. At the end of the feeding experiment, (a) hexokinase activity was measured in the homogenates of red skeletal muscle using an assay kit. (b, d) Akt, and (c, d) pAkt protein levels in whole cell lysates of red muscle tissue of mice were measured by immunoblotting and normalized to total protein contents. Values are Mean \pm SEM from 8 mice per group. SD: SD diet; HF: HF diet; HF + Kaemp: HF diet with kaempferol treatment (50mg/kg BW).

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CHAPTER SIX

Conclusions

The small molecule kaempferol has been shown to elicit various pharmacological activities in preclinical studies. However, studies exploring its anti-diabetic potential, particularly, its role in controlling glucose metabolism are limited. In this research, we first demonstrated that long-term dietary intake of kaempferol improved glucose tolerance and insulin sensitivity in HF diet-induced middle-aged obese mice. These improvements were associated with increased Glut4 and AMPK α expression in peripheral tissues. Consistently, kaempferol treatment restored chronic hyperlipidemia-impaired glucose uptake and AMPK activity in cultured C2C12 muscle cells. In the second study, dietary kaempferol supplementation preserved functional pancreatic β -cell mass and prevented hyperglycemia and glucose intolerance in obese STZ-induced diabetic mice (T2D). Similarly, in the third study, the administration of kaempferol by oral gavage to lean STZ-induced diabetic mice significantly ameliorated hyperglycemia and glucose intolerance and reduced the incidence of diabetes from 100 % to 77.8%. The improvements in glucose control might be due the changes in the activity of the key enzymes involved in the process of glucose uptake and production. In the last study, we found that administration of kaempferol by oral gavage significantly improved blood glucose control through suppressing hepatic glucose production and improving glucose intolerance in obese insulin-resistant mice. Similar to kaempferol effect in old obese mice, kaempferol enhanced whole-body insulin sensitivity. Kaempferol reduced hepatic IR by increasing Akt and hexokinase activity while decreasing PC activity. Given that insulin resistance and pancreatic β -cell dysfunction are two main defects leading to T2D, the results from these studies strongly suggest that kaempferol may be an inexpensive natural agent for the prevention and treatment of diabetes by improving insulin

sensitivity, improving glucose regulation and metabolism in peripheral tissues and the liver. As seen in Figure (1), we propose some mechanisms of action of the anti-diabetic effects of kaempferol generated from this project. More research is needed to elucidate the underlying mechanism by which kaempferol regulates the key enzymes involved in glucose regulation.

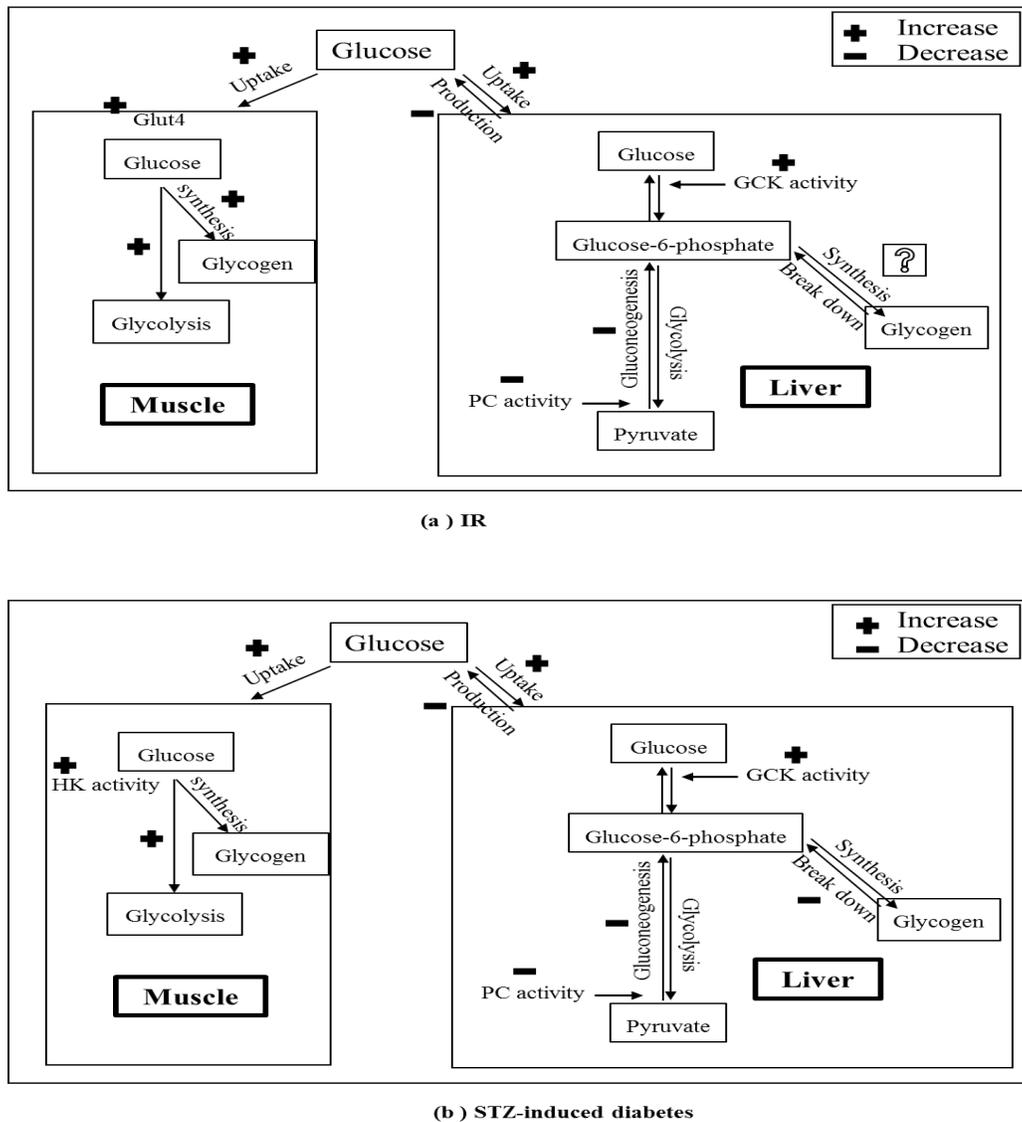


Figure 1. Proposed mechanisms of action of the anti-diabetic effects of kaempferol. Kaempferol may regulate glucose control by increasing glucose uptake and reducing glucose production in (a) IR and (b) diabetes.

Directions for future research

This research provides evidence that kaempferol exerts a broad spectrum of the antidiabetic roles in mouse models of obesity, IR, T1D, and T2D. However, more studies are needed for understanding the cellular and molecular mechanism of these kaempferol effects. Specifically, research should be focused on discovering the primary tissue and molecular targets for kaempferol to exert these actions. The following are some aspects that need to be addressed.

- 1) Determine whether kaempferol increases the activity of hexokinases in the liver and the muscle through direct modification of the enzymes *in vitro*. Further, experiments need to be conducted to determine whether this activation is associated with improved glucose uptake in the muscle and liver.
- 2) Investigate the underlying mechanism by which kaempferol reduces PC activity and further determine whether this effect primarily mediates the inhibitory effect of kaempferol on hepatic glucose production *in vivo*.
- 3) Examine the effect of different doses of kaempferol and duration of treatment in IR and STZ-induced diabetic mouse models.