

Small molecule kaempferol, a novel regulator of glucose homeostasis in diabetes

By

William T. Moore

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Advisory Committee:

Dongmin Liu, Chair

Zhiyong Cheng

Matthew Hulver

Young Ju

Andrew Neilson

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ACADEMIC ABSTRACT

Diabetes mellitus is a growing public health concern, presently affecting 25.8 million or 8.3% of the American population. 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. Type 2 diabetes (T2D) is a result of chronic insulin resistance and loss of β -cell mass and function, and it is always associated with the impairment in energy metabolism, causing increased intracellular fat content in skeletal muscle (SkM), liver, fat, as well as pancreatic islets. 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 and progression of this disease. Kaempferol is a flavonol that has been identified in many plants and used in traditional medicine. It has been shown to elicit various pharmacological activities in epidemiological and preclinical studies. However, to date, the studies regarding its effect on the pathogenesis of diabetes are very limited. In this dissertation, I explored the anti-diabetic potential of the dietary intake of kaempferol in diet-induced obese mice and insulin-deficient diabetic mice.

For the first animal study, kaempferol was supplemented in the diet to determine whether it can prevent insulin resistance and hyperglycemia in high fat (HF) diet-induced obese mice or STZ-induced obese diabetic mice. For the second animal study, kaempferol was administrated once daily via oral gavage to diet-induced obese and insulin-resistant mice or lean STZ-induced

diabetic mice to evaluate its efficacy for treating diabetes and further determining the underlying mechanism. The results demonstrated 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 SkM 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. Data from the second study show that oral administration of kaempferol significantly improved blood glucose control in obese mice, which was associated with reduced hepatic glucose production and improved whole body insulin sensitivity without altering body weight gain, food consumption, or the adiposity. In addition, kaempferol treatment increased Akt and hexokinase activity, but decreased pyruvate carboxylase and glucose-6 phosphatase activity in the liver homogenate without altering their protein expression. Consistently, kaempferol decreased pyruvate carboxylase activity and suppressed gluconeogenesis in HepG2 cells as well as primary hepatocytes isolated from the livers of obese mice. Kaempferol directly blunted the activity of purified pyruvate carboxylase. In the last study, we found that kaempferol stimulates basal glucose uptake in primary human SkM. In C2C12 mouse myotubes, kaempferol also increased insulin stimulated glycogen synthesis and preserved insulin dependent glycogen synthesis and glucose uptake in the presence of fatty acids. Kaempferol stimulated Akt phosphorylation in a similar time-dependent manner as insulin in human SkM cells. Consistent with this, kaempferol increased Akt and AMPK phosphorylation in isolated murine red SkM tissue. The effect of kaempferol on glucose uptake was blunted in the presence of chemical

inhibitors of glucose transporter 4 (Glut4), phosphoinositide 3-kinase (PI3K), glucose transporter 1 (Glut1), and AMPK. The AMPK inhibitor also prevented kaempferol-stimulated Akt phosphorylation. Further, kaempferol improved the stability of insulin receptor substrate-1. Taken together, these studies suggest that the kaempferol is a naturally occurring compound that may be of use in the regulation of glucose homeostasis and diabetes by improving insulin sensitivity and glucose metabolism, as well as by preserving functional β -cell mass.

Keywords: Kaempferol, diabetes, glucose control, skeletal muscle, β -cells, insulin resistance, gluconeogenesis.

GENERAL AUDIENCE ABSTRACT

Diabetes mellitus, more commonly referred to as diabetes, is a cause for concern in the context of public health. Currently, 25.8 million or 8.3% of the American population is affected by some type of diabetes. While the development of new drugs, techniques, and surgeries have improved the survival rate of individuals with diabetes, the number of diabetes cases continues to rise. Type 2 diabetes (T2D) is a result of the inability of tissues to respond to insulin and a loss of insulin producing β -cell mass and function. T2D is always associated with an impairment in the storage and release of energy, causing increased fat content in skeletal muscle (SkM), liver, and fat cells, as well as pancreatic islets. As such, the search for new agents that simultaneously promotes the ability of body tissues to respond to insulin and β -cell survival may provide a more effective strategy to prevent the onset and progression of this disease. Kaempferol is a flavonol that has been identified in many plants and used in traditional medicine. It has been shown to elicit various drug-like activities in incidence and distribution studies as well as in preclinical studies. However, to date, the studies regarding its effect on the onset and progression of diabetes are very limited. In this dissertation, I explored the anti-diabetic potential of the dietary intake of kaempferol in diet-induced obese mice and insulin-deficient diabetic mice.

For the first animal study, kaempferol was added to the diet to determine whether it can prevent insulin resistance and high blood glucose in high fat (HF) diet-induced obese mice or chemically-induced obese diabetic mice. For the second animal study, kaempferol was given once daily via oral gavage to diet-induced obese and insulin-resistant mice or lean chemically-induced diabetic mice to evaluate its efficacy for treating diabetes and further determining its mechanism. The results demonstrated that dietary intake of kaempferol for 5 months (mo) improved insulin sensitivity and the ability of body tissues to respond to glucose, which were associated with

increased expression of the insulin sensitive glucose transporter (Glut4) and a central regulator of metabolism (AMPK α) in muscle and adipose tissues in middle-aged mice fed a high-fat (HF) diet. In cell culture, kaempferol increased triglyceride breakdown and restored the ability of SkM cells to take up glucose and synthesize glycogen following long-term exposure to elevated fatty acids. These results were also associated with an improved AMPK α activity and Glut4 expression. In addition, kaempferol in the diet preserved functional pancreatic β -cell mass and prevented the development of high blood glucose and the inability of body tissues to respond to glucose in chemically-induced diabetic mice. Data from the second study show that oral administration of kaempferol significantly improved blood glucose control in obese mice, which was associated with reduced glucose production in the liver and an improved ability of the whole body to respond to insulin without altering body weight gain, food consumption, or fat storage. In addition, kaempferol treatment increased the activity of the final enzyme in glucose transport (Akt) and first enzyme (hexokinase) in glucose oxidation, but decreased the activity of the first and final regulatory enzymes in glucose production (pyruvate carboxylase and glucose-6 phosphatase respectively) without altering their protein expression. Consistently, kaempferol decreased pyruvate carboxylase activity and suppressed glucose production in HepG2 liver cells as well as primary liver isolated from obese mice. Kaempferol also directly blunted the activity of purified pyruvate carboxylase. In the last study, we found that kaempferol stimulates non-stimulated glucose uptake in primary human SkM. In C2C12 mouse muscle cells, kaempferol also increased insulin stimulated glycogen synthesis and prevented fatty acid impaired glycogen synthesis and glucose uptake stimulated by insulin. Kaempferol stimulated Akt phosphorylation (the active form of the enzyme) in a similar time-dependent manner as insulin in human SkM cells. Consistent with this, kaempferol increased Akt and AMPK phosphorylation in red SkM tissue from mice. The

effect of kaempferol on glucose uptake was inhibited in the presence of chemical inhibitors of Glut4, phosphoinositide 3-kinase (an enzyme in the insulin signaling pathway), glucose transporter 1 (a basal glucose transporter), and AMPK. The AMPK inhibitor also prevented kaempferol-stimulated Akt phosphorylation. Further, kaempferol improved the stability of insulin receptor substrate-1. Taken together, these studies suggest that the kaempferol is a naturally occurring compound that may be of use in the regulation of glucose homeostasis and diabetes by improving insulin responsiveness and glucose storage and breakdown, as well as by preserving functional β -cell mass.

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DEDICATION

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LIST OF ABBREVIATIONS

A

Akt: Protein kinase B

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

FBS: Fetal bovine serum

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

hSkM: Human skeletal muscle

I

IL-6: Interleukin 6

Ip: intraperitoneal

IR: Insulin resistance

IRS: insulin receptor substrate

ITT: Insulin tolerance test

K

K: Kaempferol

L

LDL: Low-density lipoprotein

N

NO: Nitric oxide

P

PC: Pyruvate carboxylase

PI3K: Phosphatidylinositol 3 Kinase

PKC: Protein kinase C

PTT: Pyruvate tolerance test

R

ROS: reactive oxygen species

S

SD: Standard diet

SGLT: Sodium-glucose linked transporter

SkM: Skeletal Muscle

STZ: Streptozotocin

T

T1D: Type 1 diabetes

T2D: Type 2 diabetes

TNF- α : tumor necrosis factor- α

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CHAPTER ONE

INTRODUCTION

Background

Diabetes is a group of metabolic disorders characterized by hyperglycemia caused by impaired insulin action, insulin secretion or both [1]. Diabetes is associated with abnormalities in carbohydrate [1], lipid [2], and protein metabolism [3]. Long term complications of diabetes include impaired function of the blood vessels, cognitive impairment, visual impairment, ischemic heart disease, renal disease, and neurological disease [1,4,5]. In 2015, diabetes was the seventh leading cause of mortality in the United states [6].

Type-2 diabetes (T2D) is the most common type of diabetes as it accounts for 90-95% of all diagnosed cases [7]. The cascade of events leading to the development of diabetes continues to be a subject of debate [8,9]. In general, T2D starts with insulin resistance. Constant insulin resistance will progress to overt diabetes when β -cells are unable to secrete adequate amount of insulin to compensate for decreased insulin sensitivity [10]. 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 (SkM), liver, fat, as well as pancreatic islets [11].

Type-1 diabetes (T1D) accounts for 5-10% of diabetes cases [7]. T1D is largely the result of autoimmune-mediated destruction of pancreatic β -cells, leading to the deficiency of insulin [12]. It may occur as a result of the interaction between a series of environmental, genetic, and autoimmune factors. While there are no known cure or prevention methods for this disease, it can be managed with the administration of insulin [13].

Despite recent advances in the understanding and management of diabetes, the prevalence of diabetes continues to rise rapidly. Global age standardized diabetes prevalence has increased from 4.3% in 1980 to 8.5% in 2014 in the adult population [14]. The U.S. is no exception as 9.4% of the U.S. population had diabetes in 2015 [6]. In addition to its impact on global health, diabetes is imposing an increasingly greater fiscal burden with costs totaling \$245 billion in the U.S. [6] and \$827 billion globally [15,16].

Therefore, investigation into the potential of low-cost, naturally occurring agents that promote insulin sensitivity and β -cell survival may provide an effectual strategy to prevent diabetes and improve the overall health of those already suffering from the disease [17]. Recently, naturally occurring polyphenolic compounds have been the focal point of medicinal research interest due to their pharmacological implications associated with human disease with considerable attention devoted to managing diabetes [18,19]. Polyphenols exist as secondary plant metabolites and are the largest source of human dietary antioxidants with a typical daily intake of roughly 1 g/day [20]. One of the most common polyphenolic subclasses is flavonols [21]. Kaempferol is a naturally occurring flavonol, of low molecular weight (286.2 g/mol), that has been identified in several plants used in traditional medicine including *Equisetum* spp., *Ginkgo biloba*, *Sophora japonica*, as well as edible plants including broccoli, cabbage, gooseberries, grapes, kale, strawberries, tea, and tomatoes [22,23]. Dietary intake of KFR-containing foods has been epidemiologically associated with a reduced risk of certain cancers and cardiovascular diseases [22]. It has indeed been shown to have anticancer [24,25], anti-inflammatory [26], antimicrobial [22], antioxidative [27], and lipolytic [26] effects, however, studies on whether kaempferol has an effect on diabetes are limited. For this dissertation research, I tested the central hypothesis that dietary supplementation of

kaempferol exerts the anti-diabetic effects in mouse models of T2D. Accordingly, three specific aims are proposed below to test this hypothesis:

Aim 1: To determine whether dietary supplementation of kaempferol prevents hyperglycemia in high fat diet (HFD)-induced obese mice and/or protects against STZ-induced β -cell dysfunction in obese diabetic mice.

Insulin resistance is a major risk factor for the pathogenesis of T2D [28,29]. Constant insulin resistance will progress to overt diabetes when β -cells are unable to secrete adequate amount of insulin to compensate for decreased insulin sensitivity [10], which is largely due to insulin secretory dysfunction and significant loss of functional β -cells [30–35]. Research in our laboratory recently showed that kaempferol prevents apoptosis and improve the biosynthesis and secretion of insulin in β -cells and human islets exposed to chronic hyperlipidemia [19].

However, whether kaempferol can preserve functional β -cell mass in vivo and/or promote insulin sensitivity while preventing T2D remains unclear. I hypothesize that dietary provision of kaempferol prevents or mitigates hyperglycemia in high fat diet (HFD)-induced obese mice and/or protects against STZ-induced β -cell dysfunction in obese diabetic mice.

Aim 2: To evaluate whether administration of kaempferol ameliorates hyperglycemia in T2D via regulating hepatic gluconeogenesis in HFD-induced obese, insulin-resistant mice.

Obesity-induced hepatic insulin resistance is a significant contributor to fasting hyperglycemia [36] as altered regulation of hepatic glucose metabolism results in decreased glycogen synthesis and concurrently increased gluconeogenesis and glycogenolysis [37]. Because of the significant contribution of the liver to whole body glucose homeostasis, the liver is a major target for preventing and treating chronic hyperglycemia [38,39]. It has been shown that kaempferol (10-

100 μ M) improved basal glucose uptake in HepG2 hepatocytes in a dose dependent manner [40]. In addition, a high dose of kaempferol (50 μ M) has been shown to inhibit glucose-6-phosphatase (G6Pase) activity in a bioassay [41]. However, the physiological relevance of these in vitro studies is unclear. Therefore, the mechanism by which kaempferol exerts the anti-diabetic effect remains enigmatic. **In the second study, I tested the hypothesis that oral administration of kaempferol can improve glucose homeostasis in obese insulin resistant mice via regulating hepatic gluconeogenesis.**

Aim 3: To explore whether kaempferol regulates glucose uptake in skeletal muscle.

It is well established that insulin resistance and β -cell dysfunction are critical components in the development of the disease [8,9]. However, approximately 80-90% of glucose disposal has been shown to be attributable to SkM in euglycemic hyperinsulinemia test [42]. Insulin resistance, specifically in SkM, is indeed considered the primary defect in T2D as it is clinically discernable decades prior to β -cell dysfunction and the development of overt diabetes making it a primary target for preventing the progression of T2D [42–44]. It has been shown that as insulin levels increase, muscle glucose uptake increases linearly with respect to time for 60 min at which point the rate of glucose uptake plateaus [45,46]. In subjects with T2D, this effect is blunted resulting in a 50% reduction of glucose uptake in muscle [46], suggesting that insulin resistance in SkM is indeed of paramount importance in the pathogenesis of T2D. 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 and that the improved insulin sensitivity was associated with increased Glut4 and AMP-dependent protein kinase (AMPK) expression in SkM [47]. Further, kaempferol was shown to improve basal glucose uptake in a

human hepatocyte cell line (Hep G2) in a dose-dependent manner (10–100 μ M) [40]. However, the means by which kaempferol exerts these effects is remains elusive. Thus, in the third study, **we tested the hypothesis that kaempferol regulates glucose uptake in skeletal muscle.**

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CHAPTER TWO

Literature Review

Abstract

Type 2 diabetes (T2D) is characterized by insulin resistance and a progressive decline in functional β -cell mass. While various environmental factors and the pathophysiological processes of insulin resistance and β -cell dysfunction in T2D have been extensively studied, with many potential new targets for the prevention and treatment of this metabolic disorder identified, the incidence of T2D is still increasing world-wide. Therefore, although there is a considerable amount of knowledge regarding the treatment and prevention of T2D, it is not applied to its greatest capacity in the context of public health. Many pharmaceutical interventions are expensive and have undesirable side-effects. Thus, there is great interest to search for safe and inexpensive naturally occurring compounds that can be used to treat and prevent T2D. Flavonoids are a class of polyphenolic compounds that have been shown to have many beneficial effects in the context of chronic disease. Of particular noteworthiness is kaempferol, which has been shown to exhibit several pharmacological activities as an anti- allergic, -cancer, -diabetic, -estrogenic, -inflammatory, -microbial, -osteoporotic, -oxidant, cardioprotective, and neuroprotective agent. The aim of this review is to summarize the current knowledge on the pathogenesis of T2D as it relates to insulin resistance and β -cell function, but the focus is on reviewing the existing evidence for the efficacy of kaempferol in the prevention or treatment of T2D and its complications. In addition, the possible mechanisms underlying its anti-diabetic effect are discussed.

1. Introduction

Metabolic syndrome is an over-arching term that encompasses such illnesses as cardiovascular disease, diabetes, fatty liver disease, renal dysfunction, neuropathy, and obesity. The growing incidence of metabolic disease is a great public health concern. As of 2015, 9.4% of the U.S. population was diabetic [1], and it is anticipated that this number will double by 2050 [2]. In addition, diabetes affects approximately 387 million people worldwide [3]. Between the years of 2007 and 2012, the cost of treatment for diabetes, and its associated complications, increased from \$174 billion to \$245 billion, thereby leading to a substantial economic burden [4,5].

T2D is a result of chronic hyperglycemia leading to progressive insulin resistance and the loss of pancreatic β -cell mass and function [6]. While the sequence of events leading to the development of T2D is a subject of debate [7,8], it has been shown that obesity is a predominant risk factor for insulin resistance due to its resultant increase in the intracellular fat content of skeletal muscle, liver, fat, and pancreatic islets [9]. Persistent insulin resistance and lipotoxicity could eventually cause pancreatic β -cell dysfunction, which subsequently leads to overt diabetes because of insufficient production of insulin to compensate for the impaired insulin sensitivity [6,10–12]. Hyperglycemia is known to play a prominent role in the pathogenesis of many secondary complications including cardiovascular, neurological, and renal diseases [13–15]. Increased glucose metabolism leads to an increase in the production of reactive oxygen species (ROS), which further contributes to insulin resistance and the deterioration of β -cell function [16].

There is a vast amount of knowledge concerning the treatment and prevention of T2D. It is well established that caloric restriction and increased physical activity improve insulin sensitivity [17–22]. Unfortunately, this knowledge is not widely translated into practice by the

public [23]. In addition, many pharmaceutical therapies have less than desirable side effects [24]. Thus there is a considerable interest and need to identify safe, naturally occurring compounds that can be used to aid in the attenuation of T2D and its complications.

Many naturally occurring compounds including those from fruits, herbs, and plants have a long history of use in traditional eastern medicine. The recent temporal interval, principally the preceding fifteen years, has fostered the relevance of naturally occurring, relatively low molecular weight substances with the aim of developing alternative and complementary therapeutic agents for multiple ailments including varied forms of neoplastic disease, inflammation, and metabolic syndrome. One study found that 908 out of 10,000 constituents of traditional Chinese Medicine and 8,000 modern drugs are structurally similar, and that 327 of them are identical [25,26]. This suggests that investigation into the efficacy of naturally occurring compounds is a worthwhile pursuit. Several of these compounds including genistein, resveratrol, epigallocatechin gallate, and quercetin have been shown to mitigate obesity and/or diabetes by promoting β -cell survival and proliferation while regulating various metabolic targets such as AMP-dependent kinase (AMPK) and PPAR [27–31]. This paper will focus on reviewing available literature from epidemiological, clinical, and experimental studies of a flavonoid compound, kaempferol, to treat and prevent various aspects and complications of the metabolic syndrome including T2D, CVD, neuropathy, oxidative stress, and inflammation.

2. Type-2 Diabetes

T2D is in essence a disruption of multiple pathways that regulate glucose homeostasis in the postprandial and post-absorptive state [32]. It is primarily brought about as a result of genetic

predisposition and lifestyle factors (which may also be genetically controlled) including physical inactivity, over-nutrition, cigarette smoking, and excessive alcohol consumption [33,34] with obesity having been found to contribute to over 50% of T2D cases [35]. It is characterized by diminished insulin sensitivity, β -cell failure, and subsequent decline in insulin secretion, [36,37]. These conditions collectively result in impaired glucose transport and disposal in the liver, fat, and muscle cells.

In a healthy individual, when food is consumed, glucose is transported via sodium glucose cotransporter-1 (SGLT1) into enterocytes lining the small intestine and subsequently transported, via glucose transporter-2 (Glut2), into the hepatic portal vein [38]. This glucose then enters the liver prior to entering the general circulation where pancreatic β -cells respond by secreting insulin into the portal vein, which stimulates glucose disposal in insulin sensitive tissues [39,40].

Among insulin sensitive tissues, skeletal muscle is a major contributor to glucose disposal [41], and responds to insulin by translocating Glut4 from intracellular vesicles to the sarcolemma and in t-tubules, which subsequently facilitates glucose uptake [42]. Once the glucose is in the cell, it is phosphorylated by hexokinase, and is then destined for either glycolysis, glycogenesis, or the hexose monophosphate shunt [43,44].

Late in the post-prandial state, glucagon levels in the blood increase, which counteracts insulin by enhancing glycogenolysis and gluconeogenesis through the modulation of the transcription of key gluconeogenic enzymes, including pyruvate carboxylase, glucose-6-phosphatase, and phosphoenolpyruvate carboxykinase [45,46].

Insulin resistance leads to reduced glucose uptake, utilization, and storage in peripheral tissues while causing excessive hepatic glucose production, thus contributing to the marked

hyperglycemia associated with T2D [47–50]. Diabetics also often experiences an impairment of renal function, which may further play an exacerbating role in blood glucose control by reabsorbing excess glucose [51].

3. Kaempferol

Flavonoids are secondary plant metabolites of phenylalanine. [52,53]. In the plant, they have been found to provide protection against microbial invasions, oxidative damage, and UV damage while providing support for the growth and development of the plant [54–58]. There have been over 9,000 flavonoids identified in plant sources [59], all of which share a common chemical structure consisting of a diphenylpropane three (A-, C-, and B-) ring structure with 15 carbon atoms; that is two aromatic rings joined by a three-carbon bridge. The flavonoids are typically divided into 6 major classes including anthocyanidins, flavan-3-ols, flavanones, flavones, flavonols, and isoflavones [60]. Subclasses of flavonoids are determined by their oxidation state and the substitution of a functional group on the heterocycle ring (C-ring) with an acetyl, glycan, hydroxyl, or methyl group [61], as well as the differential hydroxylation patterns of the phenolic rings, which influence the physiological properties of the flavonoids [62].

Of these sub-classes, the flavonols are perhaps the most ubiquitous, being found in a plethora of food sources including apples, berries, broccoli, curly kale, leeks, onions, red grapes, red wine, and teas [61,63–65]. The flavonol, kaempferol (3,5,7-trihydroxy-2-(4-hydroxyphenyl)-4H-1-benzopyran-4-one) is a yellow compound with a low molecular weight (286.2 g/mol) [66]. It is abundantly found in apples, beans, broccoli, cabbage, endive, kale, leeks, strawberries, tea, and tomatoes [67]. Kaempferol is also found in botanical products that are commonly used in

traditional medicine including *Equisetum spp.*, *Ginkgo biloba*, *Moringa oleifera*, *Sophora japonica*, *Sophora propolis*, and *Tillia spp* [66]. Despite its low molecular weight, the bioavailability of kaempferol is low, and is commonly metabolized into the forms of methyl, sulfate, or glucuronide [68–70] after dietary injection. Kaempferol has been shown to decrease the risk of cancer and cardiovascular disease [71,72] while eliciting anti-diabetic, anti-obese, anti-inflammatory, antioxidative, neuroprotective, analgesic, and antimicrobial, activities [73–78], thus substantiating its potential as a therapeutic agent for metabolic syndrome.

3.1 Kaempferol as an Anti-diabetic agent

Epidemiological studies surveying whether there is a link between kaempferol intake and diabetes are lacking, however there are a handful of studies that discuss epidemiological associations of broad flavonoid intake and diabetes. One large cohort study of 38,018 women aged 45 and up, who were free of cardiovascular disease (CVD), cancer, and diabetes, revealed that the intake of kaempferol-containing foods had a significant inverse association with T2D risk [79]. Women who consumed at least one apple per day showed a 28% reduced risk of T2D compared to those who did not consume any apples even when adjusted for the intake of other dietary factors including fiber, magnesium, and total fat [79]. In addition, women who consumed between four and six cups of tea per day had a reduced risk of T2D with multivariate RRs being 0.77 for the consumption of four to five cups of tea and 0.63 for the consumption of six cups per day [79]. However, the relative contribution of kaempferol to the observed beneficial effects by consuming kaempferol containing foods is unknown.

Studies assessing the antidiabetic effect of kaempferol in humans or animal models are scarce. We recently showed that dietary intake of kaempferol, over a five-month time span, resulted in attenuated insulin sensitivity and glucose tolerance in middle-aged mice fed a high-fat diet [80]. These effects were associated with the increased expression of Glut4 and AMPK in muscle and adipose tissues *ex vivo* [80]. In a separate cohort of diabetic mice induced with streptozotocin (STZ), dietary kaempferol administration preserved functional β -cell mass while preventing hyperglycemia and glucose intolerance [80]. These results are consistent with another study in which C57BL/6J mice were fed 0.15% dietary kaempferol glycoside [73]. This study found that mice treated with kaempferol glycoside had lower body weights and lower levels of adipose tissue and blood triglycerides compared to the control [73]. Fasting blood glucose was also diminished along with serum hemoglobin A_{1C} while insulin resistance was attenuated [73]. An *in vivo* study using kaempferol-3,7-O-(α)-dirhamnoside (kaempferitrin) also showed a decrease in blood glucose levels in rats while glucose uptake in muscle cells was increased independent of increased protein synthesis [81]. Likewise, administration of kaempferitrin (50, 100, and 200 mg/kg) had a significant blood-glucose lowering effect in alloxan-induced diabetic rats [82].

The potential anti-diabetic mechanisms of kaempferol has been explored *in vitro* using various cells. T2D is always associated with the disturbed fatty acid metabolism that leads to accumulation of lipid intermediates and triglycerols within various organs, such as liver and skeletal muscle. It was recently showed that kaempferol increased lipolysis and restored fatty acid-impaired glucose uptake and glycogen synthesis in skeletal muscle cells, which were associated with improved AMPK activity and Glut4 expression [80]. Similarly, one study demonstrated that kaempferol 3-neohesperidoside, a glycoside form of kaempferol, can stimulate

glucose uptake via the phosphoinositide 3-kinase (PI3K) and protein kinase c (PKC) pathways [83], suggesting that that kaempferol may have an insulin mimetic effect. Recently, kaempferol has been shown to increase oxygen consumption and stimulate energy expenditure as well as metabolically relevant genes such as UCP-3, PGC-1 α , CPT-1, and citrate synthase in human skeletal muscle cells via activation of cAMP/PKA-mediated mechanism [84]. However, the physiological relevance of these effects are unclear as the dose of kaempferol (20 μ M) for producing most of the observed effects in this in vitro study is unachievable via dietary supplementation.

While the hypoglycemic effects of kaempferol is still not very clear, our recent study has shown that kaempferol has cytoprotective effect on INS-1E β -cells and human islets exposed to glucotoxicity and lipotoxicity [85], thereby improving insulin synthesis and secretion in pancreatic β -cells [85]. Consistently, another study showed that kaempferol treatment prevented HIT-T15 cells from 2-deoxy-D-ribose toxicity [86]. Kaempferol may preserve β -cell viability via cAMP signaling-mediated mechanism [87]. Interestingly, it was further found that kaempferol restored lipotoxicity-attenuated cAMP signaling and β -cell survival via regulating the expression of PDX-1 [88], a transcriptional factor critical for pancreas development and islet function. Kaempferol may also affect energy metabolism in adipose tissues. It has also been shown to potentiate insulin-stimulated glucose uptake via activation of PPAR- γ in 3T3-L1 adipocytes [89]. Kaempferol also activates the insulin signaling pathway and increase Glut4 translocation in 3T3-L1 cells [90]. However, whether this kaempferol analog has similar effect in vivo remains to be determined, as the bioavailability of this compound through dietary supplementation is unknown.

3.2 Kaempferol as an Antioxidant

Oxidative stress can be defined as a condition of imbalance between cellular oxidants or pro-oxidative enzymes (i.e. NADPH oxidase or xanthine oxidase), and antioxidants or anti-oxidative enzymes (i.e. catalase, glutathione peroxidase, heme oxygenase (HO), or superoxide dismutase) with a resultant inability to clear ROS [91]. ROS are produced in a variety of cells and tissues including pro-inflammatory neutrophils and macrophages [92]. Briefly, when a molecule of O₂ gains one electron, a superoxide anion is formed, which is highly reactive, and tends to gain three additional electrons and four protons to make water. As this happens, a series of reactions may take place. The superoxide anion can be converted to hydrogen peroxide by superoxide dismutases. In the presence of reduced transition metals, hydrogen peroxide can be converted into a hydroxyl radical. Superoxide anion can also be converted to peroxynitrite by reacting with nitric oxide (NO). When ROS remain unchecked, this leads to the initiation of oxidative stress, and the excess amount of ROS then can modify and damage DNA, lipids, and proteins [93].

One of the primary contributors to oxidative stress is NO. It has been shown to cause hydrogen peroxide accumulation and toxicity while also leading to the production of peroxynitrite, a strong oxidant interacting with superoxide anions that leads to in DNA damage, LDL oxidation, mitochondrial respiration inhibition, and cell apoptosis [94–96].

Oxidative stress is thought to be one of the main factors contributing to bodily damage in the context of metabolic syndrome. Several studies have suggested that elevated ROS production and diminished antioxidant levels are strong contributors to the progression of β -cell dysfunction in the progression of T2D [97–100]. Indeed, oxidative stress has been shown to occur prior to any clinical manifestations of T2D [101–103]. It is widely accepted that flavonoids possess antioxidant

properties [104–106], and numerous studies have shown that kaempferol, kaempferol glycosides, and kaempferol-containing foods and plants display such properties [107–112].

Kaempferol has been shown to react with superoxide, NO, hydrogen peroxide, and hypochlorous acid *in vitro*, and is a well-documented radical scavenger [113] with an IC₅₀ of 0.5 μM [114]. Although the bioavailability of kaempferol is notably small (~2%) [68], this low inhibitory concentration may aid in the advancement of kaempferol as a useable antioxidant. Indeed, it has been shown to attenuate cytokine- and glutamate-induced ROS production in umbilical vein endothelial cells and neuronal HT22 cells respectively [115,116]. Kaempferol has also been demonstrated to inhibit generation of peroxynitrite, which is involved in lipid peroxidation [117]. Consistently, kaempferol treatment protects β-cells from 2-deoxy-D-ribose-induced oxidative damage through the inhibition of lipid peroxidation [86], which may be linked to the ability of kaempferol to inhibit peroxynitrite [117]. Kaempferol has also been shown to attenuate the production of hydroxyl radicals by acting as a chelating agent of ferrous or cuprous ions through the Fenton's reaction [118–120].

Kaempferol has been shown to increase the expression or activity of several antioxidative enzymes while also inhibiting the activity and expression of pro-oxidant enzymes. When stimulated, the nuclear factor erythroid 2-related factor 2 (Nrf-2) is known to regulate several antioxidative stress enzymes and phase 2 drug metabolizing/detoxifying enzymes by binding to the antioxidant response element. These enzymes include glutamate-cysteine ligase, NAD(P)H quinone oxidoreductase-1 (NQO1), sulfiredoxin-1, thioredoxin reductase-1, and hemeoxygenase-1 (HO-1), which subsequently protect against oxidative damage [121]. It has been shown that following treatment with hydrogen peroxide, kaempferol administration, in HepG2 cells induces Nrf-2 and subsequently NQO1 and superoxide dismutase-1, thus attenuating ROS formation [122].

Kaempferol has also been shown to activate HO-1 and suppress inducible nitric oxide synthase (iNOS) expression, leading to a decrease in the production of NO, thereby protecting RAW 264.7 cells from lipopolysaccharide (LPS)-induced toxicity [123]. This effect was similarly shown in J774 macrophages [124]. In addition, kaempferol can inhibit activity of xanthine oxidase, an enzyme generating ROS [125]. In addition, whether and how flavonoid in general or kaempferol exerts significant scavenging capability for ROS in vivo is unclear. Most studies that observed the potential antioxidant activity of flavonoids in vitro used concentrations far beyond those physiologically achievable by dietary intake of flavonoid containing foods or more concentrated supplements. However, the bioavailability of flavonoids including kaempferol is only between 2-3% that can reach to circulation as shown in rat study [126]. There is a possibility that the protective effects of kaempferol in vivo are exerted through suppressing the downstream pathways activated by elevated ROS or other proinflammatory mediators, as discussed below.

ROS are known to induce tissue damage by activating such stress pathways as nuclear factor kappa-light-chain-enhancer of activated B-cells (NF- κ B), p38, and Jun N-terminal kinase (JNK). These pathways can be activated by elevated glucose and free fatty acid levels, which subsequently lead to insulin resistance and ultimate β -cell dysfunction [127–129]. In addition, proinflammatory mediators such as lipopolysaccharide (LPS) is also known to activate p38, JNK, and extracellular signal-regulated kinase (ERK) by inducing their phosphorylation. Patients with T2D was found to have higher serum levels of LPS compared to those in healthy subjects [130], which further exacerbates the expression of the pro-inflammatory mediators such as myeloperoxidase and ROS.

Kaempferol treatment suppressed the activation of all three of these pathways in BALB/c mice [71]. Though the mechanism is not clear, kaempferol has also been shown to inhibit

lipoxygenase (LOX), which catalyzes the production of leukotrienes with resultant inflammatory complications [131]. Kaempferol has also been shown to decrease the accumulation of intracellular ROS and increase the survival rate of *C. elegans* [107]. In addition, mice treated with kaempferol-3-O-galactoside following a bromobenzene treatment displayed the attenuated hepatic lipid peroxidation and increased levels of reduced glutathione [109].

There have also been several *in vivo* studies showing that plant extracts containing kaempferol diminished oxidative stress in human [108], mouse [132], and rat [112] models. *Ginkgo biloba* extract has been shown to exert antioxidant effects [110]. In addition, *Capparis spinose*, of which kaempferol is a major constituent (3.28%), elicited anti-oxidant and free radical scavenging activities *in vitro*, and inhibited UVB-induced skin erythema when applied topically in humans [108]. Intraperitoneal injection of *Crassocephalum creidoides* extract exerted a strong antioxidant effect and prevented galactosamine and LPS induced hepatotoxicity in rats [112]. However, the relative contribution of kaempferol to the observed effects in these extracts are unknown.

While there is no unified mechanism by which kaempferol exerts its antioxidant activities, It is proposed that the double bond located at C2-C3 in conjugation with an oxo group at C4, and the presence of hydroxyl groups at C3, C5, and C4' are largely responsible for its antioxidant properties [104,120].

3.3 Kaempferol as an Anti-Inflammatory Agent

The inflammatory response is an important defensive mechanism in the body, however, chronic low-grade levels of inflammation are detrimental that can lead to cellular dysfunction and thereby chronic diseases [133,134]. Indeed, mounting evidence shows that a state of chronic low

grade inflammation is one of the primary contributing factors to the pathogenesis of metabolic syndrome. There are several critical signaling pathways involved in the development of metabolism-associated inflammation, including the NF- κ B, mitogen activated protein kinase p38, PI3K, and JAK/STAT-mediated inflammatory pathways. In addition, there are several enzymes that are also involved in generating proinflammatory mediators such as phospholipase A₂, cyclooxygenase (COX), lipoxygenase (LOX), and iNOS [135], as well as protein kinase C (PKC), PI3K, and tyrosine kinases [136–138]. The activation of these enzymes are involved in regulating the expression of the transcription factors NF- κ B, nuclear factor of activated T-cells (NFAT), and activator protein 1 (AP-1) [139].

Of these enzymes, COX₂, which is highly expressed in macrophages, leukocytes and fibroblasts, plays an important role in triggering inflammation by generating proinflammatory mediators prostaglandins from arachidonic acid [140–142], while LOX is attributed to the generation of leukotrienes from arachidonic acid [143,144], which are also proinflammatory molecules. Prostaglandin production is further exacerbated by excessive amounts of NO, which is augmented by iNOS expression [135]. In addition to contributing to oxidative stress, NO has also been shown to induce the production of the pro-inflammatory cytokine, TNF- α [145], which plays an important role in the development of insulin resistance [146]. In addition to the circulating factors associated with inflammation, endothelial adhesion molecules and chemokines, which include intercellular adhesion molecule 1(ICAM-1), vascular cell adhesion molecule 1 (VCAM-1), E-selectin, IL-8, and MCP-1, are essential for the interaction of endothelial cells and circulating leukocytes, and thus play a crucial role in the progression of inflammation [147,148].

Although both steroidal and non-steroidal drugs are potent attenuators of inflammation [149], they are not the best approaches to treat chronic inflammation due to adverse effects [150,151].

Thus, the search for new anti-inflammatory agents with fewer side effects is necessary to combat chronic inflammation. Several flavonoids have been shown to suppress inflammation *in vitro* and *in vivo* as they counteract many of the pro-inflammatory cascades. Kaempferol is considered to be one of the most active natural anti-inflammatory compounds [77,115,152].

MAP kinases including ERK1/2, JNK, and p38 are involved in triggering inflammation, as their activation upregulates the expression of TNF- α , IL-1 β , COX-2, and collagenase enzymes [153,154]. Kaempferol has been shown to blunt the phosphorylation of ERK and JNK induced by LPS in THP-1 cells, which leads to inhibition of the production of several pro-inflammatory cytokines [155]. It was also shown that kaempferol inhibits JNK and p38 activity in LPS- and IL-1 β -activated murine microglial BC2 and RA synovial fibroblast cells [156].

While classically considered to be primarily a part of the insulin signaling pathway, there is growing evidence suggesting that PI3K is an important component of inflammatory signaling [157,158]. When activated, it stimulates the conversion of PIP2 to PIP3, which subsequently activates Akt, leading to cytokine production [159]. Kaempferol has been shown to inhibit PI3K and Akt activation in murine microglial BV2 cells and cardiac fibroblasts exposed to LPS [160,161]. However, this effect was significant only at high concentrations (100 μ M).

Kaempferol has also been shown to suppress the production of several pro-inflammatory cytokines including IL-1 β and TNF- α [162]. This effect has been shown to be largely ascribed to its inhibitory effect on NF- κ B, which is responsible for the activation of the genes coding for these cytokines [163–165]. Another study showed that kaempferol blocked the tyrosine kinase (Tyk)-STAT signaling pathway and subsequent STAT3 transactivation in LPS-stimulated BEAS-2B cells and mice challenged with ovalbumin [166]. In line with these observations, kaempferol

treatment markedly reduced the phosphorylation of STAT3 and NF- κ B and attenuated neuroinflammation in rats [167]. Because the JAK-STAT pathway is upstream of NF- κ B, it is likely that kaempferol suppression of NF- κ B and its regulated cytokine expression is mediated via inhibition of the JAK/STAT pathway.

Kaempferol has been shown to inhibit COX in several *in vitro* studies. It dose-dependently inhibits cytokine-activated COX-2 and iNOS expression in Chang Liver cells [168], thereby suppressing the inflammatory response [123,124]. Kaempferol treatment has also been shown to decrease the level of reactive C protein (CRP) in Chang Liver cells exposed to cytokines [169]. The inhibitory effect of kaempferol on COX-2 may be mediated via Src-kinase [170]. Interestingly, it was reported that kaempferol also inhibits LOX both *in vitro* and *in vivo* [131,171].

Many of the pathways discussed in this section including the MAPK, JAK/STAT, and PI3K/Akt pathways, ultimately lead to the activation of one or more of the following transcription factors: AP-1, NFAT, and NF- κ B. NF- κ B, which is an inducible genetic element under the control of I κ B, is responsible for the activation of TNF- α , IL-6, IL-8, iNOS, and COX-2 [163–165]. Kaempferol has been shown to inhibit the expression of both NF- κ B and AP-1 induced by cytokines in umbilical vein endothelial cells [115]. In consistent with this finding, kaempferol inhibited NF- κ B and attenuated LPS and cytokines induced inflammation of BEAS-2B human airway epithelial cells [172] and ATP-induced inflammation in cardiac fibroblasts [161].

It is well recognized that inflammation plays an important role in obesity-initiated metabolic syndrome. One of the critical pro-inflammatory mediator in this process is cytokine TNF- α . TNF- α has been shown to induce inflammation by upregulating the expression of the endothelial ICAM-1 in A549 cells, an effect that was reduced by kaempferol treatment [169]. Consistently,

kaempferol attenuated cytokine-stimulated expression of adhesion molecules ICAM-1, VCAM-1, and E-selectin in HUVEC cells [115]. The inflammatory action of TNF- α is also, at least in part mediated via activation of the NF- κ B signaling pathway. NF- κ B is associated with the cytoplasmic inhibitory protein I κ B α in inactive form [173]. Cellular stimulation with NF- κ B agonists results in degradation of I κ B α , allowing the RelA (p65) and p50 subunits of NF- κ B to translocate to the nucleus, an essential step for its activation. TNF- α has been shown to activate IKK, which leads to the phosphorylation and degradation of I κ B, thereby allowing for the translocation of the p65 subunit of NF- κ B from the cytoplasm to the nucleus [174,175]. This effect has been shown to be inhibited by kaempferol [174,175]. Kaempferol also inhibited the receptor activator of NF- κ B ligand-induced immediate-early oncogene *cfos* expression in RAW264.7 monocyte/macrophage osteoclast precursor cells indicating that it may have anti-osteoclastogenic properties [174].

Kaempferol also exerted anti-inflammatory effects *in vivo*. It was reported that intra-peritoneal kaempferol administration reduced the expression of IL-6 and TNF- α in heart tissue taken from diabetic rats [176]. In BALB/c mice, kaempferol attenuated LPS-induced acute lung injury and production of some pro-inflammatory mediators by suppressing the elevated MAPKs and NF- κ B signaling [177]. Kaempferol also inhibited ovalbumin induced allergic reactions in BALB/c mice challenged with ovalbumin [172]. However, as discussed above, kaempferol has antioxidant effect that scavenges ROS, and ROS are mediators of inflammation that are critically involved in the progression of inflammatory diseases, the anti-inflammatory action of kaempferol may be partially due to a secondary action whereby kaempferol exerts an antioxidant effect. Consistent with the results from *in vitro* studies as described above, kaempferol administration was shown to suppress inflammation by blunting the phosphorylation of STAT3 and NF- κ B in transient ischemia rat models [167]. Additionally, kaempferol treatment also blocked advanced glycation end-

product-induced degradation of I κ B α and subsequent activation of NF- κ B in rats [174]. In a rabbit model with high cholesterol-induced atherosclerosis, kaempferol treatment was shown to decrease the expression of ICAM-1, VCAM-1, E-selectin, and MCP-1 in aortas [178].

There was no reported human trial evaluating the effect of pure kaempferol in inflammation; however, it was found that dietary flavonoid intake including kaempferol was inversely correlated with circulating CRP in humans, demonstrating the anti-inflammatory potential of flavonoid compounds [179].

There is a plethora of evidence that there is a strong correlation of flavonoid intake and the reduced risk of inflammation-related chronic diseases. Kaempferol has been shown to have a marked beneficial effect on several of these ailments, which are largely due to the potent inhibitory effect on the expression of pro-inflammatory cytokines in inflammatory cells. Because of the low bioavailability of kaempferol, further studies should evaluate whether it is actually kaempferol or its metabolites that exert the observed biological effect *in vivo*, and also evaluate the optimal dose for clinical trials.

3.4 Other Biological Properties of Kaempferol

3.4.1 Kaempferol as a Cardio-Protective Agent

An emerging body of evidence suggests that flavonoids can play a role in preventing CVD [180–185]. In addition, it was found that flavonoid intake was associated with reduced mortality from CVD cases [186] and incidence of myocardial infarction [181]. A cohort study consisting of 4,807 participants found high tea intake to be inversely associated with myocardial infarction

[187]. Another cohort study consisting of 66,360 women revealed that the risk of coronary heart disease was inversely related to the intake of kaempferol rich foods including apples, broccoli, grapes, onions, and tea [188]. Results from a cohort study consisting of 361 men and 394 women (aged 65-99 years) showed that kaempferol consumption was associated with decrease risk of myocardial infarction [189]. Flavonoids also inhibit LDL oxidation, platelet aggregation, and attenuate ischemic damage [190,191]. Chronic inflammation is directly involved in developing CVD and several reports indicated that kaempferol has a beneficial role in CVD, which may be attributable to its anti-inflammatory properties. It should be noted that most of these epidemiological studies assessed the effects of high flavonoid foods on CVD also contain other bioactive components such as vitamin E, vitamin C, and carotenoids. Therefore, the observed beneficial effects that are presumptively ascribed to flavonoids in the foods need to be further investigated. Although studies concerning the cardio-protective effect of kaempferol are still very limited, it is speculative that it should have a beneficial effect in preventing CVD, given that kaempferol has well established anti-oxidant and anti-inflammatory properties both *in vitro* and *in vivo*, and that inflammation [192] and oxidative stress [193] are known risk factors for CVD.

Kaempferol has been shown to decrease triglyceride and cholesterol levels and reduce body weight, which when elevated, are all additional risk factors for CVD [194–196]. As noted earlier, kaempferol decreased the expression of inflammatory adhesion molecules in cholesterol induced atherosclerotic rabbits [178]. In addition to protecting against hypercholesterolemia-induced CVD, kaempferol has also been shown to impede LDL oxidation, thus further contributing to its preventive abilities in atherosclerosis [197–201].

Angiotensin converting enzyme is responsible for converting angiotensin 1 to angiotensin 2, which leads to elevated blood pressure. Kaempferol has been shown to inhibit the activity of

this enzyme [202–205], suggesting that it may have antihypertensive effect. Further, it was demonstrated that kaempferol (100 µg/mL) exerted antiplatelet effects stimulated by arachidonic acid, collagen, and platelet activating factor (PAF) in isolated rabbit platelets, suggesting its potential as an antithrombotic agent [206]. However, the concentrations used in these studies are not physiologically achievable from an absorptive standpoint, and therefore, the biological relevance of this kaempferol effect could be questionable.

3.4.2 Kaempferol as a Neuroprotective Agent

One of the most common complications involved in T2D is pain and paresthesia from diabetic neuropathy. Roughly 50% of diabetics have noted some sort of neuropathic pain [207]. It has been shown that this complication is largely due to oxidative stress caused by mitochondrial dysfunction and NF-kB activation [208,209]. Several studies have reported that activation of glial cells by hyperglycemia release ROS and cytokines such as nitric oxide, eicosanoids, peroxynitrite, IL-1 β , and TNF- α [209–211,211–215]. ROS are known to sensitize nociceptors, which directly induces pain while also inducing central sensitization in the spinal cord leading to further indirect pain [216]. Pro-inflammatory cytokines including IL-1 β and TNF- α along with superoxide and peroxynitrite are responsible for diabetic pain and for deficiencies in nerve conduction and peripheral nerve energy [217–219]. Data from several studies showing that kaempferol decreases the generation of pro-inflammatory cytokines and oxidants by targeting NF-kB and AP-1 [74,177,220–222]. Kaempferol lowered circulating levels of cytokine and oxidants mice while also attenuating the development of diabetic neuropathy and neuropathy-associated pain sensations [162].

In addition to attenuating neuropathic pain, kaempferol has also been shown, in several *in vitro* and *in vivo* studies to protect against the development of such neurological diseases as Alzheimer's, Huntington's, and Parkinson's [76,223–230]. One study found that IV injection of kaempferol decreased ischemia-induced brain damage in rats [223]. This effect was associated with a marked reduction in apoptotic cells in the fronto-temporal and neocortex and striatum [223]. The effect was also associated with reduced oxidative stress as it diminished metalloproteinase activation, thereby blocking the accumulation of protein nitrotyrosines in the ischemic hemisphere [223]. As oxidative stress is known to be a strong contributor to neurodegenerative disorders [231,232], the antioxidant activity of kaempferol may largely responsible for its neuroprotection action [233].

Several *in vivo* studies have also shown that kaempferol glycosides and kaempferol containing plants elicit analgesic properties. In Swiss mice injected with STZ, kaempferol was shown to alleviate pain from diabetic neuropathy, at least in part, by reducing the levels of IL-1 β , NO, and TNF- α [162].

Dongmo et al., 2003 evaluated the effect of the stem bark extract of *Mitragyna ciliate* for its anti-inflammatory and analgesic properties [234]. It was found that the extract inhibited carrageenan-induced paw edema, one hour following administration, while also diminishing the sensitivity to pain [234]. Following these procedures, it was found that the extract contained significant amount of alkaloids and a kaempferol derivative [234], although it is unknown whether the analgesic effect of this plant extract is attributable to kaempferol.

Orhan et al., 2007 conducted a similar experiment in which the anti-inflammatory and antinociceptive activities of *Calluna vulgaris* L. was tested in mice [235]. The ethanolic extract of

the plant was fractioned, and orally administered to the animals [235]. After one hour, the animals were subjected to either the *p*-Benzoquinone-induced abdominal constriction test or the carrageenan-induced hind paw edema test [235]. The most effective fraction at preventing swelling and writhing was further fractioned and retested [235]. The component of the plant that was found to be most effective was indeed kaempferol-3-*O*- β -D-galactoside [235]. An earlier study by this same group also showed that kaempferitrin, which was isolated from *Tilia argentea* leaves, also had anti-inflammatory and antinociceptive properties [236]. In correlation with these studies, *Calluna vulgaris* has been shown to inhibit the activity of COX and LOX [237].

Diabetic neuropathy exists in two forms; painful and painless. One primary contributor to this phenomenon is that patients with painless diabetic neuropathy exhibit impaired c-nociceptive fiber action [238]. In addition, while serum levels of pro-inflammatory cytokines are elevated in both types of neuropathy, they elevated to a greater extent in patients with painful diabetic neuropathy [238]. There are multiple studies noting the analgesic, neuroprotective, and anti-inflammatory effects of kaempferol. Because of the relationship of these three conditions, it is likely that kaempferol elicits its analgesic and neuroprotective effects by acting as an anti-inflammatory agent. In addition, it is possible that the kaempferol-mediated inhibition of *Firmicutes* may play an additional role in attenuating inflammation in diabetics.

3.4.3 Kaempferol as an Antimicrobial Compound

As noted previously in this review, inflammation is a predominant complication and contributing factor to metabolic syndrome. It is characterized by elevated levels of pro-inflammatory cytokines in the circulation [239]. Adipose tissue and the intestine are important

reservoirs for pro-inflammatory markers [240]. There is accumulating evidence suggesting that systemic inflammation and metabolic syndrome are also regulated by a complex network of interactions between the diet and gut microbiota [241].

Data from several recent studies demonstrated that an increase in the ratio of the bacterial phyla *Firmicutes* to *Bacteroidetes* is associated with increased body weight and insulin resistance [242–244], suggesting that gut flora may be involved in energy metabolism of the host. Consistently, several other studies found that germ-free mice are resistant to HFD-induced obesity, insulin resistance, and intestinal inflammation, but develop these metabolic disorders rapidly following colonization with microbiota from obese mice [245–248], suggesting that obesity and diabetes could be a gut microbiota-related disease. Indeed, it was reported that T2D is associated with increased circulatory levels of LPS derived from gut bacteria [249]. This is thought to occur as a result of elevated dietary fat intake, which has been shown, in several mouse studies, to decrease the ratio of *Bacteroidetes* to *Firmicutes* [250–252]. Lactic acid bacteria have also been shown to be decreased in the intestinal tract of high fat diet fed mice [253]. Increased dietary fat has also been shown to diminish the diversity of the intestinal flora of mice [251]. In addition to altering the gut flora, a high fat diet has been shown to decrease the expression of enteric tight junction proteins, leading to increased permeability of the intestinal epithelium [249,254], which is the primary mechanism underlying endotoxemia. This high fat diet-induced metabolic endotoxemia provides a link of paramount importance between diet-induced alteration of the gut flora and intestinal barrier physiology along with metabolic-associated inflammation with resultant insulin resistance and glucose intolerance [255].

In plants, flavonoids are known to play a protective role against microbial invasion. Plants that are rich in flavonoids have been used in traditional medicine to treat infectious diseases for

many years [256]. Several studies have shown that kaempferol and kaempferol containing plants have antibacterial, antiviral, antifungal, and antiprotozoal properties. Interestingly, kaempferol and/or its glycosides have been shown to specifically inhibit the growth of *Staphylococcus* [257–263] *Bacillus* [260], and *Clostridium* [264], all of which are genera of the *Firmicutes* phylum. While inhibiting the growth of *Clostridium*, kaempferol does not elicit any antimicrobial activity against several species of *Bifidobacterium* which is broadly considered to be an anti-inflammatory bacterium, as it attenuates inflammation induced by TNF- α and LPS [265]. In addition, oral administration of kaempferol, twice daily for ten days, was shown to decrease the number of *Helicobacter pylori* colony forming units isolated from the stomach of Mongolian gerbils following four weeks of *H. pylori* administration [266]. While *H. pylori* is not necessarily associated with T2D incidence, it has been shown that diabetics with *H. pylori* have a significantly higher incidence of diabetic neuropathy [267]. While the research on the effects of flavonoids on gut microbiota, as they relate to inflammation and metabolic disease, is very limited, it is intriguing to speculate that the observed systemic anti-inflammatory effects of kaempferol in vivo could be partially or completely mediated via its action in the gut, where it modulates microflora and subsequent inflammatory process given that the bioavailability of this compound is very low and the observed anti-inflammatory effects in vitro were only achieved at pharmacological doses that are unattainable in vivo via dietary supplementation. This aspect should be investigated in the future.

4. Summary

T2D is a metabolic disease, characterized by insulin resistance and a progressive decline in functional β -cell mass. There are many factors contributing to the pathogenesis of T2D. Several genetic components as well as elevated fatty acids, mitochondrial dysfunction, oxidative stress, and

chronic inflammation have been identified to play a role in T2D. The flavonoid, kaempferol is a natural product present in a myriad of plants and botanicals that has been shown to possess anti-diabetic, anti-oxidative, antimicrobial, and anti-inflammatory properties. These effects have been shown in both *in vitro* and *in vivo* studies. However, many *in vitro* studies have used concentrations of kaempferol that are physiologically irrelevant due to its relatively low bioavailability. Our lab has conducted several *in vivo* studies showing that kaempferol elicits an ameliorative effect on T2D. However, the underlying mechanism for this kaempferol action is still unclear. Interestingly, kaempferol has been shown to have antimicrobial properties, specifically against several members of the *Firmicutes* phylum, which is associated with metabolic syndrome. Thus, it is possible that some of the medicinal effects of kaempferol may be partially mediated via intestinal microflora. Further research is also needed to include determining the physiological locale at which kaempferol exerts its affects.

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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 transporter-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 conditions, demonstrate that kaempferol may be a naturally occurring anti-diabetic agent that functions by improving peripheral insulin sensitivity and protecting against pancreatic β -cell dysfunction.

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

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 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 amounts of insulin to compensate for decreased insulin sensitivity, which is largely due to insulin secretory dysfunction and a 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 promote insulin sensitivity and β -cell survival may provide a more effective strategy to prevent the onset of diabetes [10].

Recently, naturally occurring polyphenolic compounds have 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 prevent 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.

Methods

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.

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%), which is roughly the human equivalent dose of 40 or 200 mg/day for an average 60 kg human, 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 (Mercodia, 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.

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.

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.

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.

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 μg/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 μg/mL) for differentiation.

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 μM kaempferol or vehicle (DMSO) along with a mixture of fatty acids or vehicle (BSA). The kaempferol concentration was chosen based on a preliminary study of optimal dose characterization. The range of concentrations used in the preliminary study were based on those used in various studies of kaempferol efficacy as are noted in the literature review. 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 CaCl₂, and 20 HEPES, pH 7.4) with the addition of 10 μM 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 μl of 0.2 M NaOH for cell lysis. Glucose uptake was calculated based on specific activity and expressed relative to protein content.

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 1.25 μ Ci/mL 14 C 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 $^{\circ}$ 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 $^{\circ}$ C for 20 min. Glycogen in the samples was precipitated with ice-cold ethanol. Following centrifugation at 4 $^{\circ}$ C (10,000 x g) 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.

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).

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.

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 $p < 0.05$.

Results

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.

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.

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.

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)).

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].

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.

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.

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 properties. While we found that pharmacological doses of kaempferol (10-50 μ M) showed significant free radical scavenging activity as was 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 be due to its potential antioxidant effect. We are presently investigating how kaempferol improves 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 the phytonutrient kaempferol may be used as a dietary supplement to prevent metabolic disorders that are associated with obesity and aging.

Figures

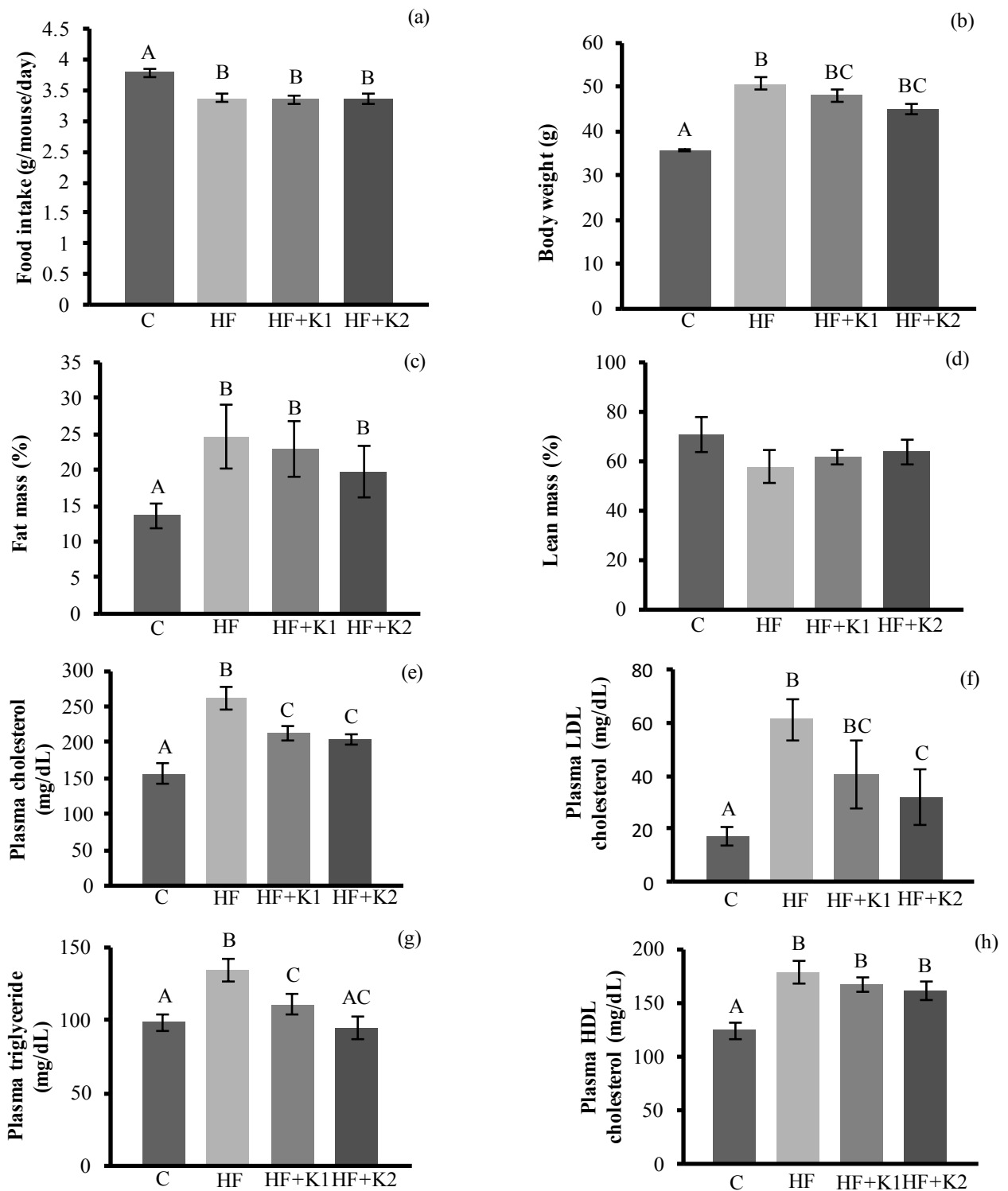


Figure 1: Kaempferol supplementation had no significant effects on food consumption, but reduced body weight gain and fat mass in HF diet-fed middle-aged mice. (a) Food intake was recorded twice a week and the average daily food intake was calculated. (b) Body weight of the individual mouse was measured 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). Letter differences denote significant difference at $P < 0.05$. C: standard diet; HF: high-fat diet; HF+K1: HF diet containing 0.01% kaempferol; HF+K2: HF diet supplemented with 0.05% kaempferol.

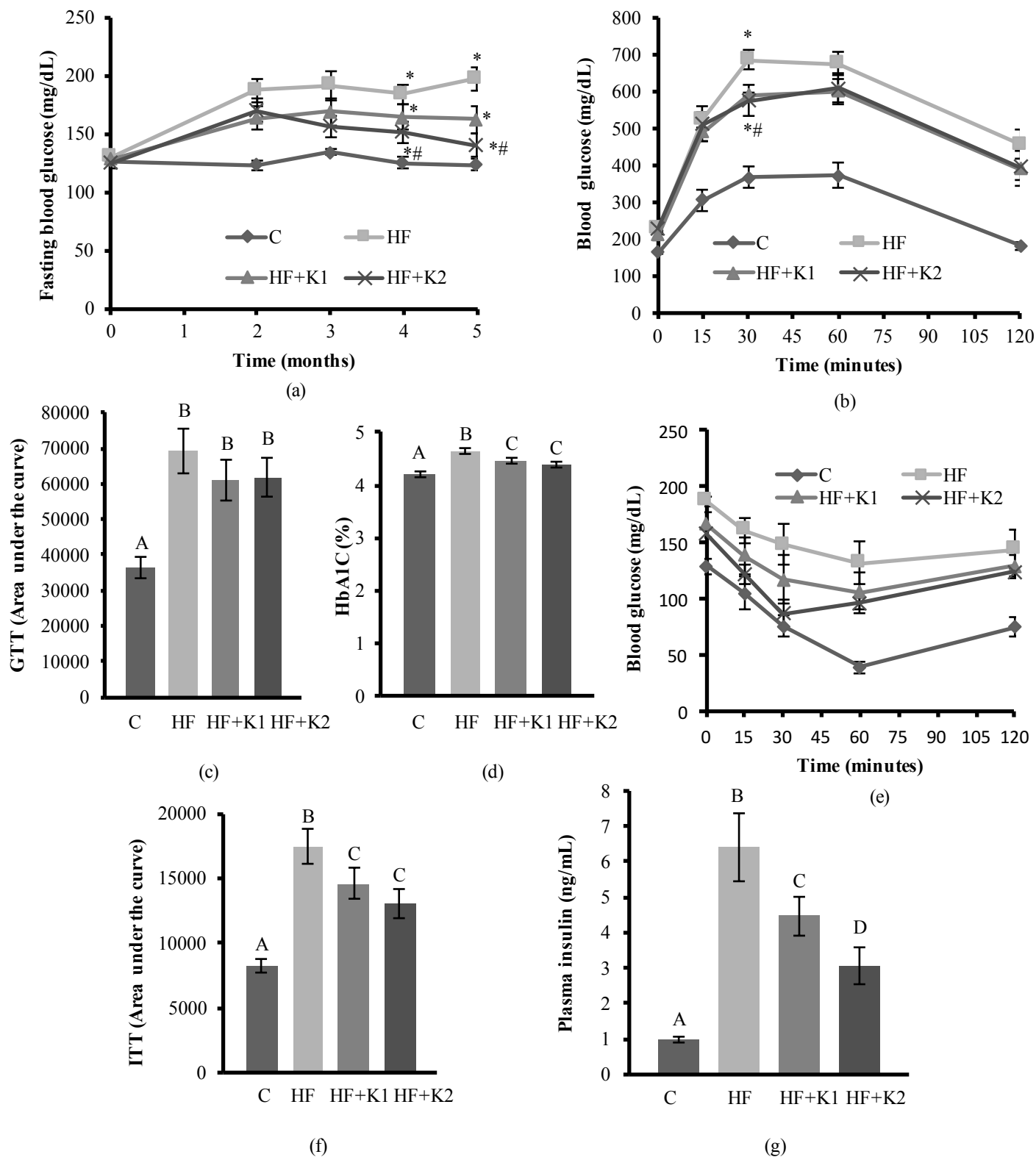


Figure 2: Kaempferol supplementation reduced blood glucose, HbA1c, and insulin concentrations, and improved insulin sensitivity in HF-diet fed mice. (a) Fasting blood glucose levels were measured at indicated time points of dietary treatment. GTT (b) and ITT (d) were performed as described in the Method 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±SE (n=12). Letter differences denote significant difference at $P < 0.05$, *, $P < 0.05$ 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.

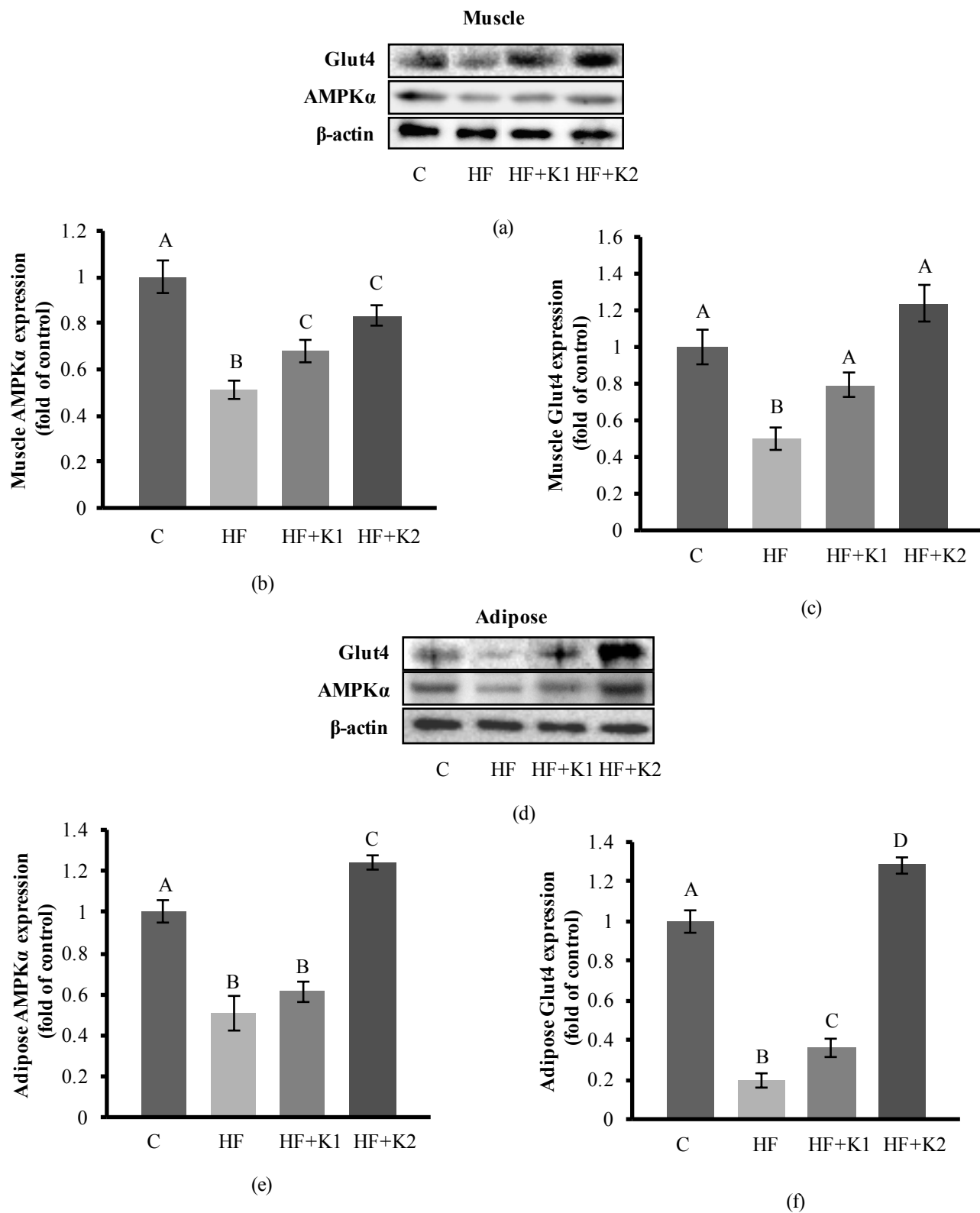
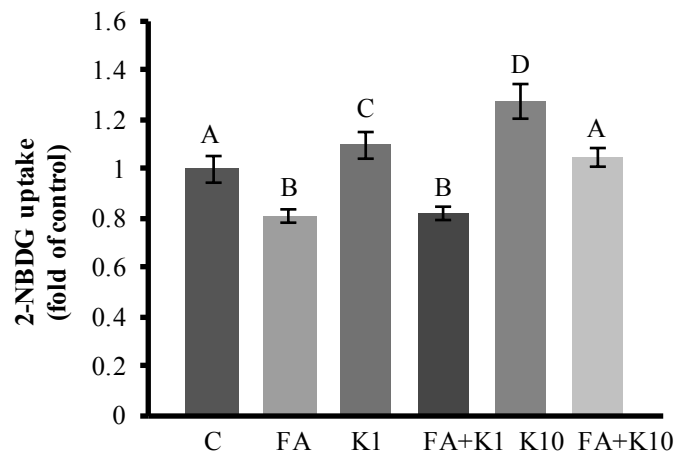
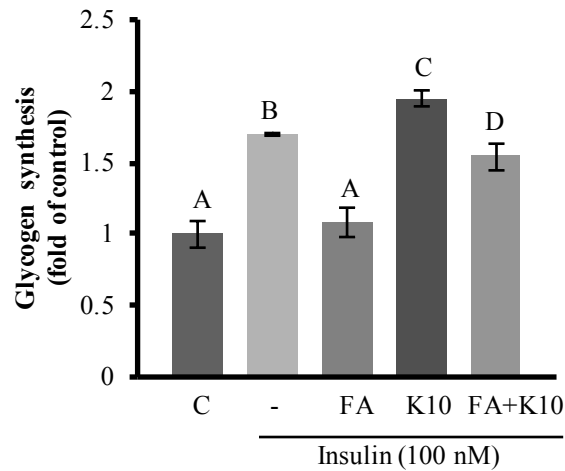


Figure 3: Kaempferol supplementation completely prevented the HF diet-induced decreases in AMPK α and Glut4 protein expression in skeletal muscle and adipose tissues of obese mice. At the end of feeding experiment, AMPK and Glut4 protein levels in whole cell lysates of skeletal muscle (a-c) and adipose 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. Letter differences denote significant difference at $P < 0.05$. C: standard diet; HF: high-fat diet; HF+K1: HF diet containing 0.01% kaempferol; HF+K2: HF diet supplemented with 0.05% kaempferol.

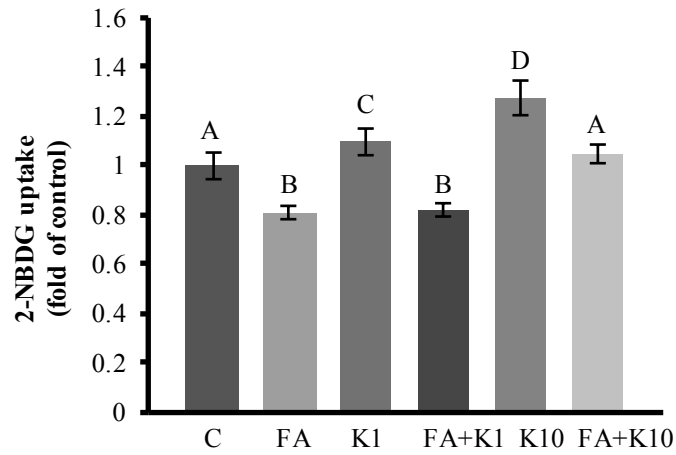


(a)

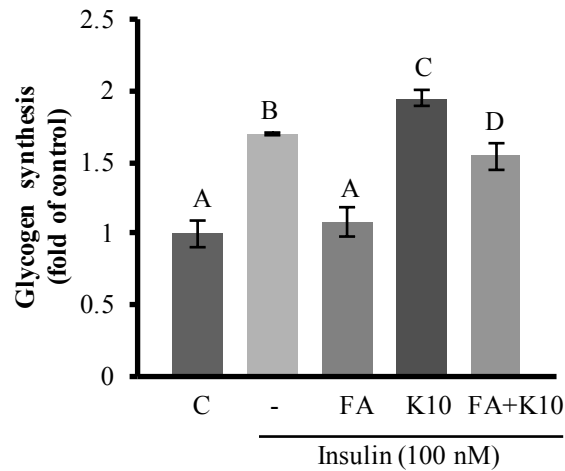


(b)

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 means \pm SEM (n=4) of duplicate or triplicated determinations each. Letter differences denote significant difference at $P < 0.05$.

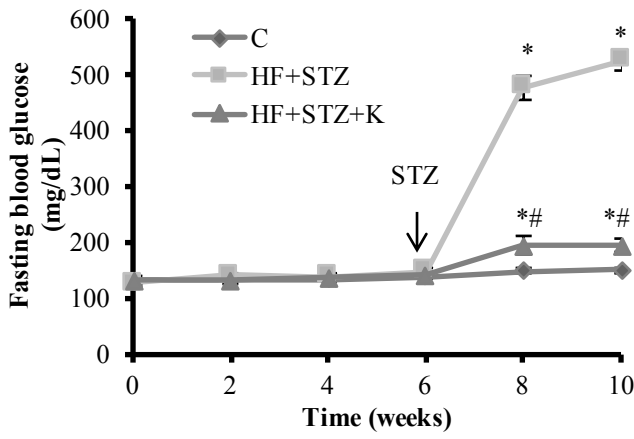


(a)

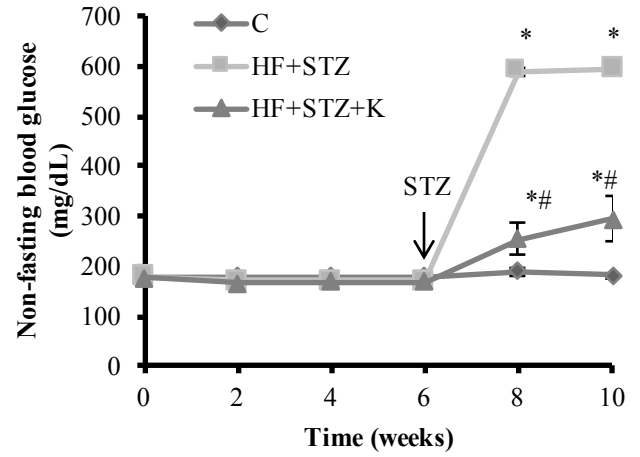


(b)

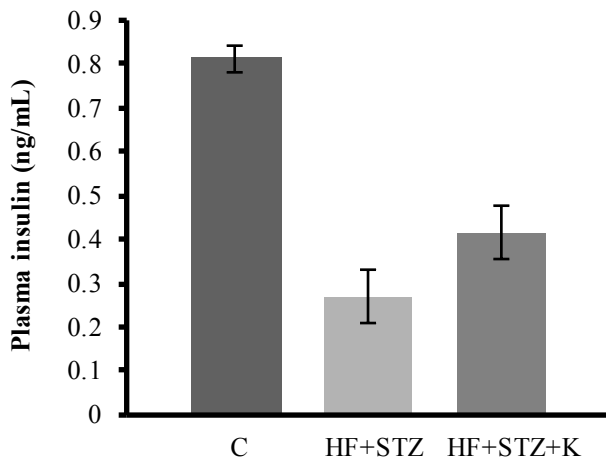
Figure 5: Kaempferol promoted lipolysis and reversed high glucose and fatty acid-impaired AMPK activity and Glut4 expression in muscle cells. C2C12 muscle cells were pre-treated with vehicle (C: DMSO) or kaempferol (K1: 1 μ M; K2: 10 μ M) for 24 h and then incubated in the presence or absence of 20 mM 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). Letter differences denote significant difference at $P < 0.05$.



(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). Letter differences denote significant difference at $P < 0.05$, *, $P < 0.05$ vs. healthy control (C); #, $P < 0.05$ vs. STZ alone-treated mice.

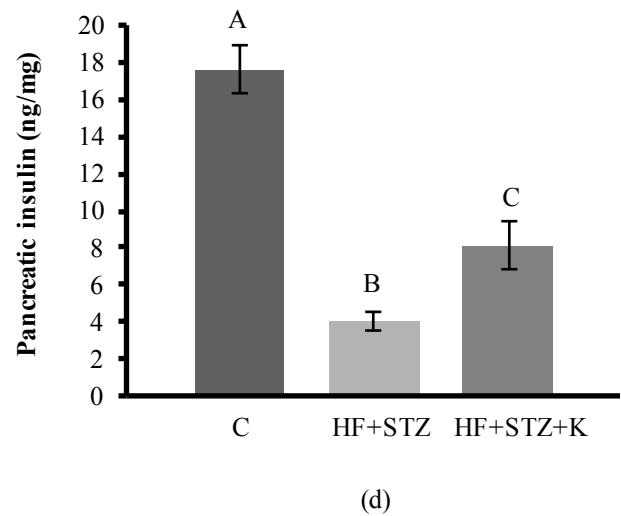
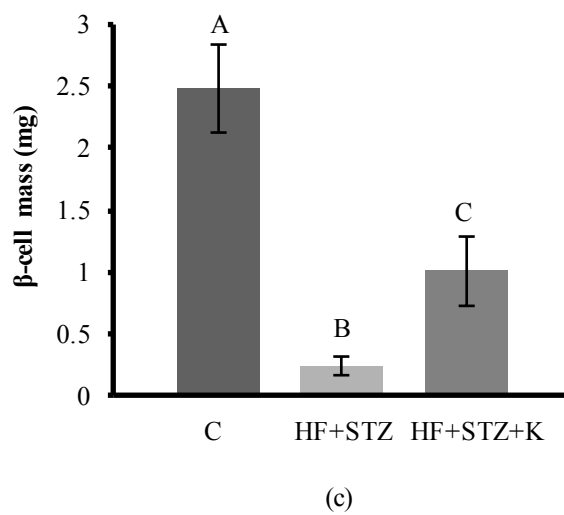
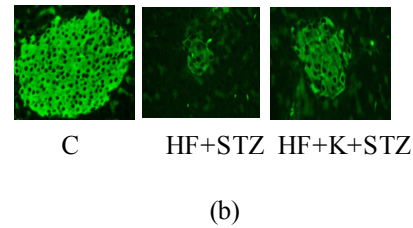
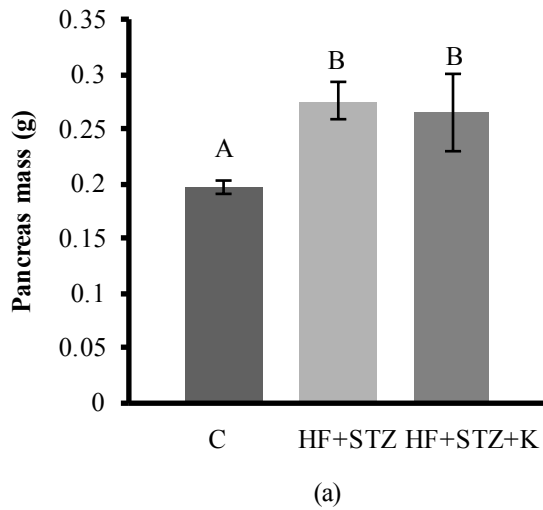


Figure 7: Kaempferol supplementation preserved pancreatic β -cell mass in STZ-induced diabetic mice. (a) Pancreas from mice given a SD diet (C), HF diet and STZ administration (HF+STZ), and HF+STZ supplemented with 0.05% kaempferol (HF+STZ+K) were isolated and weighed. (b) Fixed pancreas were then sectioned 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). Letter differences denote significant difference at $P < 0.05$.

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CHAPTER FOUR

Kaempferol ameliorates hyperglycemia through suppressing hepatic gluconeogenesis and enhancing insulin sensitivity in diet-induced obese diabetic mice.

Abstract

Obesity associated insulin resistance (IR) is a major risk factor for developing type 2 diabetes (T2D) and a wide array of other metabolic disorders. In particular, hepatic IR contributes to the increase in hepatic glucose production and consequently the development of fasting hyperglycemia. In this study, we explore the role of kaempferol, a flavonoid isolated from Ginkgo biloba, on the regulation of hepatic gluconeogenesis and blood glucose homeostasis in high-fat diet (HFD) -fed obese mice and further explore the underlying mechanism by which it elicits its effects. Oral administration of kaempferol (50 mg/kg/day), which is the human equivalent dose of 240 mg/day for an average 60 kg human, significantly improved blood glucose control in obese mice, which was associated with reduced hepatic glucose production and improved whole body insulin sensitivity without altering body weight gain, food consumption, or the adiposity. In addition, kaempferol treatment increased Akt and hexokinase activity, but decreased pyruvate carboxylase (PC) and glucose-6 phosphatase activity in the liver without altering their protein expression. Consistently, kaempferol decreased PC activity and suppressed gluconeogenesis in HepG2 cells as well as primary hepatocytes isolated from the livers of obese mice. Further, we found that kaempferol is a direct inhibitor of PC. Overall, these findings suggest that kaempferol may be a naturally occurring anti-diabetic compound that acts by suppressing glucose production and improving insulin sensitivity. Kaempferol suppression of hepatic gluconeogenesis is due to its direct inhibitory action on enzymatic activity of PC.

Keywords: Kaempferol, flavonoid, insulin resistance, type 2 diabetes, gluconeogenesis, pyruvate carboxylase.

Introduction

Type-2 diabetes (T2D) is one of the leading causes of mortality in the United States [1]. In 2015 diabetes prevalence reached 9.4% of the American population [2], and this number is anticipated to double by the year 2050 [3]. There is also a great fiscal burden imposed by diabetes-associated health-care as the estimated annual cost of diagnosed diabetes, in the U.S., is nearly \$250 billion [4]. Insulin resistance and β -cell dysfunction are two central components of T2D pathogenesis [5–7]. It is well recognized that T2D is typically prefaced by obesity, although the sequence of events leading to its development remains a matter of debate [5,6].

Obesity-induced hepatic insulin resistance (IR) is a significant contributor to fasting hyperglycemia [8] as altered regulation of hepatic glucose metabolism results in decreased glycogen synthesis and concurrently increased gluconeogenesis and glycogenolysis [9]. Because of the significant contribution of the liver to whole body glucose homeostasis, the liver is a major target for preventing and treating chronic hyperglycemia and its resultant diabetes [10,11]. As diabetes continues to develop into a greater fiscal and public health concern, investigation into the potential of novel, low-cost, naturally occurring agents that promote insulin sensitivity may be an effective strategy to prevent diabetes and improve the overall health of those already suffering from the disease [12].

Recently, naturally occurring polyphenolic compounds have drawn great interest due to their pharmacological implications, specifically those associated with human disease with considerable attention being devoted to their use in diabetes management [13,14]. 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 [15]. One of the most common

polyphenolic subclasses is the flavonols [16]. Kaempferol (3,5,7-trihydroxy-2-(4-hydroxyphenyl)-4H-1-benzopyran-4-one) is a flavonol found in many traditional medicines and edible plants, which may possess anti-diabetic activities [17]. We recently showed that dietary supplementation of kaempferol (0.05%) in a high fat diet (HFD) prevented the development of hyperglycemia and improved insulin sensitivity in middle-aged obese mice [18]. However, whether kaempferol is useful for treating T2D and how it exerts such an antidiabetic effect are still unclear. It has been shown that kaempferol (10-100 μ M) also improved basal glucose uptake in HepG2 hepatocytes in a dose dependent manner [19]. In addition, a high dose of kaempferol (50 μ M) has been shown to inhibit glucose-6-phosphatase (G6Pase) activity in a bioassay [20]. However, the physiological relevance of these in vitro studies is somewhat unclear given that the doses of kaempferol used are likely unachievable via dietary intake of this compound. In this study, we explored the mechanism underlying the anti-diabetic effect and/or insulin sensitizing effects of kaempferol.

Methods

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 diet (SD) 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, with 10% of calories derived from fat, or a HFD (Research Diets Inc., New Brunswick, NJ), with 58% of calories from fat for 8 wks, when animals gradually became obese

with significant IR, as determined by measuring their body composition and insulin tolerance. Mice were then divided into 4 groups ($n = 9$ mice/group) with blood glucose, body weight (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) via oral gavage for 6 wks. This dose was used based on an average calculation of kaempferol intake from the preceding *ad libitum* feeding studies.

Metabolic studies

Body weight 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 the 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). Blood glucose levels were then measured at 0, 15, 30, 60, and 120 min after injection of insulin. 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. Blood was immediately collected, and multiple organs were isolated, weighed, snap-frozen in liquid nitrogen, and stored at -80°C for further analyses. Plasma insulin and glucagon levels were measured using an ultrasensitive

mouse insulin ELISA kit (Merckodia, Inc., Uppsala, Sweden) and a mouse glucagon ELISA kit (Crystal Chem, Downers Grove, IL), respectively.

Pyruvate and glucose oxidation

Fresh mouse liver samples were used to analyze pyruvate and glucose oxidation as previously described [21,22] with modifications. Briefly, liver tissues were homogenized in a buffer containing 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 ¹⁴C-labeled pyruvate for pyruvate oxidation, or ¹⁴C-labeled glucose (American Radiolabeled Chemicals, St. Louis, MO) for glucose oxidation in a trapping device at 37 °C for 1 h. The ¹⁴CO₂ produced was trapped with 70% perchloric acid, and the resulting sodium hydroxide was collected to assess CO₂ production.

Glucose production

Primary mouse hepatocytes or HepG2 cells were maintained in DMEM containing 5 mM glucose supplemented with 1% pen-strep and 10% FBS. To measure glucose production, the cells were treated with kaempferol (0.1, 1, 10, 50 μM) or vehicle in FBS-free, low glucose DMEM for 5 h, washed, and then incubated in glucose production media (glucose- and phenol-free DMEM containing 20 mM sodium lactate and 2 mM sodium pyruvate) in the continued presence or absence of either the vehicle or kaempferol for 3 h. Glucose released in the media was measured using Amplex Red glucose assay kit (Life Technologies, Carlsbad, CA) and normalized to protein content of the same samples.

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 manufacturer's protocol. 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 the following equation: IU/L unit= $\mu\text{M}/(\text{Lmin}) = (\text{O.D.} \times 1000 \times 110 \mu\text{l}) / (30 \text{ min} \times 0.6 \text{ cm} \times 18 \times 10 \mu\text{L})$. Total hexokinase concentrations in the samples were determined by using a mouse hexokinase ELISA kit (Elabscience, Beijing, China).

The enzymatic activity of glucose-6 phosphatase (G6Pase) was measured as described with modifications [23]. This test quantifies liberated inorganic phosphate according to the Taussky-Shorr method [24]. Briefly, the assay buffer and substrate (200 mM glucose-6 phosphate) were mixed and allowed to equilibrate for 5 min followed by the addition of 5 μg of tissue homogenates. This mixture was incubated for 5 min at 37°C and the reaction was then stopped with 20% trichloroacetic acid, followed by the addition of 5 μg of tissue homogenate to the blank. The mixtures were incubated at 25°C for 5 min followed by centrifugation at 4,000 rpm for 10 min. The supernatant was used for color development by adding Taussky-Shorr color reagent. Following a 6-min incubation at 25°C, the $A_{340\text{nm}}$ was determined. The μmoles of inorganic phosphate was calculated according to the following equation:

$$\mu\text{moles Pi} = \Delta A_{340\text{nm}} (\text{Test}) - b/m.$$

The units per mg of enzymes was calculated according to the following equation:

$U/mg = \mu\text{mol Pi} \times \text{RXN volume} \times \text{df} / \text{volume homogenate} \times \text{volume of color development}.$

Pyruvate carboxylase (PC) activity was measured essentially as described [25]. This assay uses malate dehydrogenase to couple oxaloacetate production to the oxidation of NADH. The oxidation of NADH leads to a decline in the A_{340} , which was used to calculate the units per mL of enzyme based on the following equation:

$\text{Units/mL enzyme} = (\Delta A_{340\text{nm}} / \text{min (Test)} - \Delta A_{340\text{nm}} / \text{min (Blank)}) (\text{Assay volume}) (\text{df}) / (\text{mM extinction coefficient of } \beta\text{-NADH}) (\text{lysate or enzyme volume}).$

Western blot analysis

Tissues or cultured cells were homogenized in cell lysis buffer (Cell Signaling, Danvers, MA) supplemented with protease and phosphatase inhibitor cocktail (ThermoFisher Scientific, Waltham, MA). After centrifugation, equal amounts of protein from cell lysates 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 then probed with antibodies against phospho-Akt (Ser473) (#9271), Akt (#9272) (Cell Signaling Technology, Inc, Danvers, MA), PEPCK (H-300), G6Pase (H-60), PC (H-300), glucokinase (H-88), and glucokinase regulatory protein (N-19) (Santa Cruz Biotechnology, Inc, Dallas, TX). The immunoreactive proteins were detected and imaged using ChemiDoc™ Touch Imaging System (Bio-Rad, Hercules, CA). Proteins of interest were normalized to total protein content per lane on the blot.

Statistical analysis

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

Results

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 HFD for 8 wks to induce obesity and IR [26] 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 vs. 183.7 ± 5.6 mg/dl). After 6 wks of treatment, kaempferol reversed fasting blood glucose levels in obese mice comparable to those in SD-fed mice. Importantly, kaempferol had no effect on fasting blood glucose of SD-fed lean mice. IR contributes to the development of impaired glucose tolerance (IGT) [26,27]. Obese mice had IGT when compared to lean mice. Consistently, kaempferol treatment significantly improved glucose tolerance in obese mice but had no effect on glucose tolerance in lean mice (Figure 1 (c, d)). IGT is known to be associated with both defects in insulin secretion and/or IR [27] as β -cells have been shown to increase

insulin secretion to achieve normal glycemia in order to compensate for IR [28]. We therefore examined whether the better glycemic control in kaempferol treated obese mice was due to improved insulin sensitivity or insulin secretion [27]. Consistent with previous reports, we found that obese mice had significantly higher plasma insulin levels after 30 and 60 min of glucose injection than those detected in lean mice, suggesting that HFD-fed mice developed IR. Kaempferol did not modulate either basal insulin levels or glucose-stimulated insulin secretion (Figure 1 (e)), indicating that the anti-diabetic effect of kaempferol was not due to altering insulin secretory function of the islets. Next, we assessed whole body insulin sensitivity in mice by performing ITT. As shown in (Figure 1 (f, g)), obese mice were insulin resistant as compared with lean mice, but kaempferol treatment almost normalized insulin sensitivity in obese mice.

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

The average BW of experimental mice fed HFD (45.4 ± 1.14 g) was higher ($P < 0.05$) than mice fed SD (32.0 ± 0.89 g) throughout the study. Kaempferol had no effect on either BW (Figure 2 (a)) or food intake of obese and lean mice (Figure 2 (b)). The percentage of body fat for obese mice was significantly higher while lean mass lower (Figure 2 (d)) than those of lean mice (Figure 2 (c), (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.

As a compensatory mechanism for IR, prior to the development of overt hyperglycemia and T2D, the overall weight of the pancreas, and moreover pancreatic β -cell mass has been shown to increase [29]. Although obese insulin-resistant mice in the control group had heavier pancreas than that in 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 effect on the pancreas weight (Figure 2 (f)).

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

Glucagon regulates glucose homeostasis and counteracts insulin action by increasing glycogenolysis and gluconeogenesis, leading to increased hepatic glucose output [30]. However, circulating glucagon concentrations in fasted mice did not differ between groups (Figure 3 (a), suggesting that kaempferol improvement in glucose homeostasis is not due to modulation of circulating glucagon levels. We then evaluated Akt phosphorylation in the liver, as the activation of Akt mediates insulin suppression of gluconeogenesis while increasing glycogen synthesis [31]. We observed that Akt protein levels in the liver of obese mice were higher ($P < 0.05$) than those of lean mice (Figure 3 (b, h)). Oral administration of kaempferol increased Akt phosphorylation in obese mice (Figure 3 (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 glycolytic process and inducing glycogen synthesis [32], was significantly increased ($P < 0.05$) in kaempferol-treated obese mice (Figure 3 (d)). The increase in hepatic hexokinases activity wasn't associated with a change in protein expression of total hexokinases (Figure 3 (g)) or glucokinase (GCK) (Figure 3 (e, h)), which accounts for 90 and 95% of the total activity of glucose phosphorylation in rat and human liver, respectively [33]. Further, GCK regulatory protein (GCKRP) contents were similar between all groups (Figure 3 (f, h)). Moreover, kaempferol treatment did not modulate glucose or pyruvate oxidation in hepatic tissues (Figure 4 (a, b), suggesting that the reduction of hepatic glucose production observed in kaempferol-treated mice was not due to increased glucose glycolysis or oxidation.

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 blood glucose control. We measured skeletal muscle Akt and hexokinase protein levels and activities. Unlike kaempferol effect in the liver, neither proteins nor their activities were changed in the muscle tissues (Figure 5 (a-d)).

Kaempferol inhibits gluconeogenesis by suppressing PC activity.

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 [34], we examined whether kaempferol affected hepatic gluconeogenesis, which appears to be predominantly responsible for the excessive hepatic glucose output in T2D [35]. We show that kaempferol significantly decreased hepatic glucose production in obese mice ($P < 0.05$) as determined by pyruvate tolerance test (Figure 6 (a, b)). To determine whether kaempferol directly inhibits gluconeogenesis, we isolated and cultured mouse hepatocytes in the presence or absence of kaempferol for gluconeogenesis assay, and found that kaempferol treatment directly suppressed glucose production in hepatocytes (Figure 6 (c, d)).

To further determine how kaempferol inhibits glucose production, we studied some key enzymes for hepatic gluconeogenesis. PC (Figure 7 (a, f)) and G6Pase (Figure 7 (b, f)) protein levels were significantly higher in obese mice as compared with lean mice, whereas PEPCK protein expression did not differ between groups (Figure 7 (c, f)). Kaempferol treatment did not alter protein expression of these enzymes in the liver. Interestingly, it reduced the elevated PC

and G6Pase activity in the livers of obese mice to the levels similar to that observed in the lean mice ($P < 0.05$) (Figure 7 (d, e)). Similarly, kaempferol treatment inhibited glucose production (Figure 8a), and PC activity (Figure 8b in HepG2 cells, further confirming a direct role for kaempferol in the control of hepatic glucose production. Lastly, we found that kaempferol at the same concentrations for inhibiting glucose production and PC activity in hepatocytes, inhibited the purified PC activity in cell-free system (Figure 8c), suggesting that kaempferol is a direct inhibitor enzymatic activity of PC.

Discussion

In this study, we demonstrate that oral administration of kaempferol (50 mg/kg daily) for 4 wks restores glucose homeostasis and insulin sensitivity, independent of BW change or adiposity, in HFD-induced obese mice. Importantly, kaempferol did not exert an effect on the metabolic phenotype of SD-fed healthy mice, suggesting that the potential side effect of nutritional supplementation of kaempferol is minimal and that the health benefits of kaempferol depend on the metabolic state [36]. The observed metabolic effects of kaempferol in obese mice were associated with a robust suppression of hepatic glucose production. Impaired 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, indicating that 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, specifically its effect on PC activity. Our data from in vivo, in vitro and pure enzyme-based assays consistently show that kaempferol is an inhibitor of hepatic PC activity. In addition, we observed an increase in hepatic

Akt and GCK activities in obese mice treated with kaempferol, which are known to be associated with blunted gluconeogenesis and ameliorated insulin sensitivity respectively [37–39]. These results, together with our recent findings that long-term dietary kaempferol intake prevented diet-induced IGT and IR in middle-aged mice [18], suggest that kaempferol could potentially be a safe and inexpensive natural compound for preventing and treating T2D.

It is well recognized that obesity increases the risk for developing IR and T2D. In order to determine the potential therapeutic effect of kaempferol on obesity-related IR and glucose intolerance, C57BL/6 mice were fed a HFD for 8 wks to induce obesity, IR, and glucose intolerance prior to receiving kaempferol treatment [40]. The diet-induced obese mouse models have been widely used due to the phenotypic similarities of those induced by a Western diet in humans [26], although IR can develop at any degree of adiposity in humans [41]. In the present study, we observed that kaempferol improved glucose tolerance and insulin sensitivity without altering insulin levels, BW gain, food intake, or the degree of adiposity, suggesting that the anti-diabetic action of kaempferol is not a secondary effect whereby it modulated these metabolic parameters. These observations are consistent with previous results showing that treatment with kaempferol and some of its glycosides extracted from unripe Jinadi soybean leaves improved glucose tolerance, lowered HbA_{1c} levels, decreased fasting blood glucose levels, and improved insulin sensitivity [42,43]. These results from various mouse models and experimental designs collectively provide evidence that kaempferol indeed exerts anti-diabetic action at least in animal models.

The liver plays an integral role in maintaining glucose homeostasis [44]. Activation of GCK, the predominant hexokinase in the liver, is proposed to be a potential target for diabetes treatment due to its critical role in maintaining glucose homeostasis [45]. When activated, GCK

phosphorylates glucose and increases its clearance by diverting glucose into glycolysis and inducing glycogen synthesis in the liver [46]. Although the factors that regulate GCK are not entirely studied, several studies demonstrated that insulin and GCKRP play a role for regulating GCK [47]. In T2D, hepatic glucose uptake and transport are blunted thus decreasing its flux into hepatocytes. It is suggested that these alterations associated with IR and diabetes may be due to reduced GCK activity [48]. Therefore, targeting GCK to promote its activity has been actively explored for better glycemic control. Indeed, several studies demonstrated that the use of GCK activators led to a significant increase in glucose uptake and metabolism, thereby improving glucose tolerance in T2D mice [38]. In HFD-fed mice, chronic GCK activation improved diminished blood glucose and insulin while improving glucose tolerance despite reduced insulin secretion, which is indicative of improved insulin sensitivity [39]. Consistently, we found that kaempferol treatment increased GCK activity in HFD-fed mice, which may contribute to the improved glucose control and enhanced insulin sensitivity observed in these mice. It is possible that the increase in GCK activity might increase glycolysis and glycogen synthesis, however, in our study the increase in GCK was not accompanied by an increase in hepatic glucose oxidation. 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 GCK [49,50]. 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.

The activation of Akt by insulin contributes to the control of hepatic glucose metabolism as it has been shown to phosphorylate and inhibit PGC-1 α , which is a regulator of post-absorptive hepatic metabolism [37]. This inhibition of PGC-1 α impedes its ability to activate

gluconeogenic genes including *PEPCK* and *G6Pase* [37,51]. Akt further suppresses gluconeogenesis by phosphorylating and inactivating FOXO1 [52–55]. Induction of Akt signaling also stimulates glycogen synthesis [56,57] by phosphorylating GSK-3. In this study, we found that kaempferol increased hepatic Akt activity in obese mice.

Hepatic IR is associated with upregulated expression and activity of enzymes that control glucose production [58] including PC, PEPCK [59], and G6Pase [60], consequently leading to excessive hepatic gluconeogenesis and thus glucose output, which primarily contribute to fasting hyperglycemia [61–63]. Indeed, increased hepatic gluconeogenesis is considered one of the early pathological changes in newly diagnosed T2D subjects [61]. Our results show that G6Pase protein levels increased significantly in obese compared to lean mice, whereas PEPCK protein levels were not altered. This observation was consistent with the results of another study [64]. However, neither PEPCK nor G6Pase protein levels were altered by kaempferol treatment. It was demonstrated that neither the gene nor the protein expression of PEPCK or G6Pase was associated with fasting hyperglycemia in either rats or patients with T2D [65]. Consistently, another study showed that the elevated hepatic glucose production in morbidly obese diabetic patients was associated with an increase in hepatic G6Pase activity [66], suggesting that the increase in gluconeogenesis in insulin resistant and diabetic subjects might be primarily due to changes in the activities of one or more enzymes rather than increases in their protein expression.

While PEPCK and G6Pase play a vital role in gluconeogenesis, they display relatively weak control over its progression [67]. Instead, PC, which is responsible for the first step in gluconeogenesis, was found to be strongly associated with glycemia in humans [68], as its inhibition greatly reduced gluconeogenesis in vitro and in vivo [69]. To our surprise, we observed that kaempferol treatment had no effect on PC expression, but greatly inhibited PC

activity in the liver, primary mouse hepatocytes, and HepG2 cells. Therefore, it is conceivable that kaempferol may suppress gluconeogenesis and thus hepatic glucose output via inhibition of this gluconeogenic enzyme. To determine whether kaempferol is a direct inhibitor of PC, we measured the enzymatic activity of pure PC in response to kaempferol treatment, and found that kaempferol directly inhibited its activity. To that end, it is possible that increased hepatic Akt activity in kaempferol-treated obese mice may be secondary whereby it inhibited PC activity. Indeed, in a study where PC expression was specifically ablated in hepatic and adipose tissue by ip injection of a specific antisense oligonucleotide of PC in HFD-fed rats, it was shown that the inhibition of PC in liver and adipose tissue subsequently improved hepatic insulin sensitivity, which was reflected by the suppression of hepatic glucose production and an increase in hepatic Akt activity [68].

Metabolically inflexible individuals typically have higher levels of blood glucose in the post-absorptive state when compared to metabolically flexible individuals as has been demonstrated by elevated respiratory quotients in skeletal muscle from obese insulin-resistant [70] and T2D adults [71]. This is consistent with findings that endogenous glucose production is higher in T2D compared to healthy subjects [72]. This increased blood glucose, partially due to elevated gluconeogenesis, along with blunted insulin sensitivity, results in increased insulin secretion as a compensatory mechanism for elevated blood glucose and blunted insulin sensitivity [28]. Conversely, increased insulin sensitivity reduces pancreatic insulin release [73], as the amount of insulin that is required to maintain euglycemia is decreased [74]. Although we did not measure fasting-blood insulin levels in this study, data from our previous studies showed that kaempferol decreased circulating insulin levels [18], consistent with the improved insulin sensitivity and glucose homeostasis [75].

As we have shown in previous studies, kaempferol administration increases the expression of Glut4 in skeletal muscle [18], and we show here that kaempferol stimulates hepatic glucose uptake and also inhibits gluconeogenesis by inhibiting PC and G6Pase. Our findings that kaempferol stimulates glucose uptake, inhibits glucose production, and thereby decrease blood glucose combined with the finding that kaempferol attenuates insulin sensitivity further suggests that these effects are related. While the mechanism of this relationship remains unclear, previous studies show that insulin resistance can be improved by inhibiting glucose production and enhancing glucose disposal resulting in diminished insulin secretion.

In summary, hepatic insulin resistance increases the risk of T2D development due to increased hepatic glucose production and the subsequent development of fasting hyperglycemia. Here we show that oral administration of kaempferol ameliorated hyperglycemia, glucose intolerance, and IR in diet-induced obese mice. These effects were associated with the ability of kaempferol to increase glucose uptake and inhibit hepatic gluconeogenesis by inhibiting PC and G6Pase activity. In addition, kaempferol treatment also improved hepatic glucose metabolism by increasing Akt and GCK activity. Collectively, these findings suggest that kaempferol may be an effective anti-diabetic compound by regulating hepatic gluconeogenesis and improving insulin sensitivity.

Results

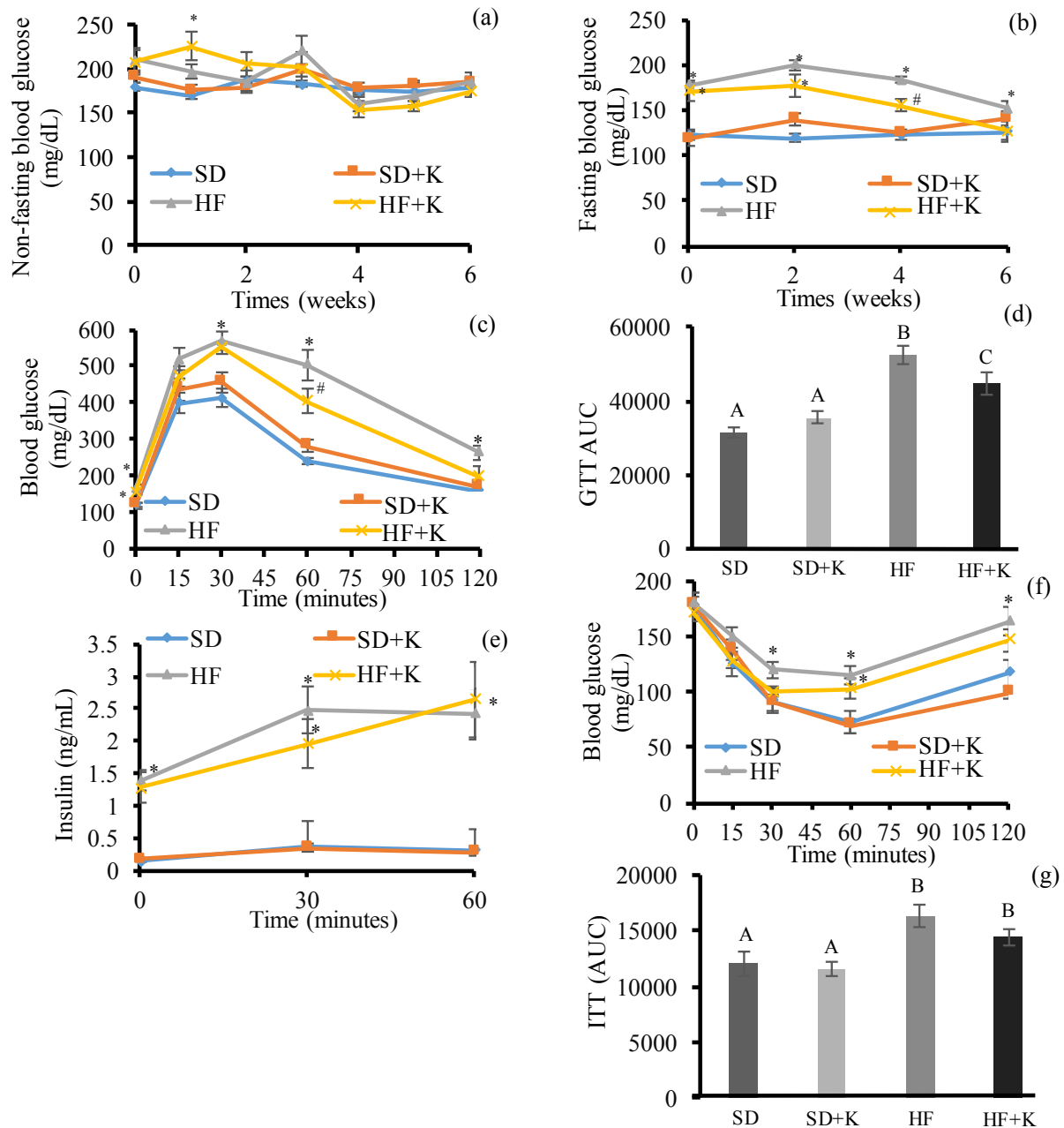


Figure 1. Kaempferol treatment reduced fasting blood glucose, improved glucose tolerance, and improved insulin sensitivity in HF diet-fed mice. (a) Non-Fasting and (b) fasting blood glucose levels were measured at indicated time points of dietary treatment. GTT (c) and (f) ITT were performed as described 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). Letter differences denote significant difference at $P < 0.05$, *, $P < 0.05$ 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 (50 mg/kg BW).

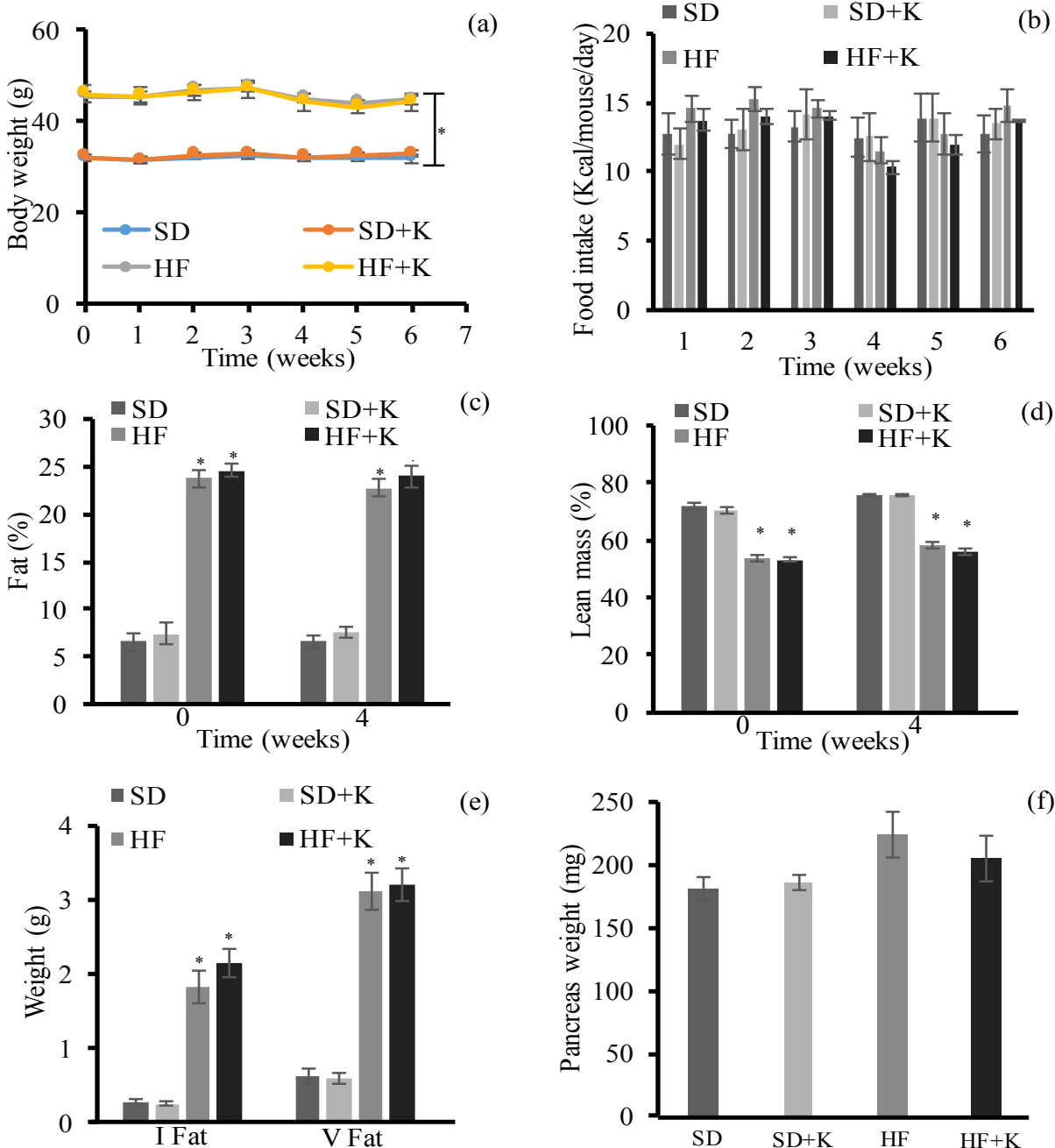


Figure 2. Kaempferol treatment had no significant effect on BW gain, food consumption, body composition, fat tissue or pancreas weight in HF diet-fed mice. (a) BW of the individual mouse was measured weekly. (b) Food intake was recorded each week, and the average daily food intake was calculated. Fat 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 (50 mg/kg BW).

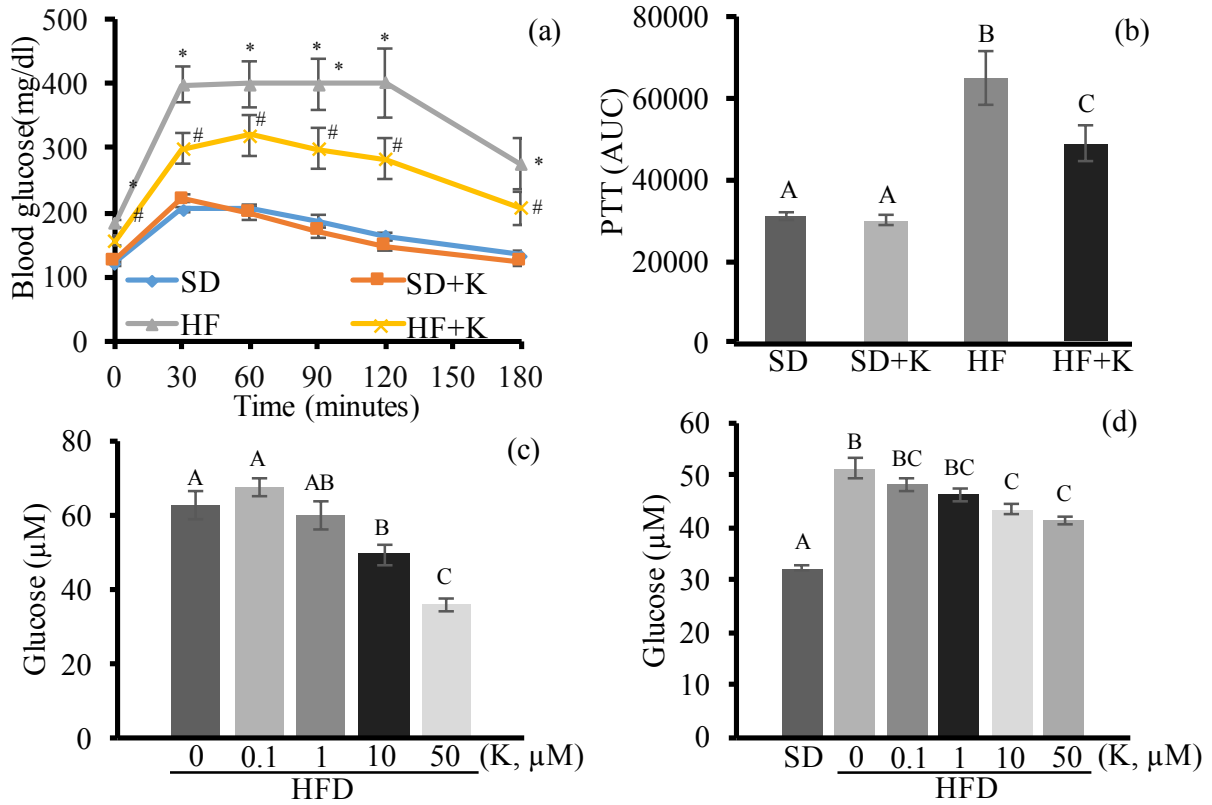


Figure 3. Kaempferol treatment increased liver Akt and GCK activity in HF-fed mice. At the end of the feeding experiment, (a) fasting plasma glucagon levels were measured using ELISA kit. (b, h) Akt, (c, h) pAkt, (e, h) GCK and (f, h) GCKRP protein levels in whole cell lysates of liver tissue of mice 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. Letter differences denote significant difference at $P < 0.05$, *, $P < 0.05$ vs. standard diet-fed mice (SD). SD: SD diet; HF: HF diet; HF + Kaemp: Hf diet with kaempferol treatment (50 mg/kg BW).

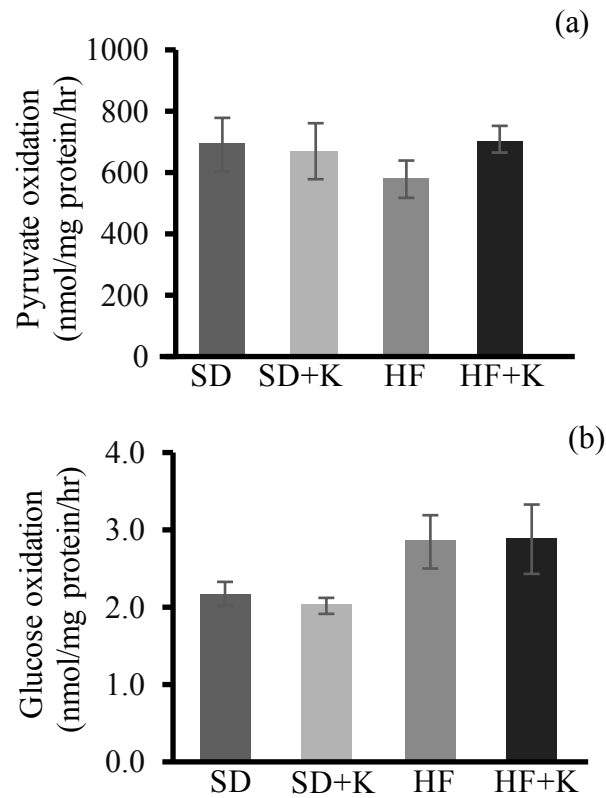


Figure 4. Kaempferol had no effect on glucose and pyruvate oxidation in the liver of HF diet-fed mice. (a) Pyruvate and (b) glucose oxidation were measured in fresh mouse liver homogenates using either ^{14}C -labeled pyruvate for pyruvate oxidation or ^{14}C -labeled glucose as described in the Method section. Values are Mean \pm SEM from 7 mice per group. SD: SD diet; SD + Kaemp: SD diet with kaempferol treatment (50 mg/kg BW); HF: HF diet; HF + Kaemp: HF diet with kaempferol treatment (50 mg/kg BW).

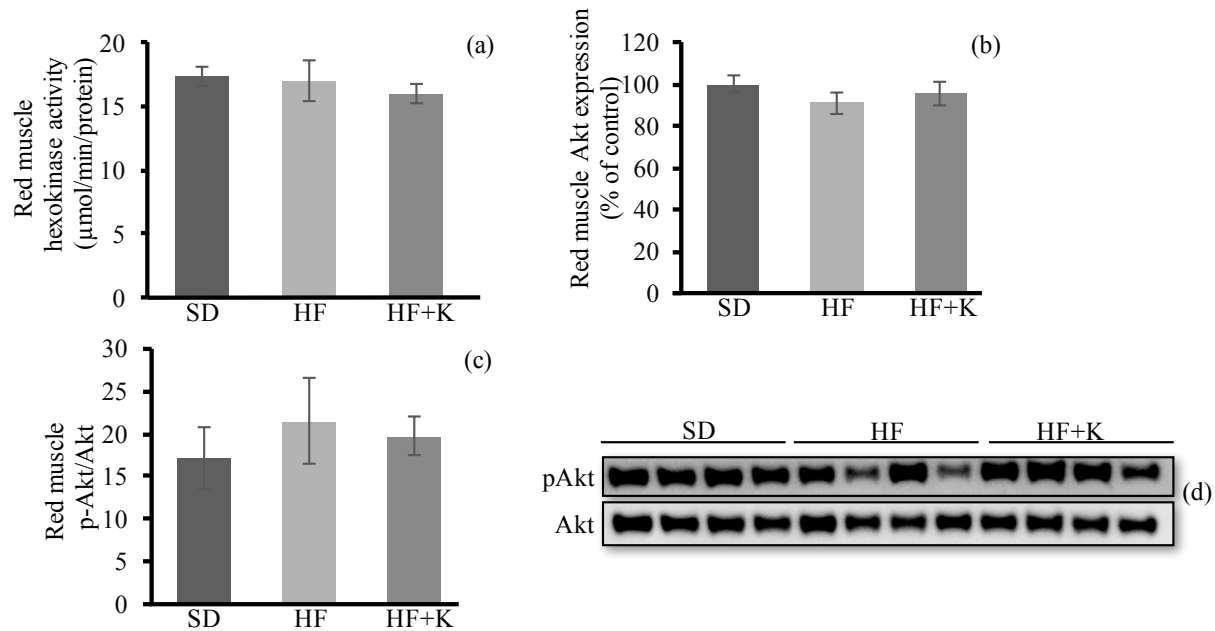


Figure 5. Kaempferol had no significant effect on Akt or hexokinase activities in red muscle of HF diet-fed mice. At the end of the feeding experiment, (a) hexokinase activity was measured in the homogenates of red skeletal muscle using an assay kit. (b, d) Akt, and (c, d) pAkt protein levels in whole 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 (50 mg/kg BW).

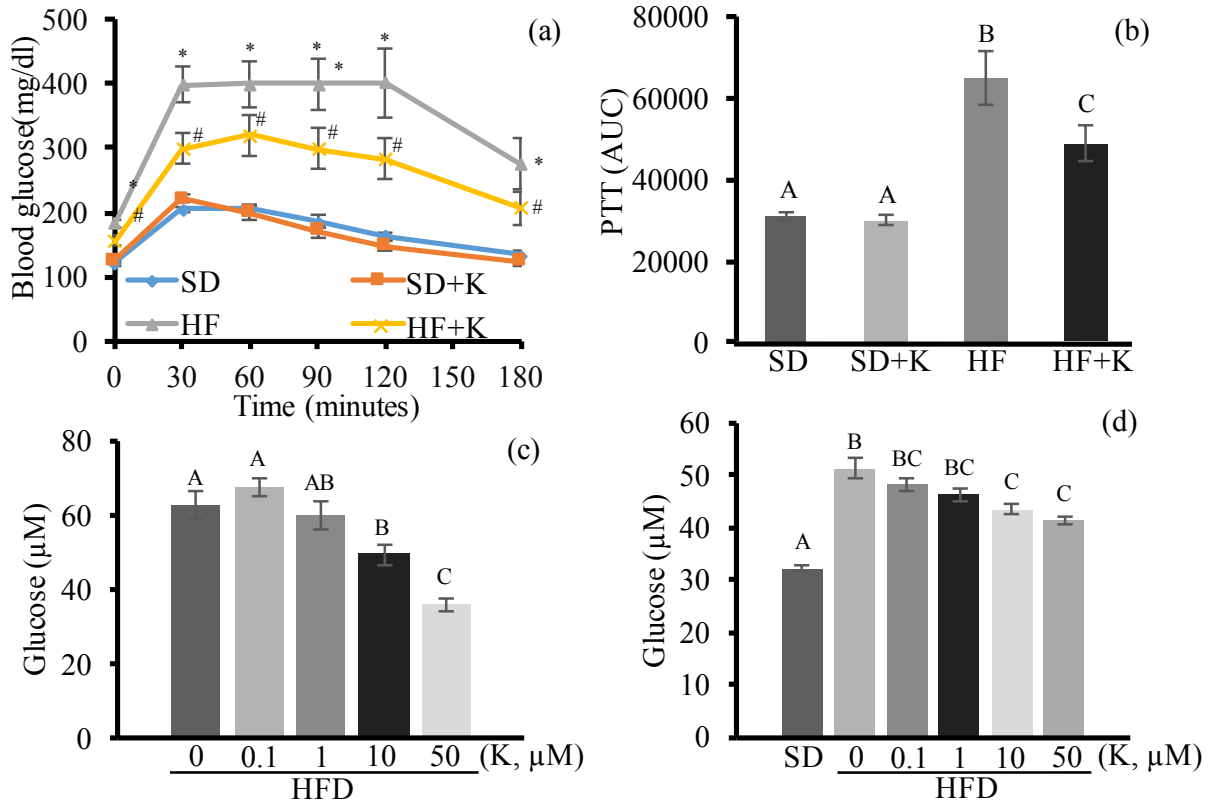


Figure 6. Kaempferol suppressed hepatic glucose production in HF diet-fed mice. (a) PTT was performed as described in the Method section and the area under the curve (AUC) for PTT (b) was calculated. Primary hepatocytes were isolated from LFD-fed mice (c) and HFD-fed mice (d). Cells were then cultured in gluconeogenic medium containing kaempferol, and its ability to suppress glucose production was assayed. Values are Mean \pm SEM from 8-9 mice per group. Letter differences denote significant difference at $P < 0.05$, *, $P < 0.05$ vs. standard diet-fed mice (SD); #, $P < 0.05$ vs. HF-fed mice (HF). Different letters denote $P < 0.05$. 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).

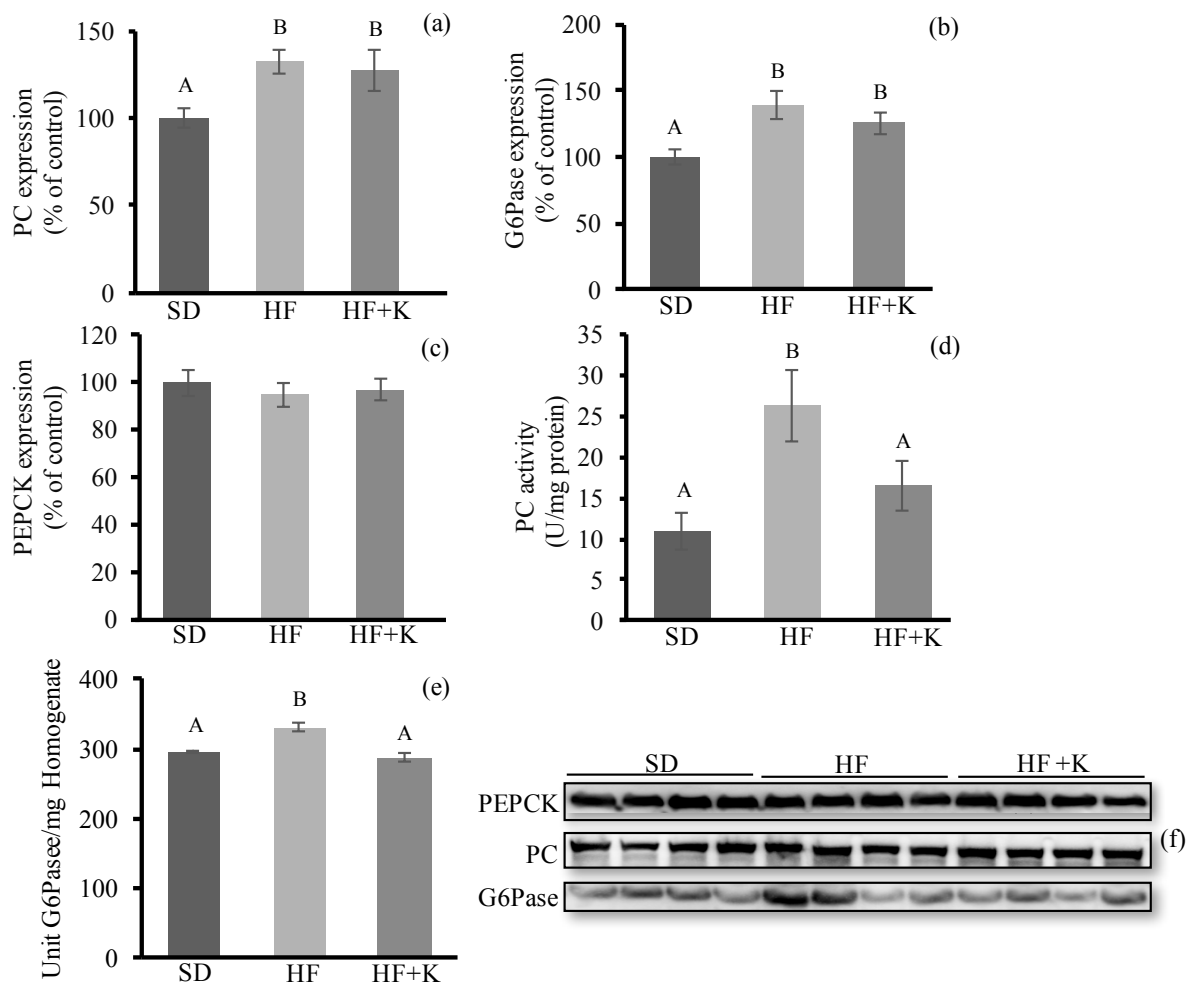


Figure 7. Kaempferol restored the impaired hepatic PC and G6Pase activity without altering their protein expression in HF diet-fed mice. At the end of feeding experiment, (a,f) PC, (b,f) G6Pase, and (c,f) PEPCK protein levels in whole cell lysates of liver tissue of mice were measured by immunoblotting and normalized to total protein, which were not altered by kaempferol. (d,e) PC and G6Pase activity was measured as described in the Method section. Values are Mean \pm SEM from 8-9 mice per group. Letter differences denote significant difference at $P < 0.05$. SD: SD diet; SD + Kaemp: SD with kaempferol treatment (50 mg/kg BW); HF: HF diet; HF + Kaemp: HF diet with kaempferol treatment (50 mg/kg BW).

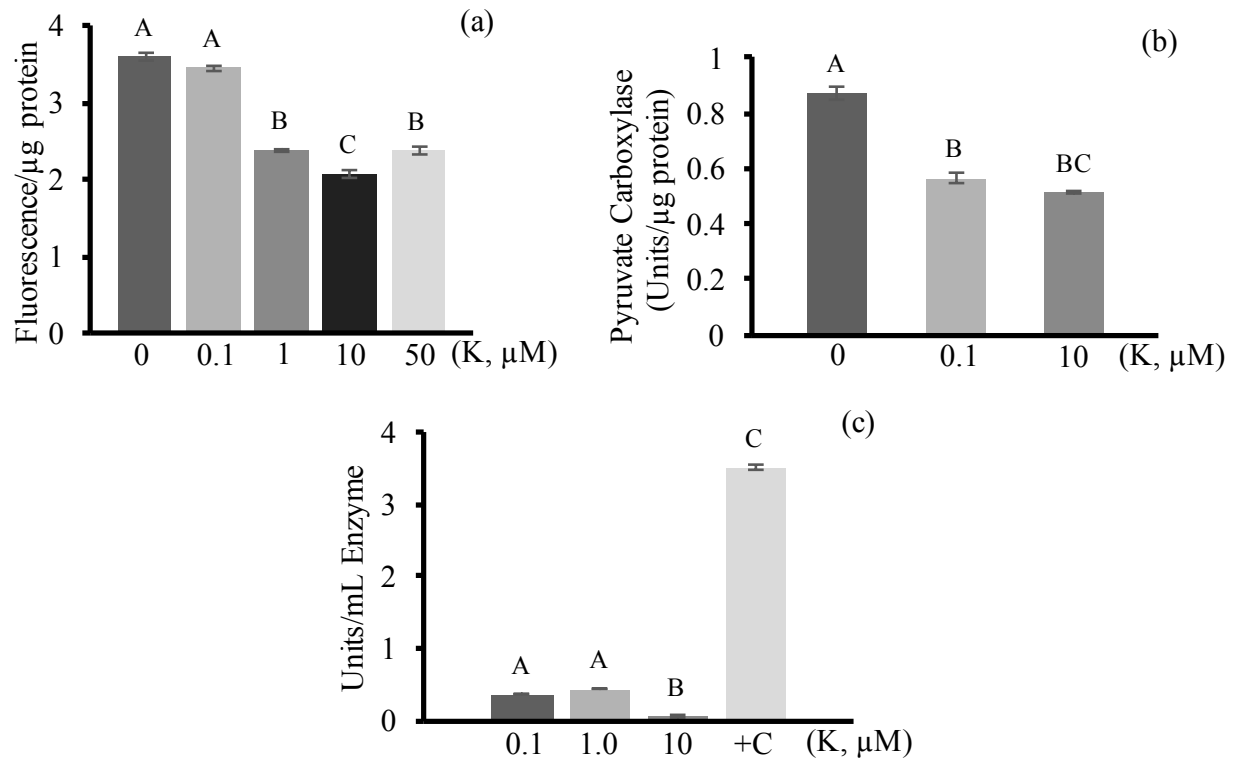


Figure 8. Kaempferol suppressed hepatic glucose production and pyruvate carboxylase activity in HepG2 cells. (a) Glucose production was determined using an assay kit. (b, c) Pyruvate carboxylase activity was determined as describe in the methods section. Values are expressed as Mean \pm SEM (n=3). Letter differences denote significant difference at $P < 0.05$.

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CHAPTER FIVE

Kaempferol improves glucose homeostasis partially by enhancing glucose uptake in skeletal muscle

Abstract

Insulin resistance is a hallmark of type-2 diabetes (T2D) pathogenesis. Because skeletal muscle (SkM) is the predominant site of glucose disposal, especially in the post-prandial state, insulin resistance in SkM is considered to be the primary clinical manifestation of the disease as it is typically evident years prior to β -cell dysfunction and subsequent overt diabetes. Thus, the identification of low-cost compounds that enhance the ability of SkM to take up glucose is a promising strategy for treating and preventing T2D. Our previous work showed that kaempferol improved insulin sensitivity in obese middle-aged mice, however, the means by which this action is achieved remains unknown. In this study, we examined whether kaempferol, a flavonol, enhances SkM glucose uptake. Kaempferol stimulated basal glucose uptake in primary human SkM (hSkM). In C2C12 mouse myotubes, kaempferol also increased insulin stimulated glycogen synthesis and preserved insulin dependent glycogen synthesis and glucose uptake in the presence of fatty acids. Kaempferol stimulated Akt phosphorylation in a similar time-dependent manner as insulin in human SkM cells. Consistent with this, kaempferol increased Akt and AMPK phosphorylation in isolated murine red SkM tissue. The effect of kaempferol on glucose uptake was blunted in the presence of chemical inhibitors of glucose transporter 4 (Glut4), phosphoinositide 3-kinase (PI3K), and AMPK. In addition, inhibition of AMPK prevented kaempferol-stimulated Akt phosphorylation. Further, kaempferol improved the stability of insulin receptor substrate-1. Taken together, these findings suggest that kaempferol stimulates glucose uptake in SkM via an AMPK/Akt dependent mechanism, and may be a viable therapeutic agent for insulin resistance.

Keywords: Type-2 diabetes, skeletal muscle, kaempferol, insulin resistance

Introduction

Insulin resistance and obesity are the central culprits in the development of a myriad of chronic diseases including cardiovascular disease [1], neoplastic disease [2], liver disease [3], and diabetes [4]. These four conditions account for over half of all deaths in the United States each year [5,6]. In 2016, diabetes remained the 7th leading cause of death in the United States, and claimed the lives of 76,488 people [7]. As of 2015, diabetes affected nearly 9.4% of the U.S. population, and with the occurrence of the disease continuing to rise, this number is expected to double by 2050 [8]. There is also a great fiscal burden imposed by diabetes-associated health-care as the estimated annual cost of diagnosed diabetes, in the United States, is nearly \$250 billion [9].

While the series of events leading to the clinical diagnosis of T2D is still a subject of debate, it is widely accepted that both insulin resistance and β -cell dysfunction are critical components of T2D pathogenesis [10–12]. However, insulin resistance is clinically discernable long before β -cell dysfunction, and is thus a primary target for preventing the progression of T2D [13,14]. Approximately 80-90% of glucose disposal has been shown to be attributable to skeletal muscle (SkM) in euglycemic hyperinsulinemia test [15]. As insulin levels increase, leg muscle glucose uptake has been shown to increase linearly with time for 60 min at which point the rate of glucose uptake reaches plateaus [16,17]. In subjects with T2D, this effect has been shown to be blunted resulting in a 50% reduction of glucose uptake in muscle [17], suggesting that insulin resistance in SkM is indeed of paramount importance in the pathogenesis of T2D. As diabetes increasingly becomes a fiscal and public health concern, investigation into the potential of novel, low-cost, naturally occurring agents that promote insulin sensitivity may provide an effective

strategy to prevent diabetes and improve the overall health of those already suffering from the disease [18].

Recently, naturally occurring polyphenolic compounds have been the focal point of medicinal research interest due to their pharmacological implications associated with human disease with considerable attention devoted to managing diabetes [19,20]. 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 [21]. One of the most common polyphenolic subclasses is flavonols [22]. Kaempferol is a naturally occurring flavonol that has been identified in several plants used in traditional medicine including *Equisetum* spp., *Ginkgo biloba*, *Sophora japonica*, as well as edible plants including broccoli, cabbage, gooseberries, grapes, kale, strawberries, tea, and tomatoes [23,24]. Dietary intake of kaempferol-containing foods has been epidemiologically associated with a reduced risk of a myriad of chronic illnesses associated with metabolic syndrome [23,25–28]. However, the studies assessing the anti-diabetic action of kaempferol are limited. We recently reported that long-term dietary intake of kaempferol supplemented in high-fat diet (HFD, 0.05%) improved insulin sensitivity and hyperglycemia in middle-aged obese mice [29]. Further, kaempferol was shown to improve basal glucose uptake in a human hepatocyte cell line (Hep G2) in a dose-dependent manner (10–100 μ M) [30]. However, the means by which kaempferol exerts these effects is remains elusive. Here, we investigate the effect of kaempferol on glucose uptake in SkM and further investigate the underlying mechanism by which this effect might occur.

Methods

Cell culture

Primary human skeletal muscle cells from a single non-obese donor (Caucasian, healthy, male, BMI < 25 kg/m², no evidence of metabolic disease, no family history of diabetes or cardiovascular disease) were grown to ~80% confluence in Dulbecco's Modified Eagle's Medium (DMEM; HyClone, Logan, UT) containing 10% fetal bovine serum (FBS; Sigma-Aldrich, St. Louis, MO), 5% fetuin bovine (Lonza, Walkersville, MD), 0.5% gentamicin (Lonza), 0.5% dexamethasone (Lonza), and 0.5% recumbent human epidermal growth factor (Lonza). At 80% confluence, the myocytes were differentiated into myotubes in low glucose (5.5 mM) DMEM containing 2% horse serum (Invitrogen, Carlsbad, CA), BSA, fetuin, and gentamicin in the same concentrations as noted above. Experiments were performed on day 7 of differentiation. C2C12 mouse cells (ATCC, Manassas, VA) were grown in DMEM supplemented with penicillin (50 IU/mL), streptomycin (50 µg/mL), and 10% FBS. The cells were grown to 80% confluence and the growth medium was replaced with a differentiation medium consisting of DMEM, 2% horse serum, penicillin (50 IU/mL) and streptomycin (50 µg/mL). Experiments were performed on day 7 of differentiation.

Glucose uptake

Cells were treated with kaempferol (0.1, 1.0, 10 µM) or vehicle (DMSO) in serum-free medium for 30 min, and then in glucose uptake assay buffer containing 15 µL/mL of 2-(N-(7-Nitrobenz-2-oxa-1,3-diazol-4-yl)Amino)-2-Deoxyglucose (2-NBDG) for 10 min. The cells were then centrifuged at 400 x g for 5 min at room temperature. In some experiments, cells were pre-incubated with compound C (Sigma Aldrich, St. Louis, MO), indinavir (Sigma Aldrich, St.

Louis, MO), or LY24009 (Cell Signaling, Danvers, MA) prior to the addition of kaempferol. Glucose uptake was measured using an assay kit from (Cayman Chemical, Ann Arbor, MI) according to manufacturer's protocol. The fluorescence was measured at excitation/emission wavelengths of 485 and 528 nm with a plate reader (BioTek Synergy 2 plate reader, Winooski, VT) and expressed as fold-induction compared to the control.

Glycogen synthesis

C2C12 myotubes were incubated with either 0.4 mM fatty acid cocktail or BSA in the presence or absence of 10 μ M kaempferol or vehicle (DMSO). The fatty acid cocktail contained a 2:1 ratio of palmitate to oleate. After 24 hours, the cells were incubated with 1.25 uCi/mL 14 C glucose (American Radiolabeled Chemicals, Saint Louis, MO) in the presence or absence of insulin (100 nM; Eli Lilly, Indianapolis, IN) for 3 hours. Following this treatment, the cells were washed twice with PBS at 4°C and solubilized with 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 of distilled water. After 20 min of shaking, the precipitated glycogen labeled with 14 C was counted in a liquid scintillation spectrometer (LS 6500, Beckman Coulter, Brea, CA). Glycogen synthesis was calculated based on specific activity and was expressed relative to protein content.

AMPK activity assay

C2C12 myotubes were exposed to kaempferol (0.1, 1.0, 10 μ M) for 10 min and phospho-AMPK in the cell lysates was measured using an ELISA kit (Cell Signaling, Danvers, MA).

Western blot analysis

Cultured primary hSkM cells were lysed in cell lysis buffer (Cell Signaling, Danvers, MA) containing HALT protease and phosphatase inhibitor cocktail (ThermoFisher Scientific, Waltham, MA). Following centrifugation at 13,000 RPM for 10 min, equal amounts of protein from the supernatants were mixed with 2X Lameli Buffer (Biorad, Hercules, CA) and separated by SDS PAGE. The protein was then transferred onto nitrocellulose membranes and blocked with 5% milk protein in Tris-buffered saline containing 0.1 % Tween-20 for one hour. The membranes were then probed overnight with antibodies specific for Akt, phospho-Akt (Ser473), and IRS-1 in 5% BSA (Cell Signaling, Danvers, MA). The protein levels were normalized to total protein per lane by using Image Lab Software (Biorad, Hercules, CA).

Animal study

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 HFD (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. Non-fasting blood glucose and insulin levels were measured at 0, 60, and 120 min following kaempferol administration. In a separate experiment, the mice received either kaempferol or vehicle by oral gavage, and euthanized 30 min later. The gastrocnemius and quadriceps muscle were harvested and manually separated into red and white tissues for Western blot analysis.

Statistical analysis

The data were analyzed by one-way ANOVA using Microsoft Excel (Microsoft Inc., Redmond, Washington). 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).

Results

Kaempferol increases glucose uptake and prevents fatty acid impaired glucose homeostasis

To determine whether kaempferol can increase glucose disposal in muscle, we first treated primary hSkM cells with kaempferol (0.1, 1.0, and 10 μ M). The cells were exposed to kaempferol for 30 min followed by a 10-min co-incubation with 10 μ L/mL 2-NBDG.

Kaempferol treatment significantly increased glucose uptake as compared to the control ($P < 0.05$) (Figure 1) with the 10 μ M dose increasing glucose uptake by over 30% ($P < 0.01$). There is a plethora of evidence that circulating free fatty acids impair insulin signaling and impede muscle glucose transport and glycogen synthesis [31–38]. We further used C2C12 myotubes to

determine whether kaempferol can attenuate fatty acid impairment of glucose uptake and insulin-stimulated glycogen synthesis. To do this, we incubated muscle cells with palmitate and oleate in the presence or absence of kaempferol. We found that fatty acid exposure impaired glucose uptake (Figure 2A) as well as insulin-stimulated glycogen synthesis (Figure 2B), but kaempferol (10 μ M) partially rescued these detrimental effects (Figure 2 A, B).

Kaempferol-induced glucose uptake is mediated by the PI3K/Akt-dependent mechanism

It is well recognized that Akt plays an important role in insulin-stimulated glucose uptake. Next, we determined whether kaempferol is an insulin mimetic by measuring Akt phosphorylation. In that regard, SkM cells were treated with kaempferol (10 μ M) for 0, 10, 30, and 60 min.

Incubation of SkM cells with kaempferol induced a rapid increase in Akt phosphorylation detected by immunoblotting. The level of Akt phosphorylation peaked at 10 min of incubation with kaempferol and returned to basal level over 60 min (Figure 3A). Total Akt expression in the cells was not altered by kaempferol over the time course of these studies.

In order to determine whether kaempferol also induces Akt phosphorylation in SkM cells in vivo and whether there is a discriminatory effect on the basis of muscle type, we administered kaempferol via gavage. 30 min later, mice were euthanized, and the quadriceps and gastrocnemius muscles were collected and snap frozen. These tissues were later separated into red and white components and homogenized for analyzing for Akt phosphorylation via Western blot. Interestingly, kaempferol induced Akt phosphorylation in red muscle, while it had no effect on Akt phosphorylation in white muscle (Figure 3B).

Given that kaempferol induced Akt phosphorylation, which mediates insulin-stimulated glucose uptake via inducing Glut4 translocation to the sarcolemma, we then determined whether

kaempferol-stimulated glucose uptake is mediated via Glut4. We found that indinavir (50 μ M), a chemical inhibitor of Glut4, inhibited kaempferol-stimulated glucose uptake (Figure 4A). We then further examined whether Akt is involved in mediating kaempferol effect on glucose uptake. Skeletal muscle cells were incubated in the presence of LY294009, a chemical inhibitor of PI3K, for 30 min, followed by kaempferol treatment. Inhibition of PI3K blocked kaempferol-stimulated glucose uptake in muscle cells (Figure 4B).

Kaempferol-induced glucose uptake is mediated by activation of AMPK

It has been previously shown that activation of α stimulates the conversion of PIP2 to PIP3, which subsequently activates PI3K and then Akt [39]. To examine whether AMPK plays a role in kaempferol-stimulated glucose uptake, we determined the effects of compound C, a chemical inhibitor of AMPK on kaempferol-induced glucose uptake, and found that kaempferol-stimulated glucose uptake was inhibited by compound C (250 nM) (Figure 6). Next, we determined whether kaempferol stimulates AMPK phosphorylation in muscle cells. To that end, cells were exposed to kaempferol for 10 min, followed by analyzing AMPK phosphorylation by ELISA. As shown in Figure 7, kaempferol at the same dose for increasing glucose uptake significantly increased AMPK phosphorylation. To determine whether the effect of kaempferol observed in vitro is physiologically relevant, we then evaluated whether oral administration of kaempferol has acute effects on glucose disposal and muscle AMPK phosphorylation in vivo. In this regard, diet-induced obese mice were given kaempferol via oral gavage, followed by measuring their non-fasting blood glucose and insulin level as well as AMPK phosphorylation in red and white SkM. We found that kaempferol induces rapid glucose disposal while insulin

levels were not changed (Figure 8). Consistent with in vitro finding, kaempferol increased AMPK phosphorylation in red muscle, but not white muscle when (Figure 9).

Discussion

In this study, we demonstrate that kaempferol (0.1, 1.0, and 10 μ M) stimulates glucose disposal in SkM. It also rescues SkM from fatty acid impaired glucose uptake and insulin-stimulated glycogen synthesis. We observed that kaempferol stimulates Akt phosphorylation as well as AMPK phosphorylation. The observed effect on glucose uptake was inhibited in the presence of indinavir and LY294009 suggesting that kaempferol-stimulated glucose uptake requires, in some capacity, the action of Glut4 and PI3K.

Glucose transport in SkM can be stimulated through at least two signaling cascades dependent on whether the stimulus is insulin or muscle contraction [40]. The insulin-stimulated glucose uptake is dependent on PI3K/Akt-mediated Glut4 trafficking [41,42], however, it has also been shown that activation of AMPK stimulates the conversion of PIP2 to PIP3, which subsequently activates PI3K/Akt signaling [43]. Thus insulin is not necessarily required for the activation of this pathway. The role of AMPK in the enhancement of insulin sensitivity and the amelioration of insulin resistance has been well established [44,45] although its net effect on insulin signaling is complex and involves multiple targets. Multiple lines of evidence have shown that activation of AMPK induced Akt activity, whereas the overexpression of a dominant negative AMPK mutant construct inhibited Akt activation [46–50]. In addition, AMPK activation of Akt phosphorylation can be inhibited by the PI3K inhibitor wortmannin [43], demonstrating that AMPK is upstream of the PI3/Akt pathway. These findings suggest that AMPK can stimulate glucose disposal via the PI3K/Akt-mediated pathway. Consistently,

activation of AMPK was shown to enhance Glut4 translocation and its glucose transport activity [51,52]. In the present study we found that kaempferol increases AMPK phosphorylation, and that inhibition of AMPK, by compound C, blunted kaempferol-stimulated glucose uptake, which further supports the important role for AMPK in mediating the kaempferol effect on glucose disposal. In addition, kaempferol-stimulated glucose uptake was blunted in the presence of a Glut4 inhibitor. While not determined in this study, due to previous reports of the relationship of AMPK with Glut4, it is conceivable that kaempferol-stimulated glucose uptake and Akt phosphorylation may be mediated via an AMPK-dependent mechanism.

Circulating free fatty acids (FFAs), which are significantly elevated in obese people, are known to contribute to insulin resistance [58]. Excess amounts of FFAs increase intramyocellular accumulation of triglycerides that may lead to impairment of IRS/PI3K/Akt insulin signaling [59]. In addition, lipotoxicity plays a role in producing low-grade inflammation in skeletal muscle and other organs mediated via inducing the release of various pro-inflammatory and/or oxidative stress mediators, which are involved in the pathogenesis of insulin resistance [60]. In this study, we show that kaempferol rescued fatty acid-impaired glucose uptake and insulin-stimulated glycogen synthesis. While the means by which free fatty acids cause insulin resistance is not completely understood, our findings together suggest that kaempferol may have insulin mimicking effects in SkM that has an implication in promoting insulin sensitivity.

In summary, we presented data in this study that kaempferol stimulates rapid glucose uptake via activation of the AMPK/PI3K/Akt pathway in SkM cells. This result, together with our other works showing that kaempferol improved insulin sensitivity and blood glucose control in obese diabetic mice, provide solid basis for further studies elucidating the molecular

mechanism underlying its effect on glucose homeostasis, and for designing a clinical trial to test its ameliorating insulin resistance and antidiabetic potential in humans.

Figures

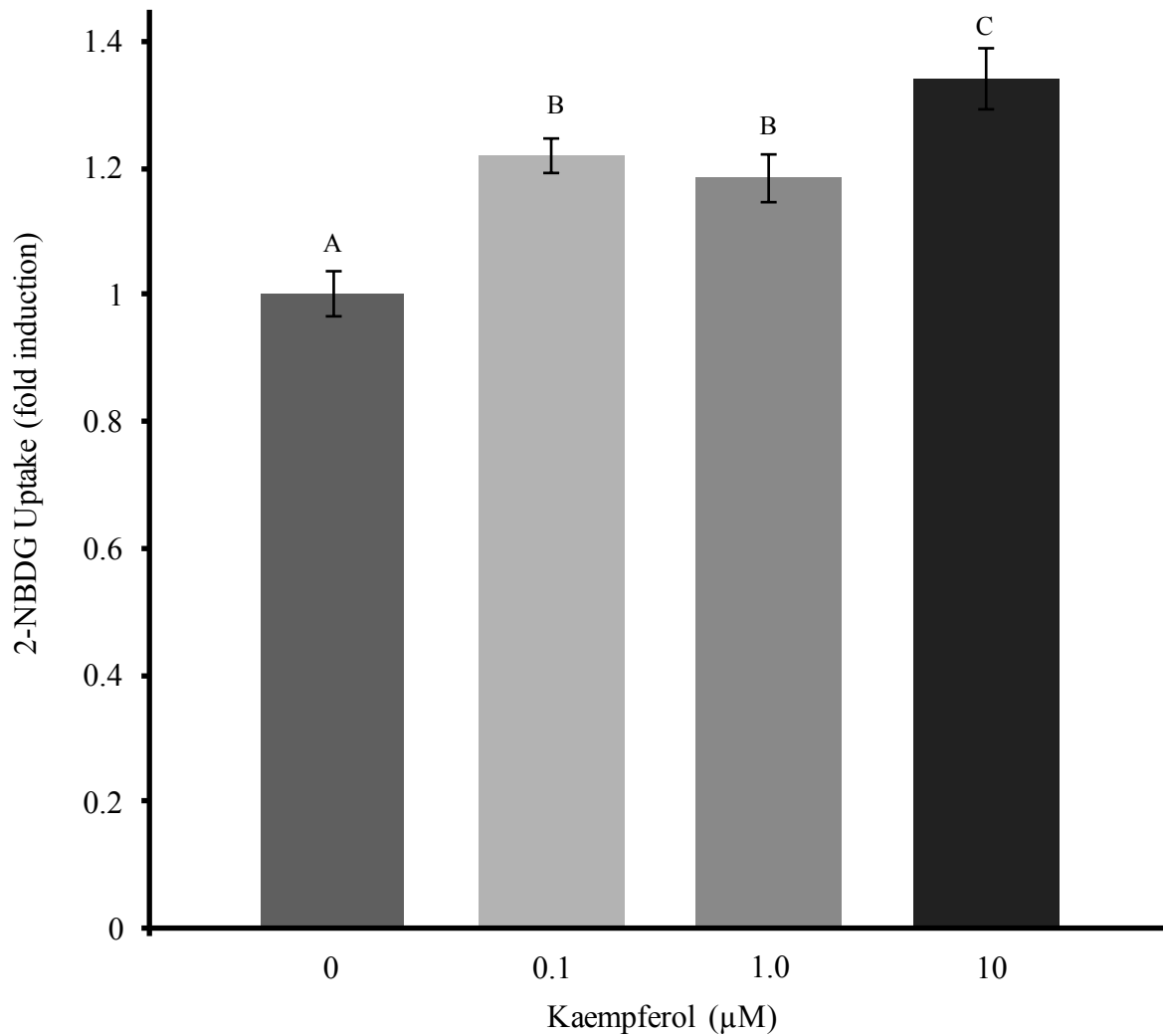


Figure 1. Glucose uptake in primary hSkM cells. Cells were preincubated with kaempferol for 30 min followed by addition of 2-NBDG for 10 min. 2-NBDG in the cells were measured as described in “Methods section”. Values are expressed as mean fold induction \pm SEM.; $n = 4$ in triplicate for each group. Letter differences denote significant difference at $P < 0.05$.

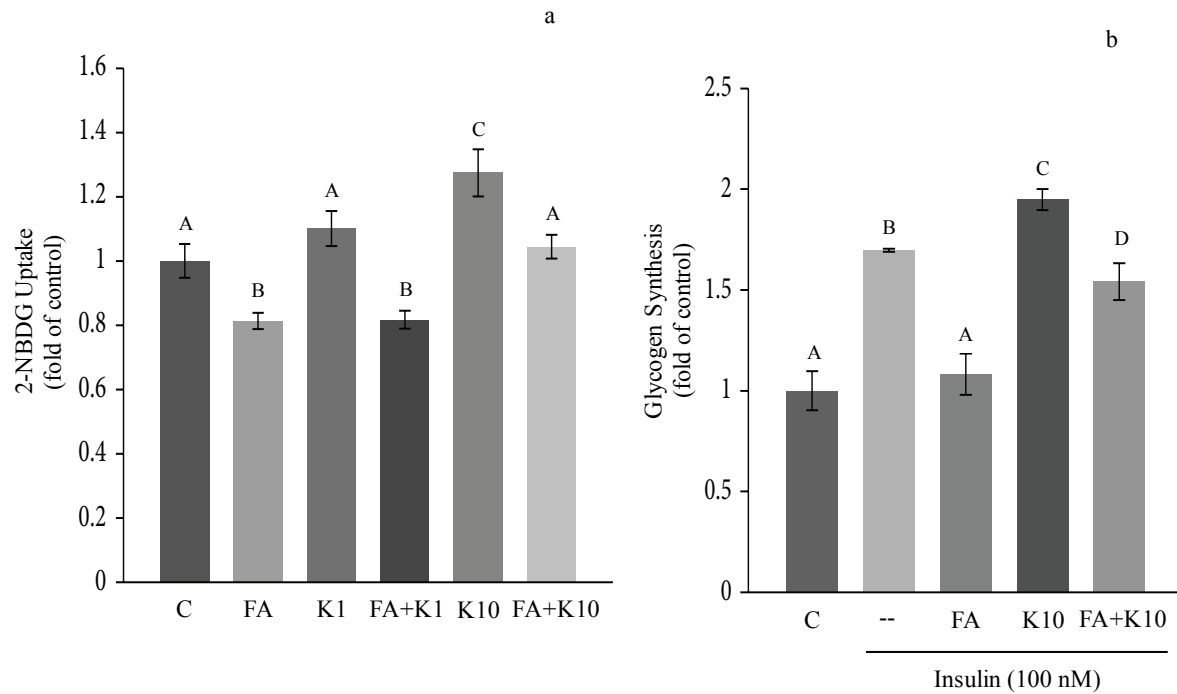


Figure 2. Kaempferol treatment reversed high fatty acid-impaired glucose uptake (a) and glycogen synthesis (b) in muscle cells. C2C12 myotubes were pretreated for 30 min with kaempferol (K1: 1 μ M; K2: 10 μ M) or DMSO followed by addition of 0.4 mM fatty acid mixture (FA: 2:1 ratio of palmitate to oleate) or vehicle (C:BSA). 24 h later, (a) glucose uptake and (b) insulin-stimulated glycogen synthesis were measured. Values are expressed as means \pm SEM. Letter differences denote significant difference at $P < 0.05$.

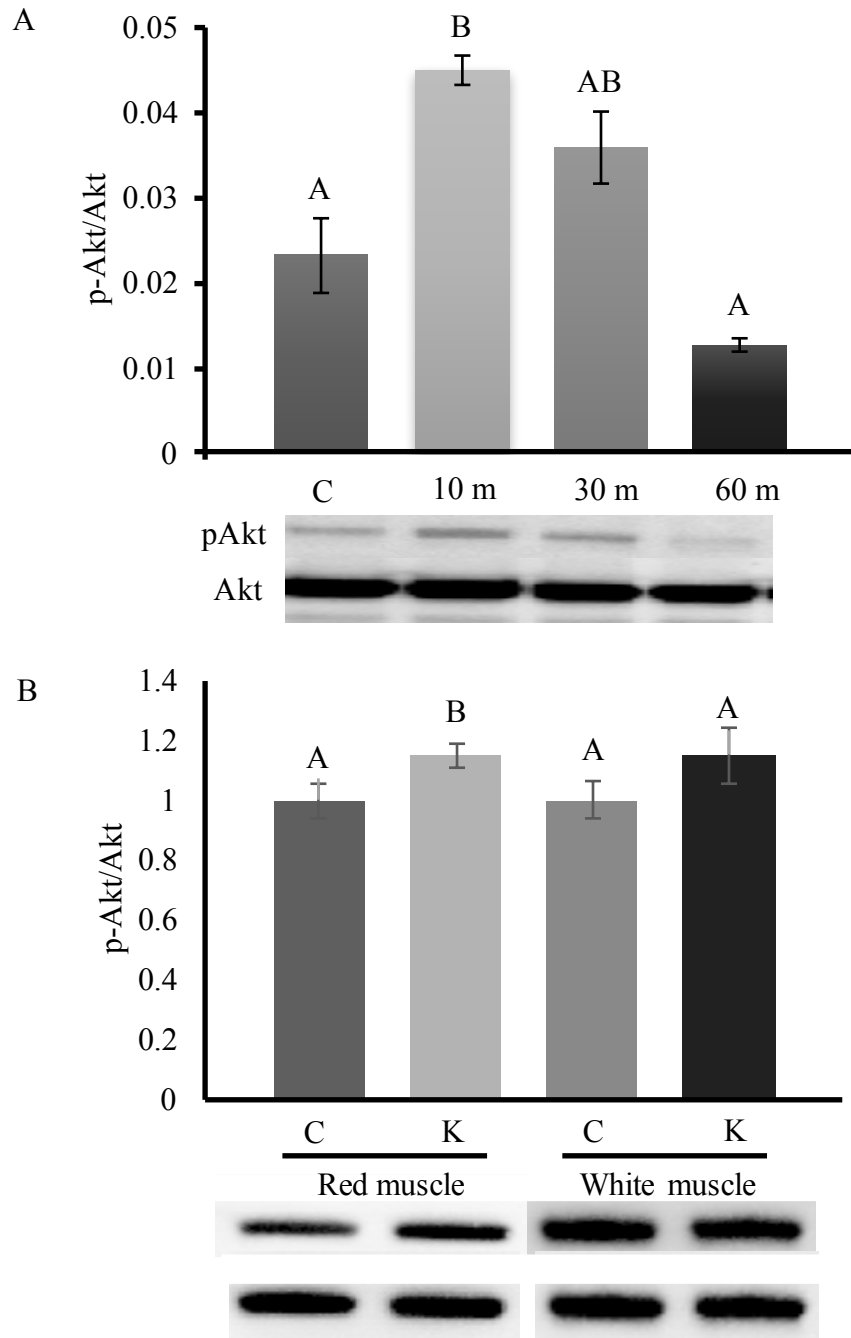


Figure 3. Kaempferol induces rapid Akt phosphorylation in skeletal muscle cells. (a) hSkM cells were treated with kaempferol (10 μ M) for 10, 30, or 60 min and phosphorylated (p-Akt) and total Akt in the cell lysates were detected by Western blot. (b) Kaempferol (250 mg/kg bw) was administered via oral gavage. 30 min later, SkM was isolated for determining Akt phosphorylation. As shown, kaempferol significantly increases Akt phosphorylation in red SkM, but not white SkM. All levels are normalized to total protein content. Data are expressed as a ratio of pAkt to Akt. Values are expressed as means \pm SE. Letter differences denote significant difference at $P < 0.05$.

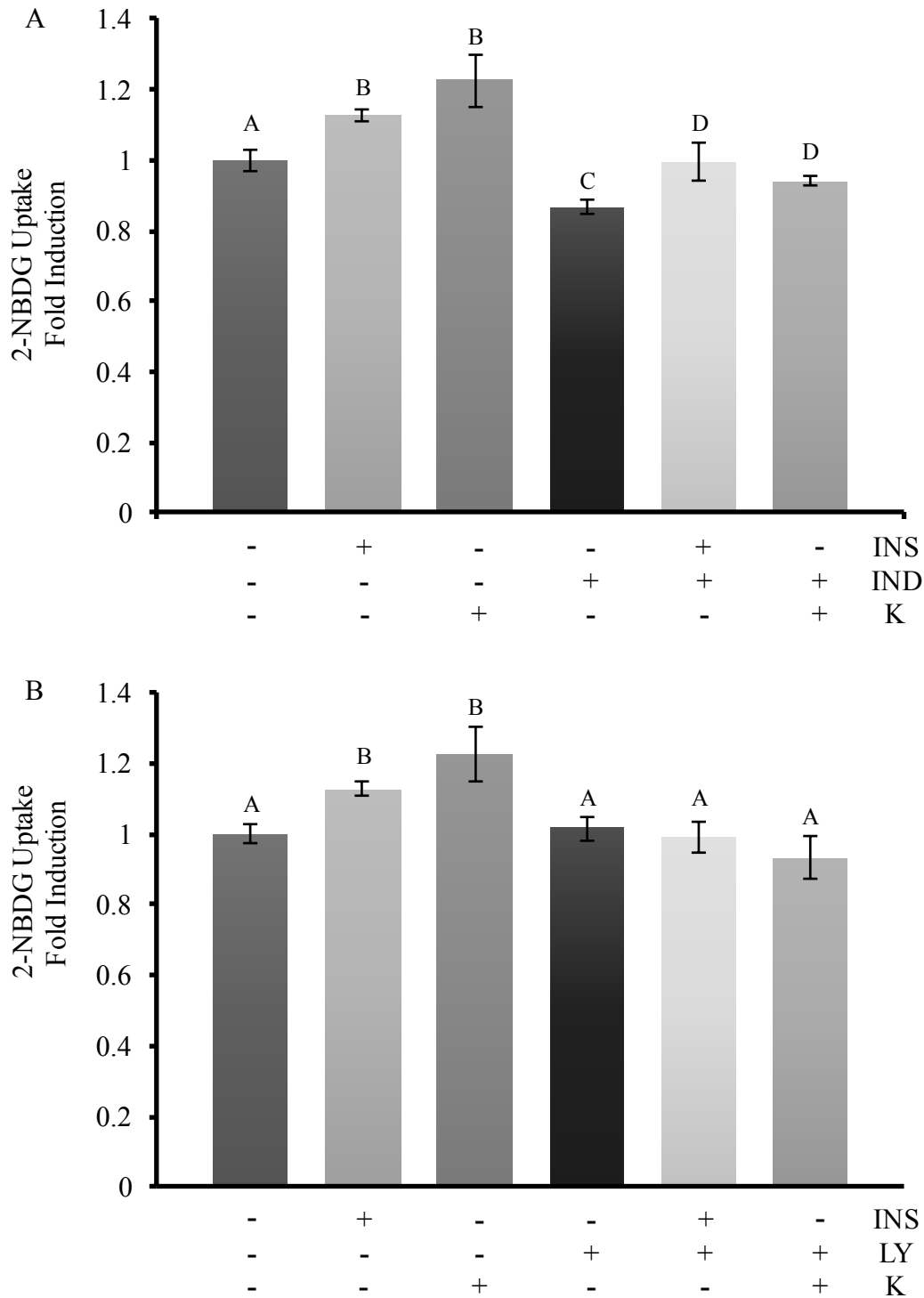


Figure 4. Kaempferol-stimulated glucose uptake is completely inhibited by the Glut4 inhibitor, indinavir (IND) (A) or the PI3K inhibitor, LY29004 (LY, 50 μ M) (B). hSkM cells were preincubated with insulin (INS, 100 nM), IND (50 μ M) or LY (50 μ M) for 60 min before addition of kaempferol (K, 10 μ M) for 30 min, as described in Figure 1. Data are expressed as mean \pm SE. Different letters denote significant difference at $P < 0.05$.

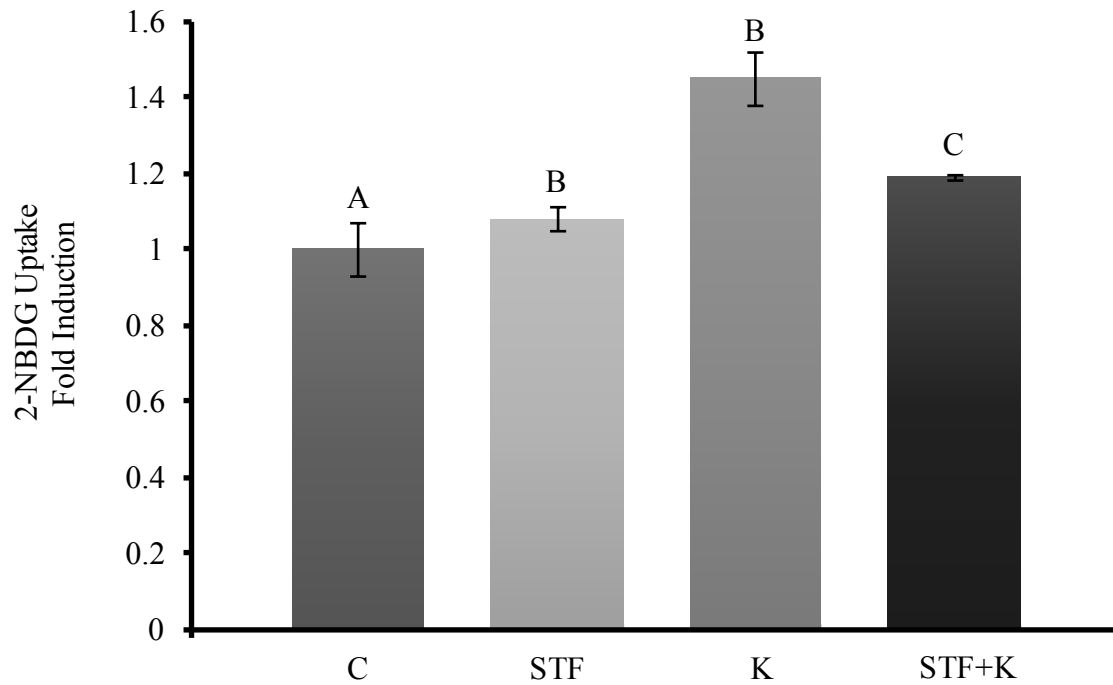


Figure 5. Effects of AMPK inhibition on Kaempferol-induced glucose uptake was attenuated by inhibition of AMPK. Primary hSkM cells were treated with AMPK inhibitor compound C (250 nM) for 24 h, followed by addition of 10 μ M kaempferol (K) for 30 min. Data are mean \pm SEM (n=3). Letter differences denote significant difference at $P < 0.05$.

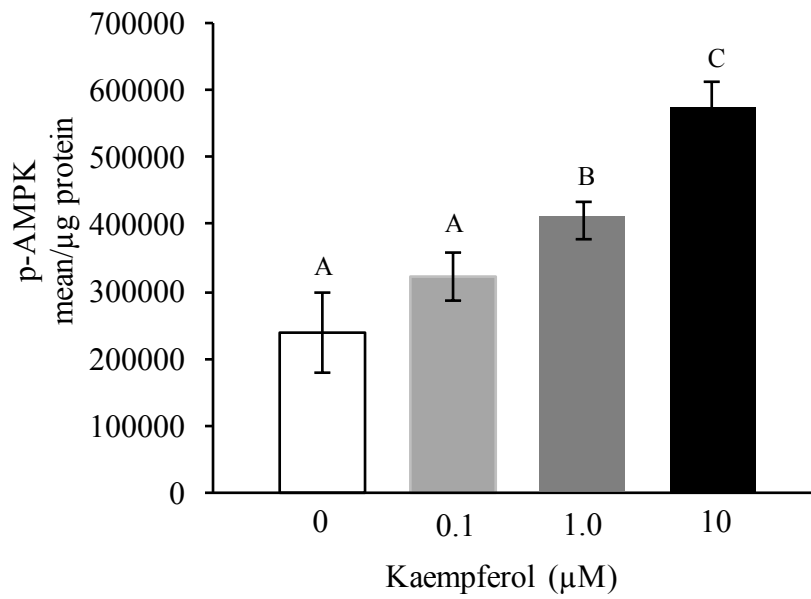


Figure 6. Kaempferol induces rapid AMPK activity in C2C12 myotubes. Cells were exposed to kaempferol for 10 min, followed by measuring AMPK phosphorylation in the cells by an ELISA assay. Letter differences denote significant difference at $P < 0.05$.

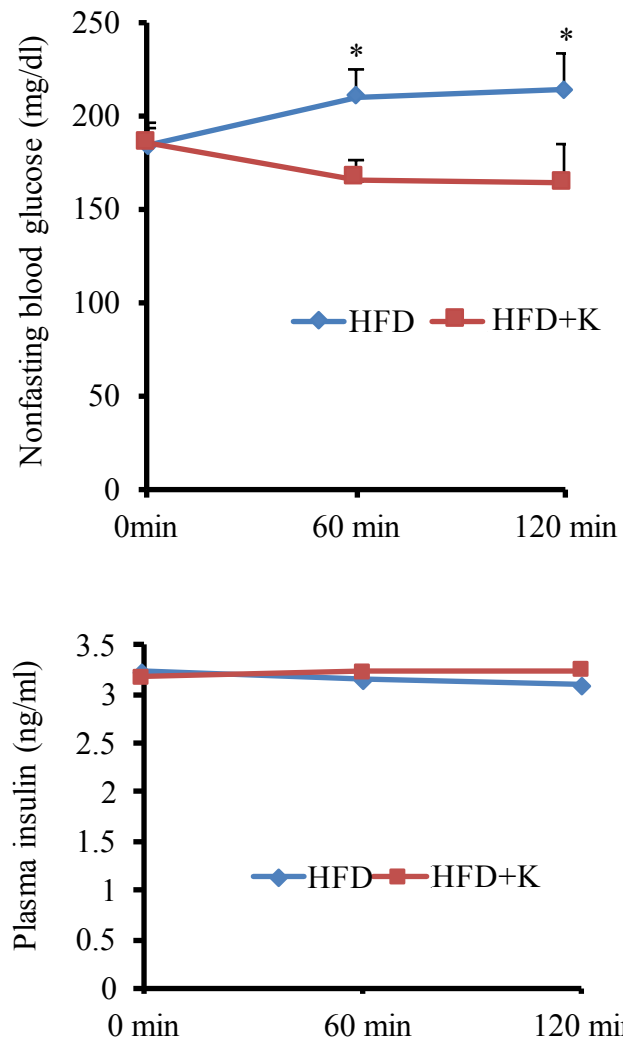


Figure 7. Kaempferol administration induces rapid glucose disposal without affecting insulin secretion in obese mice. Sixteen wks old male mice were fed a HFD for 8 wks and then given either vehicle (cellulose) or kaempferol (50 mg/kg oral gavage. Non-fasting blood glucose and insulin levels were measured at 0, 60 and 120 min after kaempferol administration. Values are expressed as Mean \pm SE (n=9). *, $P < 0.05$.

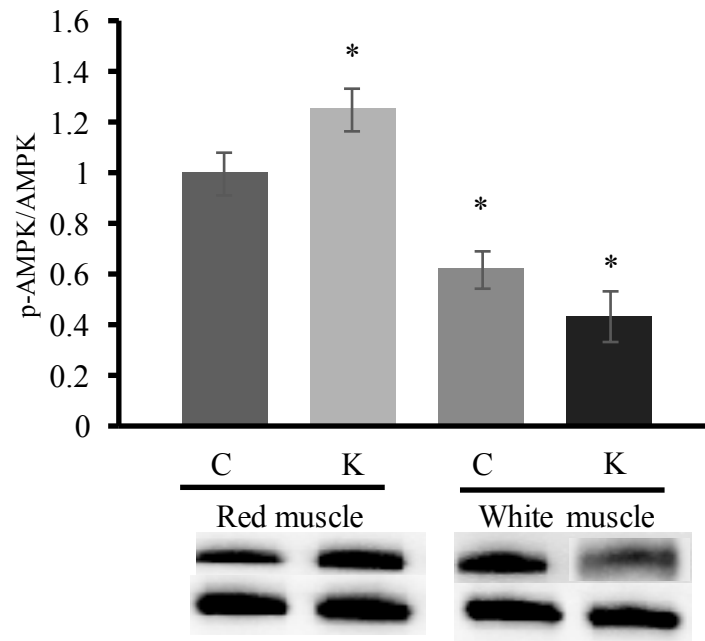


Figure 8. Oral administration of kaempferol increased AMPK phosphorylation in red SkM, but not in white SkM. Kaempferol was provided orally as described in Figure 8. The phosphorylated and total AMPK in SkM were determined. All levels are normalized to total protein content. Data are expressed as mean \pm SE. Letter differences denote significant difference at $P < 0.05$.

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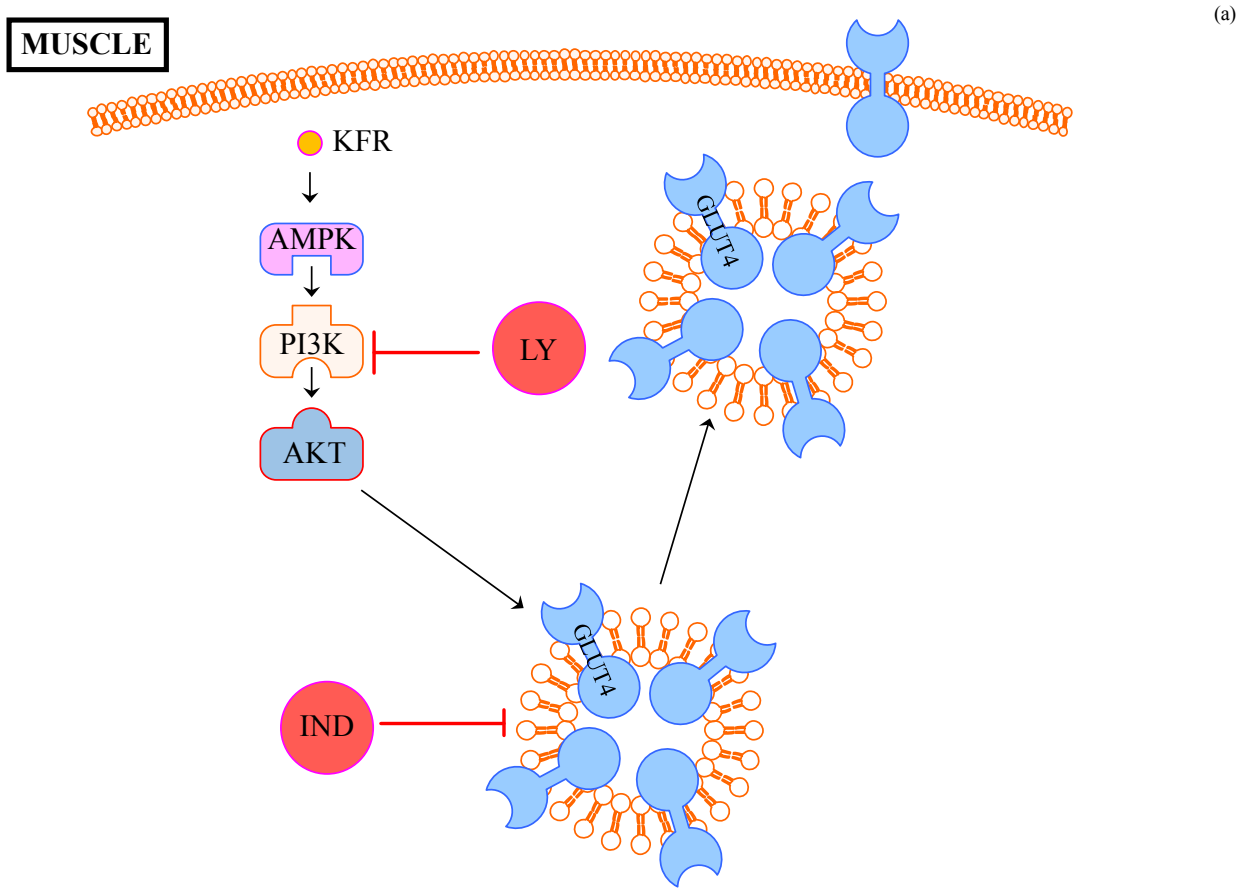
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CHAPTER SIX

Conclusions

In this dissertation research, we first showed that dietary supplementation of kaempferol prevented the development of glucose intolerance and insulin sensitivity in high fat diet (HFD)-fed mice, and protected against streptozotocin (STZ)-induced beta-cell dysfunction in obese diabetic mice. While glucose transporter 4 (Glut4) expression is not compromised in diabetes or obesity, increased Glut4 expression does ameliorate insulin resistance. AMP-activated protein kinase (AMPK), activity is increased during exercise and is associated with increased Glut4 expression. The improvements from our animal studies were associated with increased Glut4 and AMPK expression in skeletal muscle (SkM) and adipose tissues. Consistent with these findings, we also demonstrated that kaempferol treatment restored chronic hyperlipidemia-impaired glucose uptake and AMPK activity in cultured C2C12 mouse myotubes. However, whether the ameliorative effects of kaempferol on glucose metabolism are due to a direct effect on AMPK requires further investigation. The pathogenesis of T2D is typically associated with elevated glucose production and insulin resistance. We also showed that oral administration of kaempferol to obese diabetic mice ameliorated hyperglycemia and insulin resistance, which was associated with decreased hepatic glucose production and improved insulin sensitivity. We also found that Akt and hexokinase activity were increased by kaempferol treatment, but that the activities of glucose-6 phosphatase (G6Pase) and pyruvate carboxylase (PC) were diminished independent of protein expression. Consistently, we found that kaempferol treatment decreased PC activity and suppressed gluconeogenesis in HepG2 as well as primary hepatocytes isolated from the livers of obese mice. However, the molecular mechanism underlying this action of kaempferol needs

further investigation. Insulin stimulated glucose uptake is impaired in T2D, however, exercise-stimulated glucose uptake is not. AMPK is associated with increased glucose uptake, however, its role in insulin- and exercise-stimulated glucose uptake remains enigmatic. At the cellular level, kaempferol stimulates basal glucose uptake as well as insulin stimulated glycogen synthesis in C2C12 cells and consistently augments glucose uptake in human SkM cells. Kaempferol also stimulated Akt phosphorylation in a similar time dependent manner as insulin in human SkM cells. Consistent with this, Akt and AMPK phosphorylation were increased in isolated murine red SkM tissue. Chemical inhibitors of Glut4, phosphoinositide 3-kinase (PI3K), and AMPK were found to blunt the effect of kaempferol on glucose uptake. Based on the results, the mechanisms of action of the anti-diabetic effects of kaempferol are proposed below. However, more research is needed to elucidate the means by which kaempferol regulates the key enzymes involved in glucose regulation.



Abbreviations: AKT, protein kinase B; AMPK, adenosine activated protein kinase; CC, compound C; GLUT, glucose transporter; IND, indinavir; KFR, kaempferol; LY, LY492002; PI3K, phosphoinositide 3-kinase; SGK1, serum- and glucocorticoid-inducible kinase.

LIVER

(b)

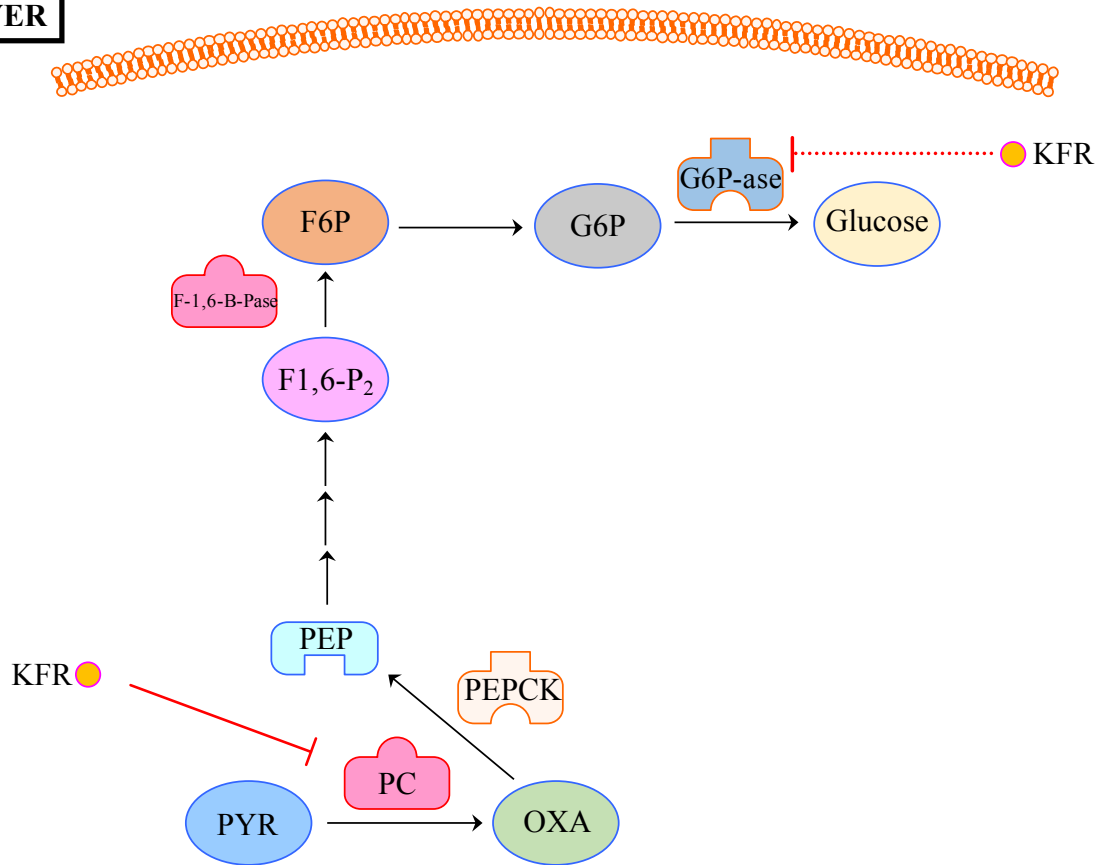


Figure 1. Proposed mechanisms of action of the anti-diabetic effects of kaempferol. Kaempferol may regulate glucose control by increasing glucose uptake in SkM through and AMPK-mediated mechanism (a). In the liver, kaempferol reduces glucose production by inhibiting PC and G6Pase (b).

Abbreviations: F-1,6-B-Pase, fructose-1,6-bis-phosphatase; F-1,6-P₂, fructose-1,6-bis-phosphate; G6P, glucose-6 phosphate; G6P-ase, glucose-6 phosphatase; KFR, kaempferol; OXA, oxaloacetate, PC, pyruvate carboxylase; PEP, phosphoenolpyruvate; PEPCK, phosphoenolpyruvate carboxykinase; PYR, pyruvate.

Directions for future research

This research provides evidence that kaempferol may have useful applications in the context of metabolic syndrome as it exerts a variety of antidiabetic roles in mouse models of insulin resistance, obesity, type-1 diabetes, and type-2 diabetes. However, more studies are needed to understand the molecular mechanism by which kaempferol exerts these effects. Although this research demonstrates an effect in both muscle and hepatic tissue, future research should determine whether the effects that improve glucose homeostasis are predominantly due to its effect on either of these tissues. 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 should be conducted to determine whether this activation is associated with improved glucose uptake in muscle and liver.
- 2) Investigate the molecular interaction that allows kaempferol to diminish PC activity and further determine whether this effect is the primary mediator of the kaempferol-mediated inhibition of glucose production.
- 3) Determine an optimal dose and therapeutic duration in obesity- and STZ-induced diabetic mouse models.