ALLEVIATION OF METABOLIC SYNDROME BY GREEN TEA POLYPHENOL EGCG: MOLECULAR MECHANISM INVOLVING AMP-ACTIVATED PROTEIN KINASE

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ABSTRACT OF THE THESIS

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Metabolic syndrome (MetS) is a clustering of several cardiometabolic risk factors, including abdominal obesity, hyperglycemia, dyslipidemia and elevated blood pressure. More and more attention has been paid on MetS due to many reasons. For example, a quarter of the world's adults have MetS, especially in developed countries; people with MetS has a five-fold greater risk of developing type II diabetes and three-times as likely to have a heart attack or stroke; and over 80% of the 200 million people with diabetes globally will die of cardiovascular diseases. In recent years, tea which is brewed from the plant *Camellia sinensis* has become increasingly popular for research because of its possible beneficial effects on human health, in particular in the area of MetS prevention. These beneficial qualities have mainly been attributed to catechins.

In the present study, green tea polyphenols, Polyphenon E (PPE) and EGCG, were investigated in *db/db* and wild type mice to determine their effects on suppression of blood

glucose level and activation of AMP-activated kinase (AMPK). Treatment of *db/db* mice with PPE (0.1% *i.g.*) or EGCG (200 or 400 mg/kg) decreased the level of blood glucose and increased the level of insulin both in blood or pancreas. Further, EGCG at 100, 200, and 400 mg/kg time- and dose-dependently up-regulated the phosphorylation of AMPK in liver, but it did not increase the oxidative stress. Melatonin, a strongly anti-oxidative compound, at 50 mg/kg (*i.g.*) was unable to block the effects of EGCG on activating AMPK. Together, these data suggest that green tea polyphenols or EGCG can alleviate MetS possibly through the activation of AMPK in liver, and the activation of AMPK by EGCG appears not to involve the reactive oxygen species in mice.

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1. Introduction

1.1 Metabolic Syndrome

Metabolic syndrome (MetS) refers to the clustering of several cardiometabolic risk factors, including abdominal obesity, hyperglycemia, dyslipidemia and elevated blood pressure, that are likely to be linked to insulin resistance [1]. In clinic, MetS is associated with the increased long-term risk of cardiovascular diseases and type II diabetes; therefore, it may provide an opportunity for preventive lifestyle interventions [2, 3]. There are at least three characteristics to identify patients who may have the MetS. The first feature is insulin resistance which occurs in most people with the diseases and is strongly correlated with other features such as hypertension, glucose intolerance, and obesity [2]. Excess circulating fatty acids strongly contribute to the development of insulin resistance by inhibiting the anti-lipolytic effects of insulin [4]. Perhaps one of the most recognized features of MetS worldwide is obesity, as its prevalence has doubled in the past three decades. Obesity, as reported by the World Health Organization, is defined as having a BMI of 30 and over. Roughly one-third of adults twenty years and over are considered overweight or obese [5]. Excess visceral fat and central obesity in particular are believed to be causative factors of MetS. Other factors identified in MetS are atherogenic dyslipidemia (in which elevated levels of triglycerides and LDL particles in the blood arise while levels of HDL cholesterol are reduced), hypertension, increased pro-thrombotic (blood clotting) factors, pro-inflammatory cytokines such as TNF-α, and decreases in anti-inflammatory cytokines such as adiponectin [2, 4].

In the last couple of decades, there has been increasing attention on MetS due to several reasons: 1). A quarter of the world's adults have metabolic syndrome, especially in US

over 34% of American adults are suffering the MetS; 2). People with the MetS are twice as likely to die from, and three times as likely to have a heart attack or stroke compared with people without the syndrome; 3). People with MetS have a five-fold greater risk of developing type II diabetes; 4). Up to 80% of the 200 million people with diabetes globally will die of cardiovascular disease; 5). This put MetS and diabetes way ahead of HIV/AIDS in morbidity and mortality terms, yet the problem is not as well recognized.

1.2 AMP-activated Protein Kinase (AMPK) and Its Regulation on Metabolism

As a cellular "fuel gauge", AMPK plays a major role in cellular energy homeostasis. It consists of three proteins (subunits), α, β and γ, which are all required for a functional enzyme. AMPK is widely expressed in mammalian tissues, including the liver, adipose tissues, skeletal muscle, and brain. AMPK is activated by an increased ratio of AMP: ATP resulted from an accelerated ATP consumption or inhibited ATP synthesis under various cellular stresses. Common metabolic stresses include hepatic fatty acid oxidation and ketogenesis, inhibition of cholesterol synthesis, lipogenesis, and triglyceride synthesis, inhibition of adipocyte lipolysis and lipogenesis, stimulation of skeletal muscle fatty acid oxidation and muscle glucose uptake, and modulation of insulin secretion by pancreatic β cells [7-11]. AMPK is also activated under some pathological state, including hypoxia, ischemia, and oxidative and hyper osmotic stresses [13-15]. Furthermore, exercise and glucose deprivation also increase AMPK activity, suggesting that AMPK is also involved in exercise adaptations and β cell function. Overall, activation of AMPK in these energy-starved states changes the cellular metabolism from an anabolic to a catabolic state in order to elevate the ATP levels [12].

In theory, AMPK activity can be regulated in three ways: 1) allosterically through the binding of AMP or ADP to the γ subunit, 2) through the phosphorylation of the threonine 172 residue in the α subunit by upstream kinases, and 3) through the binding of glycogen to the β subunit [8]. The phosphorylation by upstream kinases is the most potent activator of AMPK, with an over one hundred-fold increase in activity. The displacement of ATP with AMP or ADP results in a two to five-fold increase in AMPK activity, while indirectly suppressing dephosphorylation of the Thr172 site [16]. On the other hand, the binding of glycogen to AMPK inhibits its activity.

Upon activation, a conserved threonine within the AMPK "activation loop" of the kinase domain is phosphorylated by upstream kinases. The primary upstream kinases that phosphorylate Thr172 include tumor suppressor kinase LKB1 and the Ca^{2+}/calmodulin-activated protein kinase kinase-β, CaMKKβ. As a predominant AMPK kinase in cells, LKB1 regulates the phosphorylation of AMPK in response to the changes in energy state during muscle contraction [17]. Under such stresses, LKB1 is activated by forming a complex with two regulatory proteins, STRAD and MO25, and then induces AMPK phosphorylation [18]. Many studies show that the deletion of LKB1 results in ablated T172 phosphorylation of the α2 subunit, leading to a decreased AMPK activity and a lowered glucose uptake [17, 19]. In contrast, CaMKKβ activates AMPK by sensing a rise in intracellular calcium ions even without detectable changes in the AMP/ATP ratio [20]. CaMKKβ is widely expressed in many tissues, with high abundance in the central nervous

system and lower levels in liver and skeletal muscle, suggesting that the AMPK activity is regulated by different mechanisms that are likely to be tissue specific [21].

As it is highlighted in many studies, AMPK plays a major role in regulating lipid metabolism. For example, fatty acid oxidation in skeletal muscle is heavily regulated by AMPK-ACC pathway via carnitine palmitoyltransferase 1 (CPT1). As a rate-controlling transporter, CPT1 transfers long-chain acyl-CoA into the mitochondria for β-oxidation. When the cellular energy level is high, CPT1 is inhibited allosterically by malonyl-CoA to inhibit fatty acid oxidation [22]. The production of malonyl-CoA derived from excess acetyl-CoA is catalyzed by acetyl-CoA carboxylase (ACC) [23]. ACC is a downstream substrate of AMPK, and phosphorylation of ACC decreases the enzyme activity [24, 25]. During the exercise and skeletal muscle contraction, AMPK is activated to lower ACC activity, leading to a reduced malonyl-CoA production, relieving the CPT1 inhibition and thereby increasing fatty acid oxidation to restore energy balance [26]. Based on the roles of AMPK in inducing lipid oxidation and lowering skeletal muscle and liver lipid accumulation, AMPK activation is considered an important feature for the insulin sensitizing effect [27].

Since its activation is closely related to an elevation in AMP and a reduction in creatine phosphoate energy stores, AMPK is considered as an attractive candidate for contraction-induced skeletal muscle glucose uptake [28]. Also, a lot of drug-induced activation of AMPK has the effect of increasing glucose uptake [29], together with an observation of glucose transporter 4 (GLUT4) fusion with plasma membrane [30, 31]. Similar to the

exercise effect, this increase in glucose uptake is not affected by inhibition of the insulin signaling and is additive to insulin-stimulated glucose uptake. On the other hand, in the absence of AMPK, glucose uptake is only partially suppressed during muscle contraction, indicating that other AMPK-independent pathways may be involved in this process [32]. For example, in a genetic knockout mouse model, overexpression of an α2 kinase-dead subunit in skeletal muscle abolishes AICAR effects on inducing glucose uptake (AICAR is a compound activating AMPK by mimicking the effects of AMP by forming ZMP after it is metabolized in cells), but the glucose uptake level is still responsive to contraction [32]. Furthermore, ablation of skeletal muscle AMPK α2 and γ3 subunit expression does not affect contraction-stimulated glucose uptake, despite the blunted AICAR's effect [33-36].

In addition to the glucose uptake by peripheral tissues, hepatic glucose production is also important in maintaining glucose homeostasis. Elevated glucose production by the liver is a major cause of fasting hyperglycemia in type II diabetes [37, 38]. As two critical enzymes in liver gluconeogenesis, phosphoenolpyruvatecarboxykinase (PEPCK) and glucose-6-phosphatase (G6Pase) are regulated by AMPK activity [39]. The mRNA levels of PEPCK and G6Pase is downregulated by AMPK activation in hepatoma cells [40], providing clues for the role of AMPK in gluconeogenesis. Furthermore, AMPK α2 liver-specific knockout mice display fasting hyperglycemia with increased PEPCK and G6Pase activity, suggesting an elevated gluconeogenesis level associated with AMPK function loss [41]. Recent data have shown that inhibition of the gluconeogenic program by AMPK requires a transcriptional coactivator, transducer of regulated CREB activity 2 (TORC2) [42]. Glucagon and fasting condition induce hepatic TORC2 nuclear translocation, which

enhances CREB-dependent transcription of the gluconeogenic program, including PGC-1α. Conversely, AMPK activation leads to TORC2 phosphorylation, trapping the coactivator in the cytoplasm and thus inhibiting the expression of gluconeogenic program. Deletion of hepatic LKB1 abolishes AMPK activation and leads to nuclear accumulation of TORC2, which in turn increases gluconeogenesis [43]. Consistently, liver LKB1 knockout mice on a high-fat diet exhibit fasting hyperglycemia and glucose intolerance and are unresponsive to metformin treatment, which inhibits gluconeogenesis by activating AMPK [43].

The role of AMPK has been implicated in regulation of liver lipogenesis, lipid oxidation, and cholesterol synthesis to restore energy balance in liver. For example, in order to reduce lipid deposition, AMPK suppresses glucose-induced expression of lipogenic genes, such as fatty acid synthase (FAS) [44], ACC, and pyruvate kinase [45], as well as increasing lipid oxidation. In rat primary hepatocytes, activation of AMPK by AICAR or metformin increases fatty acid oxidation via inhibition of ACC [45]. Consistently, in rats treated with metformin, liver triglyceride levels are reduced, concomitantly with an increase in β-hydroxybutyrate, suggesting elevated hepatic lipid oxidation [45]. Moreover, the lipid-lowering effects of metformin in cultured hepatocytes require AMPK activity [46]. These enhanced metabolic profiles are accompanied by down-regulation of lipogenic genes such as SREBP-1, FAS, and S14, supporting an overall effect of decreasing lipid accumulation [45].

AMPK is also shown to be involved in regulating pancreatic insulin secretion. As one of the critical steps in insulin exocytosis, the glucose increases the intracellular ATP/ADP ratio in β cells [47, 48], closing ATP-sensitive potassium channels [49] and allowing influx of calcium ions [50, 51]. Meanwhile, AMP and ADP levels in β cells decrease in response to increased glucose concentration [52, 53], suggesting that AMPK could affect insulin release by sensing glucose-mediated AMP level. Under normal condition, increased glucose level stimulates insulin secretion in β cell lines with a suppressed AMPK activity [53-56], whereas this glucose-stimulated insulin release is markedly reduced by AICAR-induced activation of AMPK in primary pancreatic islets [53, 55] and β cell lines [55, 57]. Furthermore, overexpression of a constitutively active form of AMPK results in repressed glucose-induced insulin release from β cell lines with reduction in calcium influx [55, 58], whereas overexpression of a dominant-negative form of AMPK leads to increased insulin release without apparent changes in glucose metabolism and calcium influx [55]. Given the AMPK-activating effect of antidiabetic drugs (such as metformin), the regulation of insulin release by AMPK is proposed to be a promising target for type II diabetes treatment [59, 60]. Recently more evidence is emerging to support this hypothesis. Incubation of either human islets or cultured β cells with metformin leads to AMPK activation and inhibition of glucose-stimulated insulin secretion. From a physiological point of view, the suppression of insulin release in response to AMPK activation could also be important in maintaining glucose homeostasis during glucose deficiency.

In addition to its effect on fatty acid oxidation in skeletal muscle, AMPK also plays an important role in adipose lipid metabolism. As one of the common characteristics of type

II diabetes, high circulating levels of lipids are in part caused by impaired insulin-mediated suppression of lipolysis in adipose tissue [61]. Under normal condition, the release of FFAs from TG in adipose tissue is inhibited in response to increased insulin level via hormone-sensitive lipase, a rate-limiting enzyme of lipolysis. With impaired insulin sensitivity, elevated circulating FFAs released from adipocytes are not only associated with ectopic fat accumulation in liver and skeletal muscle, but also affect insulin secretion by β cells [62]. Interestingly, AMPK is thought to inhibit lipolysis in adipose, based on the evidence that treating isolated rat adipocytes with AICAR reduces isoprenaline-induced lipolysis [64]. Furthermore, expression of a constitutively active form of AMPK reduces isoproterenol-stimulated lipolysis, whereas reduced AMPK activity increases lipolysis in adipocytes [65]. A possible theory is that HSL is phosphorylated by AMPK without changing enzyme activity, but this phosphorylation allosterically inhibits subsequent activation by protein kinase A [63]. On the other hand, AMPK is also shown to inhibit lipogenesis in isolated adipocytes via increased ACC phosphorylation. With lipolysis and lipogenesis both regulated by AMPK in response to different energy state or cellular stress, it is not surprising that AMPK also controls whole-body adiposity, as it is shown that AMPK knockout mice developed markedly higher fat mass than the wild-type mice [66].

1.3 Tea and Tea Polyphenols

Tea is made from leaves of the plant *Camellia sinensis*, which has been used in ancient days for medicinal purposes and now as a popular beverage. Depending on the processing of tea leaves, tea is classified into two major types: green tea and black tea. While black

tea is the major type of tea produced and consumed worldwide, green tea is more popular than black tea in China and Japan.

The most avtive compound in tea leaves are polyphenols, which are natural chemical compounds found in many plants [67]. In the human diet, polyphenols serve primarily as antioxidants, and the intake is higher than any other class of phytochemicals, with consumption being roughly ten time higher than vitamin C intake and one hundred times higher than vitamin E intake [68].

Tea contains characteristic polyphenolic compounds known as catechins, which majorly include: (-)-epigallocatechin gallate (EGCG), (-)-epicatechin gallate (ECG), (-)-epigallocatechin (EGC), and (-)-epicatechin (EC) (Figure 1). Catechins account for about 30-40% of the dry weight of brewed green tea, and EGCG is the major form of tea catechin. Tea leaves also contain lower quantities of other polyphenols, such as quercetin, kaempferol, and myricetin as well as alkaloids, such as caffeine and theobromine. Black tea is commonly referred to as "fermented tea", but it does not involve fermentation by microorganisms. In its manufacturing, the tea leaves are withered, crushed, and allowed to undergo enzyme-mediated oxidation. During this process, most of the catechin are oxidized, dimerized to form theaflavins (TFs) and polymerized to form thearubigins (TRs) [69, 70]. TFs are produced from the dimerization of two catechin molecules and exist in four major forms (theaflavin, theaflavin-3 gallate, theaflavin-3'-gallate, and theaflavin-3,3'-digallate), which contribute to the red-orange color and characteristic taste of black tea. TRs are mixtures of heterogeneous polymers with red-brown color and the structures are poorly

understand [70]. In brewed black tea, catechin, TFs, and TRs each account for 3-10%, 2-6%, and >20% of the dry weight, respectively.

The health effects of tea depend on the biochemical properties and bioavailability of the constituents in tea. Tea catechins, especially EGCG, have received most of the attention. It is commonly recognized that tea catechin are strong antioxidants, efficiently scavenging free radicals and also preventing the formation of reactive oxygen species (ROS) by cheating metal ions. *In vivo*, EGCG (and perhaps other catechins) may also cause the formation of ROS in the mitochondria [71, 72]. The ROS may activate nuclear factor erythroid 2-related factor 2 (Nrf2) mediated antioxidants and other cytoprotective enzymes [72-74], and this may be referred to as an "indirect antioxidant" effect. EGCG is also known to undergo superoxide-catalyzed auto-oxidation *in vitro* to produce ROS [75]. Nevertheless, such auto-oxidation *in vitro* to produce unlikely occurs in internal organs because of the lower oxygen partial pressure (than in solution *in vitro*) and the presence of antioxidant enzymes in animal tissues.

The phenolic groups in catechins can be donors for hydrogen bonding; the multiple H-bonds enable tea polyphenols to bind strongly to proteins, lipids, and nucleic acids. The binding of EGCG to many proteins, such as 67-kDa laminin receptor [76] and propel cis/trans isomerase [77], has been proposed to be a key mechanism for its anticancer activities [78]. Black tea polyphenols may bind to biomolecules and biomembranes with even higher affinity than EGCG.

The bioavailabilities of tea polyphenols follow the prediction of "Lipinski's Rule of 5" [79-81]. Both human and animal studies have shown that the bioavailabilities of epicatechin and catechin (molecular weight 290 and five phenolic groups) are higher than that of EGCG (molecular weight 458 and eight phenolic groups). The plasma bioavailability of EGCG in mice following intragastric administration of EGCG (75mg/kg) was low, with more than 50% of EGCG existing as glucuronide conjugates. Levels of EGCG in the small intestine and colon were 20.6 and 3.6 ng/g, respectively [80]. In humans, following oral administration of the equivalent of two or three cups of green tea, the peak plasma levels of tea catechins (including the conjugated forms) were usually 0.2-0.3 μM [80]. On the other hand, theaflavin and tehaflavin-3-3'-digallate (molecular weights of 564 and 868 and containing 9 and 14 phenolic groups, respectively) have extremely low or no bioavailabilities when administered orally [82]. It was reported that following consumption of 700 mg of pure theaflavins mixture, equivalent to about 30 cups of black tea by an individual, the maximum concentrations of theaflavin in plasma and urine were only 1 μg/L (1.8 nM) and 4.2 μg/L, respectively [82]. Apparently, galloped theaflavins were not detected. The bioavailability of may polyphenolic compounds is regulated by active efflux. The multi drug resistance-associated protein-2, located on the apical surface of the intestine and liver, mediates the transport of some polyphenolic compounds to the lumen and bile, respectively. Therefore, EGCG is predominantly effluxes from the enterocytes into the intestinal lumen, or to be effluxes from the liver to the bile and excreted in the feces, with little or no EGCG excreted in the urine.

1.4 Animal Studies on Tea Preventing MetS

The effects of tea and tea polyphenols on body weight and MetS have been studied extensively in animal models. Most of the studies showed that the consumption of green tea extracts (GTE) or EGCG significantly reduced the gaining of body weight and/or adipose tissue weight, lowered blood glucose or insulin levels, and increased insulin sensitivity or glucose tolerance. These studies used rodents on high-fat diets or genetically obese/diabetic animal models. For example, in mice fed with a high-fat (60% of the calories) diet, we found that dietary EGCG treatment (0.32 % in diet) for 16 weeks significantly reduced body weight gain, body fat, and visceral fat weight compared to mice without EGCG treatment [83]. These results were also reproduced in a second study using a high-fat/Western style diet [84]. EGCG treatment also attenuated insulin resistance, plasma cholesterol, and monocyte chemoattractant protein concentrations in mice on the high-fat diet [83, 84]. Similar results were also observed in several recent studies [85-87]. For example, treatment of male Swiss mice with GTE (50 mg/kg, *i.g.*, daily) for 8 weeks decreased body weight and white adipose tissue weight. In another study, EGCG administration (20 mg/kg, *i.p.*, 3 times weekly) to C57BL/6J mice that were fed a high-fat diet significantly reduced body weight and liver fat accumulation at 42 and 66 weeks. In a genetic diabetes model, the *db/db* mice, dietary supplementation of EGCG (1% in diet) prevented the progression of glucose intolerance and reduced the number of pathologically altered islets of Langerhans. A recent study in diabetic mice showed that both GTE and black tea extract (BTE), administered at 0.01% in drinking water, lowered blood glucose levels, and GTE was more efficacious [88]. GTE was also more effective in lowering body weight gain and histological liver deterioration, but only BTE significantly elevated serum

insulin levels [88]. The doses of GTE and BTE used in this study were very low and the mechanism of BTE action is unknown.

Green tea polyphenols also alleviate MetS in other insulin-resistant animals. For example, in insulin-resistant rats, treatment with green tea polyphenols significantly decreased blood glucose, insulin, triglycerides, total cholesterol, LDL cholesterol, and free fatty acids [89]. In insulin-resistant beagle dogs, oral administration of GTE (80 mg/kg daily, before the daily meal) for 12 wk also markedly increased insulin sensitivity index [90].

Diet-induced liver steatosis, which predisposes to liver cancer, is becoming a common disease, and its possible prevention by tea consumption warrants more investigation. We have shown that EGCG treatment reduces the incidence of hepatic steatosis, liver size (48% decrease), liver triglycerides (52% decrease), plasma alanine aminotransferase concentration (67% decrease), and liver pathology in mice fed with a high-fat diet [91, 92]. Tea catechins have been reported to also reduce hepatic steatosis and liver toxicity in rodents treated with ethanol, tamoxifen, or endotoxins, or rodents with liver ischemia/reperfusion injury. These findings may have potential for practical applications.

Tea and its major constituents have been demonstrated to inhibit tumorigenesis in many animal models for different organ sites, including the lung, oral cavity, esophagus, stomach, small intestine, colon, skin, liver, pancreas, bladder, prostate, and mammary glands. Treatment with green or black tea extracts for 60 weeks inhibited the spontaneous formation of lung tumors as well as rhabdomyosarcomas in A/J mice. In addition, oral

administration of green tea infusion reduced the number of lung colonies of mouse Lewis lung carcinoma cells in a metastasis model (93). These results demonstrate the broad activities of tea preparations in the inhibition of lung neoplasia at different stages of carcinogenesis. EGCG also inhibited tumorigenesis in rat stomach and forestomach induced by *N*-methyl-*N*-nitro-*N*-nitrosoguanidine. The inhibitory effects of tea and tea polyphenols on intestinal tumorigenesis in mice have been consistently observed in different laboratories. For example, Yang et al. showed that administration of EGCG at 0.02% to 0.32% in drinking fluid dose-dependently inhibited small intestinal tumorigenesis in $Apc^{Min/+}$ mice (94). Shimizu *et al.* also demonstrated the inhibition of azoxymethane (AOM)-induced colon aberrant crypt foci (ACF) formation in C57BL/KsJ- *db/db* mice by 0.01% and 0.1% of EGCG in drinking water. Administration of a green tea polyphenol infusion (0.1% in drinking fluid) to TRAMP (transgenic adenocarcinoma of the mouse prostate) mice for 24 weeks markedly inhibited prostate cancer development and distant site metastases (95). The inhibition was associated with decreased cell proliferation, increased apoptosis, decreased insulin-like growth factor (IGF) signaling, and decreased levels of angiogenic and metastatic markers.

1.5 Human Studies on Tea Preventing Metabolic Syndrome

The effects of tea consumption on body weight and biomarkers of MetS have been studied in many short-term randomized controlled trials (RCTs) during the past decade. Systematic reviews and meta-analysis covering more than 26 earlier RCTs indicated the beneficial effects of tea consumption in reducing body weight and alleviating MetS [96, 97]. Most of these studies used green tea or green tea extracts with caffeine, in studies for 8-12 weeks,

on normal weight or overweight subjects. Some of the more recent RCTs also showed that daily consumption of 458–886 mg of green tea catechins by moderately overweight Chinese subjects for 90 days reduced body fat [98], and that intake of Polyphenon E capsules (containing 400 or 800 mg EGCG and lower amounts of other catechins, but negligible amounts of caffeine) daily for 2 months by postmenopausal women in the United States decreased blood levels of LDL cholesterol, glucose, and insulin [99]. In another study, GTE supplementation (379 mg per day) to obese patients for 3 months decreased body weight and waist circumference [100]. Improvements in lipid pro- files, including the decrease in levels of total cholesterol, LDL cholesterol, and triglycerides were also observed in this study [100]. However, a recent RCT with obese Caucasian women, after an energy-restricted diet intervention, showed that supple- mentation with EGCG (200 mg daily) for 12 weeks did not cause changes in body weight, fat mass, energy and fat metabolism, insulin resistance, total or LDL cholesterol levels, and other biomarkers [101]. The lack of beneficial effects of the supplement is possibly due to the relatively low dose of EGCG used, especially in subjects who were previously treated for weight reduction.

A recent study with healthy male subjects demonstrated that green tea extract supplementation (1200 mg catechins and 240 mg caffeine daily) for 7 days increased lipolysis, fat oxidation and citric acid cycle activity under resting conditions without enhancing adrenergic stimulation [102]. The role of caffeine in these studies was inconsistent among the different studies. A meta-analysis of metabolic studies showed that both a catechin-caffeine mixture and caffeine alone dose-dependently stimulated daily

energy expenditure, but only the catechin-caffeine combination significantly increased fat oxidation [103].

Two recent epidemiological studies also suggested the beneficial effects of green tea consumption on MetS [104, 105]. One study on elderly males in a rural community indicated that tea drinking, especially for individuals who drank 240 mL or more tea daily, was inversely associated with incidence of MetS [104]. The second, a cross-sectional study of US adults, showed that intake of hot (brewed) tea, but not iced tea, was inversely associated with obesity and biomarkers of MetS and CVDs [105].

The lowering of body weight and alleviation of MetS by tea should lead to the reduction of type II diabetes. Such an association was found in some, but not all, human studies. For example, a prospective cross-sectional study with US women aged 45 years and older showed that consumption of more than four cups of tea per day was associated with a 30% lower risk of developing type II diabetes, whereas the intake of total flavonoids or flavonoid-rich foods was not associated with reduced risk [106]. A retrospective cohort study of 17 413 Japanese adults aged 40–65 years indicated that daily consumption of more than six cups of green tea (but not Oolong or black tea) lowered the risk of diabetes by 33% [107]. The effects of caffeine in these epidemiological studies are unclear. A meta-analysis based on seven studies (286 701 total participants) showed that individuals who drank three to four or more cups of tea per day had lower risks of type II diabetes than those consuming no tea [108]. A more recent RCT in patients with obesity-related hypertension showed that consumption of GTE (379 mg daily) for 3 months reduced fasting serum glucose and

insulin levels [109]. Several clinical intervention studies have yielded inconclusive results concerning the effects of tea on insulin resistance and blood glucose control, but there are reported changes in certain biomarkers such as an increase in satiety, a reduction of hemoglobin A1c, and a decrease in diastolic blood pressure [110-113].

The alleviation of MetS by tea logically leads to the reduction of the risks for CVDs (reviewed in [114-116]). The lowering of plasma cholesterol levels and blood pressure as well as improvement of insulin sensitivity and endothelial function by green tea have been reported by many investigators [116]. In a review of 11 RCTs, both green and black teas decreased LDL cholesterol and blood pressure [117]. The strongest evidence for the reduction of CVD risk by the consumption of green tea is provided by large cohort studies in Japan. In the Ohsaki National Health Insurance Cohort Study (n = 40 530), deaths due to CVDs were decreased dose de- pendently by tea consumption at quantities of 1 to >5 cups of tea per day [118]. In another study with 76 979 Japanese adults, the consumption of green tea was also associated with decreased CVD mortality, but daily consumption of >6 cups of tea was needed to manifest the effect [119]. In a recent study in Japan, consumption of three to four cups of tea was associated with a decreased incidence of stroke [120]. A case-control study in China also showed a correlation between the consumption of green (or Oolong) tea and a de- creased risk of ischemic stroke [121]. A meta-analysis of 14 prospective studies, covering 513 804 participants with a median follow-up of 11.5 years, found an inverse association between tea consumption and risk of stroke, and the protective effect of green tea appeared to be stronger than that of black tea [122].

1.6 Possible Mechanisms of Metabolic Syndrome Alleviation by Tea

Ingestion of green tea polyphenols has been shown to increase fecal lipid and total nitrogen contents, suggesting that polyphenols can decrease digestion and absorption of lipids and proteins [123]. On the other hand, tea may affect gut microbiome in mice. For example, green tea powder and *Lac-tobacillus plantarum* feeding affected gut microbiota, lipid metabolism, and inflammation in high-fat fed C57BL/6J mice [124]. Green tea reduced the body fat content and hepatic triglyceride and cholesterol accumulation, and the reduction was correlated with the amount of *Akkermansia* and/or the total amount of bacteria in the small intestine [124]. The abundance of *Akkermansia muciniphila* has been shown previously to be increased in prebiotic-treated *ob/ob* mice, which had lower fat mass compared to the control *ob/ob* mice [125]. Recently, evidence for a gut–brain–liver axis mechanism has been provided for the acute glucose lowering effect by intraduodenal infused metformin in diabetes rat models [126]. According to this mechanism, metformin activates AMPK in duodenal mucosa, and subsequently lowers hepatic glucose production (without involving hepatic AMPK) through a neuronal-mediated gut– brain–liver pathway.

The observations found in many studies that ingestion of tea catechins suppressed gluconeogenesis and lipogenesis and enhanced lipolysis in a coordinated manner suggest that these actions of tea catechins are mediated by energy-sensing molecules, especially for the regulation on AMPK. The possible mediation of the action of EGCG by AMPK in the liver, skeletal muscle, adipose tissue, and pancreas is shown in Fig. 2. The activation of AMPK by EGCG and different types of teas has been demonstrated *in vivo* and *in vitro*

[127-132]. For example, it has been shown that at 1, 3, and 6 h after *i.g.* administration of GTE (100 mg/kg body weight) to mice, the phosphorylation of AMPK and its upstream kinase, LKB1, increased in the liver (by 2-3 and 1.5-2 fold, respectively) [127].

However, the detailed mechanism by which AMPK is induced EGCG is still unclear, although the involvement of ROS has been suggested. It remains unknown how ROS change the ATP/ADP/AMP ratio. We propose that most of the reported effects of EGCG or green tea on metabolism can be explained by the AMPK hypothesis (Figs. 2 and 3). A hint is that EGCG is able to cause the production of reactive oxygen species (ROS) in mitochondria, probably by interrupting the electron transfer in the respiration chain. Another possibility is that glulathione (GSH) and NADPH are required for the detoxification of ROS in the mitochondria, and mitochondrial proton gradient is required to drive the transhydrogenase to reduce $NADP^+$ to NADPH by NDAH. EGCG may serve as a mitochondrial uncoupler of oxidative phosphorylation. Both these two type of actions would result in a decrease of cellular ATP synthesis and thus increase the ADP: ATP ratio and activate AMPK, but these possibilities remain to be investigated. Therefore, in this study, our goal is to further investigate how EGCG regulates the phosphorylation of AMPK in vivo, and whether the phosphorylation of AMPK induced by treatment of EGCG explains the mechanism of EGCG in metabolic syndrome prevention.

2. Goals and Specific Aims

2.1 Rationale

The main goal of this study is to investigate the preventive effects of tea polyphenols on MetS and further determine the mechanism of action of EGCG in MetS prevention. Most studies in this area demonstrated the effect of tea polyphenols or EGCG the most abundant catechin found in tea leaves in MetS prevention. For example, in one study, mice fed a high-fat diet containing EGCG were found to have a decreased body weight gain, percentage of body fat, and visceral fat weight compared to control mice. Furthermore, it was shown that at 1, 3, and 6 h after *i.g.* administration of GTE (100 mg/kg body weight) to mice, the phosphorylation of AMPK and its upstream kinase, LKB1, increased in the liver (by 2-3 and 1.5-2 fold, respectively), suggesting that the regulation of AMPK phosphorylation may play a crucial role in the metabolic syndrome prevention.

However, the detailed mechanism by which AMPK is induced by EGCG is still unclear, although the involvement of ROS has been suggested based on experiments *in vitro*. It remains unknown how ROS change the ATP/ADP/AMP ratio and activate AMPK. We therefore further investigated how EGCG increases the phosphorylation of AMPK *in vivo*, and whether the phosphorylation of AMPK induced by treatment of EGCG contribute to MetS prevention.

2.2 Specific Aims

1. To investigate the mechanism by which EGCG regulates AMPK phosphorylation and prevents MetS in C57BL/6J mice.

2. To investigate the effects of different types of tea polyphenols, Polyphenon E (PPE) from green tea and theaflavins (TFs) from black tea in metabolic syndrome prevention in *db/db* mice.

3. Materials and Methods

3.1 Animal treatments

Mice were obtained from Jackson Laboratories (BarHarbor, Maine). All animal experiments were carried out under protocol 02-027 approved by the Institutional Animal Care and Use Committee at Rutgers University (Piscataway, NJ). Mice were fed at controlled temperature (25℃) and humidity (50%) and in a 12-h day/night cycle with access to diet and water ad libitum.

In the first experiment, 8-week old wild type, *db/+*, and *db/db* mice were *i.g.* administered EGCG at 200mg/kg (n=6 at each group). Blood glucose levers were monitored at 0, 1, 3, and 6 hours.

In the second experiment, 12 hours after a starvation period, 8-week old C57BL/6J mice were *i.g.* administered either water or EGCG at 100, 200, and 400 mg/kg, respectively, (n=4 at each group). After 1, 3, and 6 hours, mice were anesthetized by carbon dioxide and the livers were quickly dissected, frozen in dry ice, and stored at -80℃ until the biochemical assay.

In the third experiment, 12 hours after a starvation period, 8-week old C57BL/6J mice were giver either EGCG alone (700 or 1000mg/kg), or EGCG (700 or 1000mg/kg) combined with melatonin at 50 mg/kg under anesthesia (n=4 at each group). Control groups mice were given water or melatonin at 50 mg/kg (n=4 at each group). After 1, 3, and 6 hours,

mice were anesthetized by carbon dioxide and the livers were quickly dissected, frozen in dry ice, and stored at -80℃ until the biochemical assay.

In the fourth experiment, 8-week old *db/db* mice were fed AIN-93M, AIN-93M containing 0.1% PPE, and AIN-93M containing 0.1% TFs diet (n=12 each group), respectively, for 6 weeks. Blood insulin and glucose level were monitored at each week. At the end of experiment, all mice were sacrificed and various tissues and blood were harvested, and stored at -80℃ until the biochemical assay.

3.2 Western blotting

Gels for electrophoresis were prepared using 1.5 mm glass plates from Bio-Rad (Hercules, CA). A 10% gel resolving gel was made using deionized water (w/v), BioAcryl-P (30%; Acrylamide: Bis-acrylamide 39:1), (w/v) 1X separating/resolving buffer (1.5 M Tris Buffer, pH 8.8) (Boston BioProducts, Ashland, MD), 10% (w/v) Sodium dodecyl sulfate (SDS), 10% (w/v) ammonium persulphate (APS) (Bio-Rad, Hercules, CA), and N,N,N,N'-tetramethylethylenediamine (TEMED) (Sigma-Aldrich, St. Louis, MO) to polymerize the gel. The resolving gel was poured between the glass plates. Bubbles were removed using ethanol. The gel was left to solidify for 30 minutes. A 5% stacking gel was made using the same ingredients listed above, but using (w/v) 1X stacking buffer (1M Tris Buffer, pH 6.8) in place of the separating/resolving buffer (Boston BioProducts, Ashland, MD). After pouring the stacking gel to the glass plates, plastic combs were added. The gel was left to polymerize for another 30 minutes before being loaded to the electrophoretic cells.

Samples containing 20 µg of protein were mixed with 1X Laemmli's SDS-sample buffer (Boston BioProducts, Ashland, MD) and then heated at 95°C for 10 minutes. The proteins were loaded onto the gel alongside the Precision Plus Protein Dual Color Standards marker from Bio-Rad (Hercules, CA) and run in 1X Running Buffer (Boston BioProducts, Ashland, MD) at 110V for 90 minutes using the Power Pac Basic from Bio-Rad (Hercules, CA). After the proteins had been separated on the gel, they were transferred to a 0.45 µm PVDF membrane pre-activated in methanol (Life Sciences, Grand Island, NY) at 100V for 60 minutes in 1X Transfer Buffer (Boston BioProducts, Ashland, MD). Transfer of the proteins was verified by Ponceau S staining (Sigma-Aldrich, St. Louis, MO). The membranes were cut into strips at the predicted weights of each protein being detected. Blots were blocked for 1 hour at room temperature in Odyssey Infrared Imaging System Blocking Buffer (LI-COR Biosciences, Lincoln, NE).

Blots were incubated overnight at 4°C in either specific primary antibodies. The primary antibodies were diluted 1:1000 in 5% (w/v) bovine serum albumin (BSA) (Sigma-Aldrich, St. Louis, MO) and 1X Tris Buffer Saline (TBS) (Boston BioProducts, Ashland, MD). Following primary incubation, blots were washed with 2% (w/v) Non-fat dry milk (Lab Scientific, Highlands, NJ) in TBS three times for 10 minutes. The blots were then incubated in 1:5000 anti-rabbit Hrp-linked IgG or anti-mouse Hrp-linked IgG (Cell Signaling Technology, Danvers, MA) in 5% BSA (w/v) and 1X TBS for one hour at room temperature. The same washing procedure was followed after the secondary antibody incubation. The membrane was developed by using SuperSignal West Femto Maximum Sensitivity Substrate from Thermo Scientific (Rockford, IL). Substrates A and B in the kit

were mixed in a 1:1 ratio. The blots were imaged using the enhanced chemiluminiscence detection system from Amersham Pharmacia (Piscataway, NJ) and Quantity One software from Bio-Rad (Hercules, CA). Quantification of protein bands was done by using ImageJ version 1.48.

3.3 Measurement of blood glucose and insulin levels

To measure blood glucose levels, a blood glucose monitor was used (Contour, Bare Healthcare, Mishawaka, IN, USA). Insulin plasma levels were measured by performing an insulin ELISA (Millipore, Billerica, MA, USA).

3.4 Immunohistochemistry (IHC)

IHC was performed on the pancreas by using specific antibodies to quantify the levels of positive staining and to detect the localization. The section of pancreas was deparaffinized in xylene and rehydrated in 100%, 95%, 70%, 50%ethanol and distilled water serially. The tissue antigens were unmasked in antigen unmasking solution (Vector Laboratories Inc, Burlingame, CA) in a microwave oven for antigen retrieval for 20 minutes. Endogenous peroxidase was quenched with 3% H_2O_2. The sections were incubated in PBS containing 5% normal serum for blocking nonspecific bindings for 1 hour at room temperature. The slides were incubated with the primary antibody overnight at 4℃. The slides were incubated in biotin-conjugated secondary antibody (1:200) and avidin-biotin peroxidase (Vector Laboratories Inc, Burlingame, CA) at room temperature for 1 hour each. Negative controls followed the protocol described earlier but without primary antibody incubation. Antibodies against insulin (1:100, Cell Signaling, Danvers, MA), 8-OHdG (1:100, Cell

Signaling, Danvers, MA), and γ-H2AX (1:100, Cell Signaling, Danvers, MA) were used for analysis. The results of the IHC analysis were quantified with an Aperio ScanScope Scanner (Aperio Technologies, Inc., Vista, CA) and calculated by the Images Scope software (Aperio Technologies, Inc.).

3.5 RNA isolation and RT-PCR

Total RNA was isolated using the RNeasy Mini Kit (Qiagen, Hilde, Germany) according to the manufacturer's protocol. RNA samples having a ration of A_{260nm}: A_{280nm} more than 1.8 were used for RT-PCR. cDNA was generated using 50 ng of total RNA, oligo dT primer, and PrimeScript RT enzyme Mix according to the manufacturer's instructions (SuperScript First-Stand kit, Invitrogen, Life Science) in a total volume of 20 μL.

3.6 Real-Time PCR.

Real-time PCR performed with a CFX system (Bio-Rad) according to the manufacturer's protocol. △CT values were determined by normalizing to GAPDH. Fold changes were calculated using $2^{-\triangle\triangle CT}$. The primer sequences (5'- 3') are: CGACTCGCTATCTCCAAGTGA (G6pc-F), GTTGAACCAGTCTCCGACCA (G6pc-R), CTGCATAACGGTCTGGACTTC (PCK-1-F), and CAGCAACTGCCCGTACTCC (PCK-1-R).

3.7 Data presentation and statistics

ALL results are presented as means ± SEM. The differences between groups were examined by one-way ANOVA post hoc through either Tukey's or Dunnett's multiple

comparison upon the result of Bartlett's test for equal variances, two-way ANOVA using GraphPad software 6.0 (Prism, San Diego, CA, USA). A *P* value of < 0.05 was considered statistically significant.

4. Results

4.1 EGCG suppresses the blood glucose level in *db/db* mice

In the first experiment, we investigated whether EGCG suppressed the elevated blood glucose level in a few hours. Mice were *i.g.* administered EGCG at 200 mg/kg, and after 3 hours we found that blood glucose level was significantly decreased in *db/db* mice ($P < 0.01$), but the blood glucose level had no changes in wild type mice (Fig. 4A). Similar results were obtained when the concentration of EGCG was increased to 400 mg/kg; EGCG significantly decreased the blood glucose level in *db/db* mice ($P < 0.001$), but did not affect the blood glucose levels in wild type and *db/+* mice (Fig. 4B).

4.2 EGCG induces the phosphorylation of AMPK in mouse liver

Many enzymes and transporters are involved in gluconeogenesis, some of which are regulated by AMPK. Therefore, we hypothesized that the action of EGCG in blood glucose suppression was resulted from the activation of AMPK. To test this hypothesis, after 12 hours fasting, C57BL/6J mice were orally administered EGCG at 100, 200, and 400 mg/kg. Results showed that, in each dosage of EGCG treatment, mouse hepatic AMPK was time-dependently activated, and at the same time there was no change of the AMPK phosphorylation in control mice, suggesting that fasting itself would not affect the phosphorylation of AMPK in mice during the 6 hours investigated (Fig. 5A). We also found that EGCG also dose-dependently induced the phosphorylation of AMPK (Fig. 5A). AMPK phosphorylation can cause the suppression of the transcription of its downstream enzymes. Therefore, we measured two AMPK downstream enzymes mRNA levels: G6Pase and PEPCK, which are involved in hepatic glucogeogenesis. Results in Fig. 5B

and C showed that EGCG significantly suppressed these two enzymes mRNA levels in 1 hour. However, we have no data to account why the mRNA of these enzymes were recovered in 6 hours (More information will be discussed in the section of Discussion and Future Direction).

4.3 EGCG dose not increase the oxidative stress during the activation of AMPK

Many *in vitro* results showed that the induction of AMPK phosphorylation by EGCG was resulted from the pro-oxidation of EGCG: the production of ROS in cell culture medium. For example, Collins *et al*. reported that the EGCG activation of AMPK was mediated by the CaMKK, and the suppression of hepatic gluconeogenesis induced by EGCG was dependent on production of ROS, because catalase and STO-609, two strong anti-oxidants, abolished this effect in isolated hepatocytes in culture. In order to determine whether EGCG increased the oxidative stress during the activation of AMPK in mice, we measured the levels of HO-1 and NQO-1 (two Nrf-2 regulated enzymes), which are induced in response to increased oxidative stress. However, we found that EGCG at 100, 200, and 400 mg/kg (*i.g.* administration) did not increase the Nrf2-mediated anti-oxidative enzymes levels (Fig. 6A). In addition, the levels of 8-OHdG and γ-H2AX expression, two markers for DNA oxidative damage, were not changed in mouse liver (Fig. 6B and C). Together, these data suggested that EGCG did not increase the oxidative stress even though it was able to up-regulate the phosphorylation of AMPK in mouse liver.

4.4 Melatonin has no effects on the activation of AMPK induced by EGCG

In vitro results showed that anti-oxidative compounds could block the action of EGCG in the AMPK activation. Therefore, we used melatonin which is known as a powerful antioxidant with a particular role in the protection of nuclear and mitochondrial DNA to further investigate whether ROS was involved in the regulation of AMPK phosphorylation in mice. In this experiment, melatonin at 50 mg/kg was *i.g.* administrated into C57BL/6J mice combined with EGCG at 400, 700, and 1000 mg/kg, respectively. Results showed that EGCG could dose-dependently induce the phosphorylation of AMPK, but melatonin failed to affect this action. Meanwhile, both in EGCG alone or EGCG combined with melatonin treatment groups mice, there was no significant changes for the oxidative stress level, as being of the hepatic expression of TrxR1, NQO-1and GPx-2 (Fig. 7A). Moreover, 8-OHdG and γ-H2AX levels were same in each group mouse liver (Fig. 7B and C).

4.5 PPE protects pancreatic β cells from damage in *db/db* mice

PPE has been demonstrated to alleviate diabetes both in animal and human studies. Thus, we further investigated whether this effect of PPE was resulted from the protection on pancreatic β cells and maintaining the secretion of insulin into blood in *db/db* mice. In another experiment (conducted by Dr. Shili Sun *et al.* in Dr. C. S. Yang's lab), it was found that only did the treatment of 0.1% PPE for 6 weeks significantly maintain the level of insulin in *db/db* mice plasma compared with other mice in control and 0.1% TFs treatment groups (Fig. 8A), and the mice blood glucose level in PPE treatment group was significantly lower during 1, 3 and 6 weeks, respectively, (Fig. 8B). We further did the insulin IHC staining and found that in control and 0.1% TFs treatment mice the insulin levels in pancreatic β cells were significantly lower (Fig. 3C), which may account for the

reason why insulin levels were decreased in plasma in these two groups mice; however, 0.1% PPE could significantly protect pancreatic β cells and maintain its function during 6 weeks ($P < 0.01$).

5. Discussion and Future Direction

5.1 Phosphorylation of hepatic AMPK is not resulted from the overnight fasting

In vivo studies have demonstrated that fasting or a low calorie diet lead to improvement of insulin sensitivity, which is mediated via AMPK. One of the reasons is that AMPK can be activated following an increase in the AMP/ATP ratio, and switch on catabolic pathways and switch off ATP-consuming pathways. Kajita *et al*. reported that overnight fasting resulted in an elevated AMP/ATP ratio and activated AMPK activity in adipose tissues, and decreased the expression of PPARγ mRNA levels [133]. Wijngaarden *et al.* found that prolonged fasting (48 hours) even declined the AMPK activity in skeletal muscle [134]. However, in contrast to these studies, we found that after an overnight fasting the ratio of p-AMPK/t-AMPK and the mRNA levels of AMPK downstream enzymes PEPCK and G6Pase were not changed in mouse liver, but EGCG could up-regulate the phosphorylation of AMPK in 1, 3 and 6 hours and down-regulate these two above enzymes mRNA levels in 1 hour (Fig. 5). Therefore, these results suggest that the change of AMPK phosphorylation was induced by the treatment of EGCG, and overnight fasting would not increase the phosphorylation of hepatic AMPK.

5.2 ROS are not involved in the activation of hepatic AMPK

ROS are widely generated in a variety of cells and organs in response to exercise, hormones, and growth factors, and act as useful signaling molecules to regulate cellular function, growth, and differentiation at physiologic concentrations [135]. Recent studies demonstrated that ROS and RNS, such as hydrogen peroxide and peroxynitrite, have an important role in the activation of AMPK [136, 137]. In addition, AMPK activation by

metformin, a well-known anti-diabetic drug, and statins, a class of hypolipidemic drugs, is reported to be mediated by RNS [138, 139]. Furthermore, Hwang *et al.* reported that EGCG treatment of HT-29 colon cancer cells inhibited cell proliferation and stimulates hydrogen peroxide-mediated phosphorylation of ACC [140], suggesting a role for ROS as an upstream regulator of AMPK. Similar results were found that the stimulatory effects of EGCG on LKB1/AMPK phosphorylation were markedly diminished by a membrane-permeable catalase, but to by the NOS inhibitor L-NAME, suggesting that ROS (hydrogen peroxide), but not RNS, mediated the LKB1 phosphorylation and subsequent AMPK activation by EGCG *in vitro.* Yang *et al.* reported that EGCG is oxidized by molecular oxygen (auto-oxidation) to form superoxide anions, hydrogen peroxide and EGCG dimers in cell culture conditions; therefore, hydrogen peroxide generated by the oxidation of EGCG under culture cells might be involved in LKB1/AMPK phosphorylation. However, it is not clear whether auto-oxidation of EGCG and subsequent AMPK phosphorylation occur *in vivo*, because under normal conditions oxygen partial pressure in the internal organs is much lower than under cell culture conditions, and cells contain various anti-oxidant enzymes. Nrf2 is a positive regulator of the human antioxidant response element (ARE) that drives expression of antioxidant enzymes such as NOQ-1, HO-1 and TrxR1. Keap1, a cysteine-rich protein that is anchored to actin in the cytosol, interacts with Nrf2, acting as an adaptor protein an ubiquitin ligase complex. Under the normal conditions, Keap1 promotes ubiquitination and eventual degradation of Nrf2. However, as the increase of ROS, it yields an increase in the oxidation or conjugation of key Keap1 cysteines, which weakens its activity. Thus, during the cellular oxidative stress, Keap1 is less effective at promoting Nrf2 degradation. As Nrf2 is stabilized, it enters the nucleus where is activates

transcript of downstream enzymes genes, including the components of an antioxidant system that can balance high ROS levels. Therefore, in the second experiment (as shown in Fig. 6), we further investigated whether ROS or higher oxidative stress was involved in the activation of AMPK induced by EGCG. Fig. 6B and C revealed that, even though EGCG at 100, 200, and 400 mg/kg, respectively, was able to induce the phosphorylation of AMPK at 1, 3, and 6 hours, it failed to change the expression of two essential antioxidant enzymes: HO-1 and NQO-1, which means the Nrf2 system was not activated. Melatonin is a powerful antioxidant with a particular role in the protection of nuclear and mitochondrial DNA. Wang *et al.* proved that melatonin at 50 mg/kg (*i.p.* injection) could attenuate the oxidative damage induced by high concentration of EGCG (75 and 125 mg/kg *i,p,* injection), suggesting that, first, high concentration of EGCG was able to overproduce the ROS which subsequently aggravated the oxidative stress and triggered hepatic toxicity; second, melatonin played a role as an antioxidant protecting liver against such oxidative damage. Therefore in the third experiment (as shown in Fig. 7), we orally administered mice with EGCG (400, 700, and 1000 mg/kg) and EGCG (400, 700, and 1000 mg/kg) combined with melatonin (50 mg/kg), and found that melatonin did not influence the action of AMPK, and increase the expression of antioxidant enzymes, like TrxR1, HO-1, and GPx-2. Taken together, these results demonstrated that EGCG inducing the phosphorylation of AMPK was not associated with the overproduction of ROS and increased oxidative stress *in vivo*. There are three possible reasons to explain these results which are different compared with the *in vitro.* findings: 1) EGCG bioavailability is extremely low which may result in a lower accumulation of EGCG in organs compared within the cell culture medium (0.1-1 nM *in vivo* VS. 1-10 μM *in vitro.*); 2) Under normal

conditions oxygen partial pressure in the internal organs is much lower than under cell culture conditions, thus EGCG may not be able to produce such high concentrations of ROS being observed *in vitro.* conditions; 3). It is much easier to eliminate the ROS produced through the EGCG auto-oxidation in organs, in that the anti oxidative system is more complex and integrity compared with the conditions in cell culture medium, which may therefore cause a lower accumulation of ROS in organs.

5.3 Mechanisms of the action of EGCG in inducing AMPK phosphorylation

Although we found that ROS was not involved in the activation of hepatic AMPK, AMPK could be activated through the change of the ratio of AMP/ATP. In mitochondria, the electrochemical energy inherent in the difference in proton concentration and the separation of charge across the inner mitochondrial membrane- the proton-motive force- drives the synthesis of ATP as protons flow passively back into the matrix through a proton pore associated with the ATP synthase. Through this way, ADP is converted to the form of ATP. Weak acids with hydrophobic properties could be uncouplers. They can uncouple oxidation from phosphorylation, which inhibits ATP synthesis in mitochondria. After entering the matrix in the protonated form, weak acids may release a proton, thus dissipating the proton gradient and across the membrane. We hypothesize that EGCG may serve as an uncoupler in mitochondria and suppress the synthesis of ATP. To test this assumption, we need to measure the mitochondrial membrane potential and the levels of ATP and ADP in mitochondria.

5.4 Concluding remarks

In this work, we investigated the mechanism of green tea polyphenols activating hepatic AMPK, as well as the effects of EGCG on pancreatic β cells protection. We found that ROS was not involved in the AMPK phosphorylation by EGCG, even though it could induce AMPK phosphorylation in mice. There are several limitations to this study. First, we did not determine the activity of hepatic AMPK. Second, the dosage of EGCG used in this study was higher than what we consumed daily. Recent studies reported the hepatic toxicity of high doses of EGCG in mice.

It is important to understand the action and the mechanism behind AMPK phosphorylation induced by EGCG treatment. In metabolic syndrome prevention, to up-regulate the AMPK phosphorylation has been a promising strategy. From this work, we conclude that EGCG could alleviate metabolic syndrome by decreasing the blood glucose levels, protecting the pancreatic β cells from damage and maintaining the secretion of insulin into blood, and strongly inducing phosphorylation of hepatic AMPK which was not associated with oxidative stress.

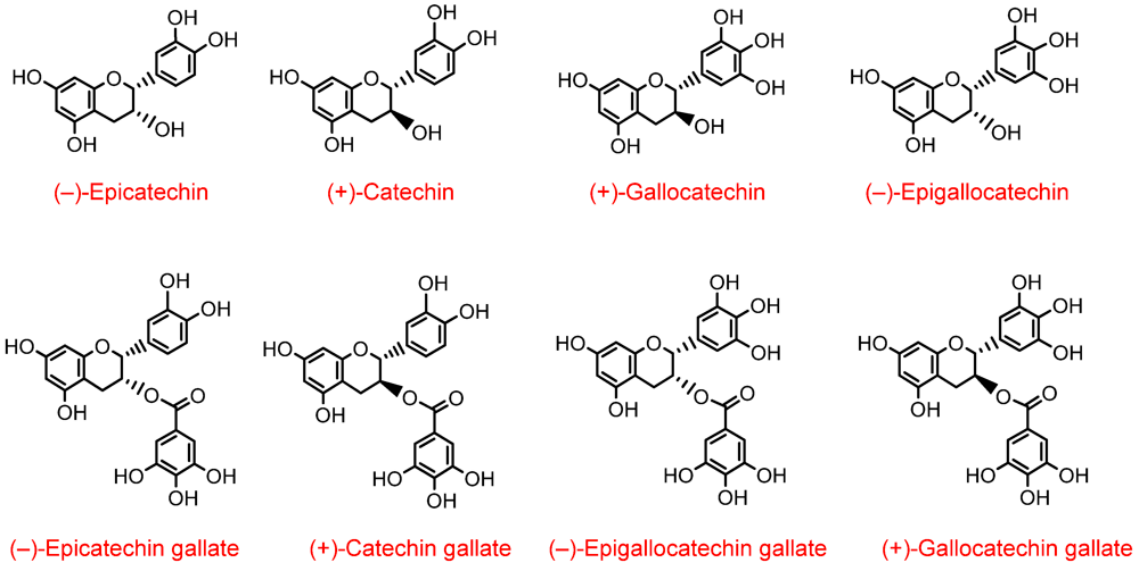


Figure 1. Chemical structures of major tea catechins.

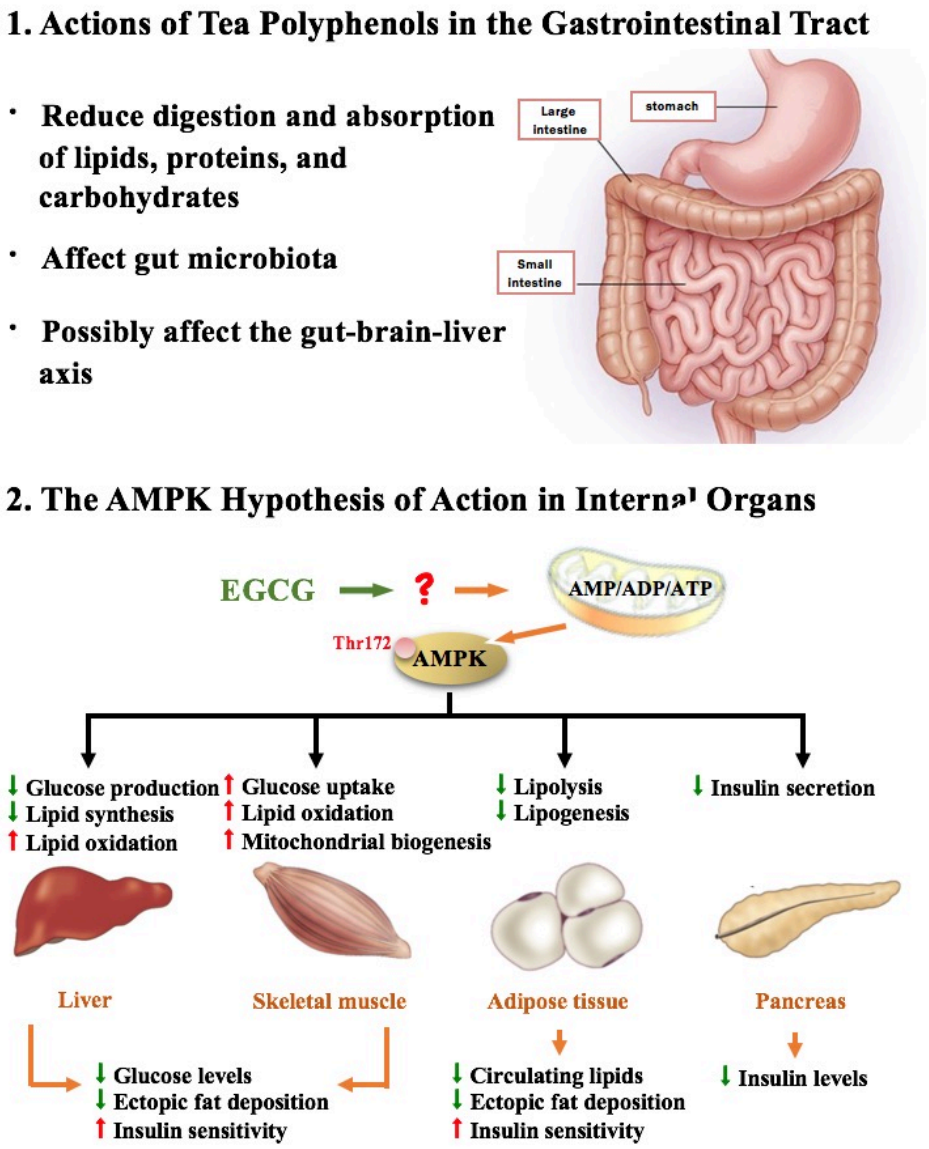


Figure 2. Proposed mechanisms for the actions of tea constituents in lowering body weight and alleviating MetS. (1) Actions of tea polyphenols in the gastrointestinal tract. (2) A hypothesis on the central role of AMPK in metabolic regulation by EGCG. EGCG is proposed to active AMPK through affecting the ratios of AMP/ADP/ATP. The activated (phosphorylated) AMPK regulates metabolism in different organs toward the direction of reducing gluconeogenesis, fatty acid synthesis, insulin secretion, and ectopic fat deposition

in muscle and liver. These are accompanied by increased insulin sensitively and the oxidation of glucose and fatty acids [93].

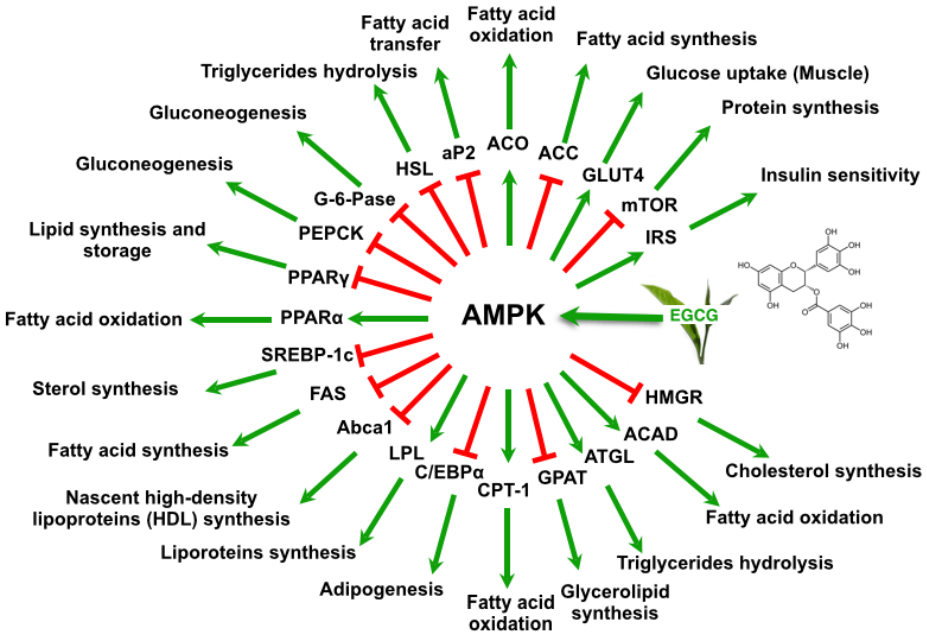


Figure 3. EGCG induces changes in proteins and genes that are mediated by AMPK. mTOR, homolog of target of rapamycin; ACC, acetyl-CoA carboxylase; ACO, acyl-CoA oxidase; aP2, adipocyte protein 2; HSL, hormone sensitive lipase; PEPCK, phosphoenolpyruvate carboxykinase; SREBP-1c, sterol regulatory element binding protein-1c; Abca1, ATP-binding cassette superfamily of transporter proteins 1; LPL, lipoprotein lipase; C/EBP , CCAAT enhancer binding protein ; GPAT, glycerol phosphate acyltransferase; ATGL, adipose triglyceride lipase; ACAD, acyl-CoA dehydrogenase [93].

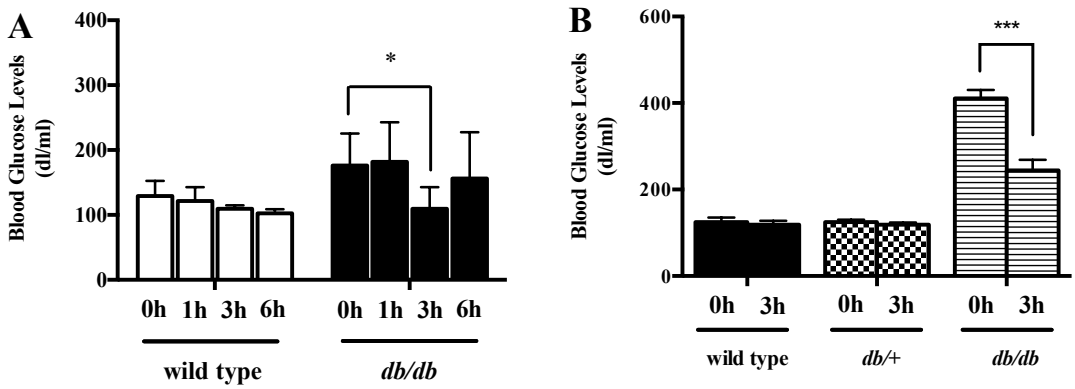


Figure 4. Suppression of blood glucose levels in *db/db* mice. (A) Wild type and *db/db* mice were treated with EGCG at 200 mg/kg (*i.g.* administration). Blood glucose levels were monitored after the treatment at 0, 1, 3 and 6 hours. (B) Wild type, *db/+*, and *db/db* mice were orally treated with EGCG at 400 mg/kg, and blood glucose levels were tested after 0 and 3 hours. Data are presented as mean ± SEM (n=4). *, $p < 0.05$. ***, $p < 0.001$.

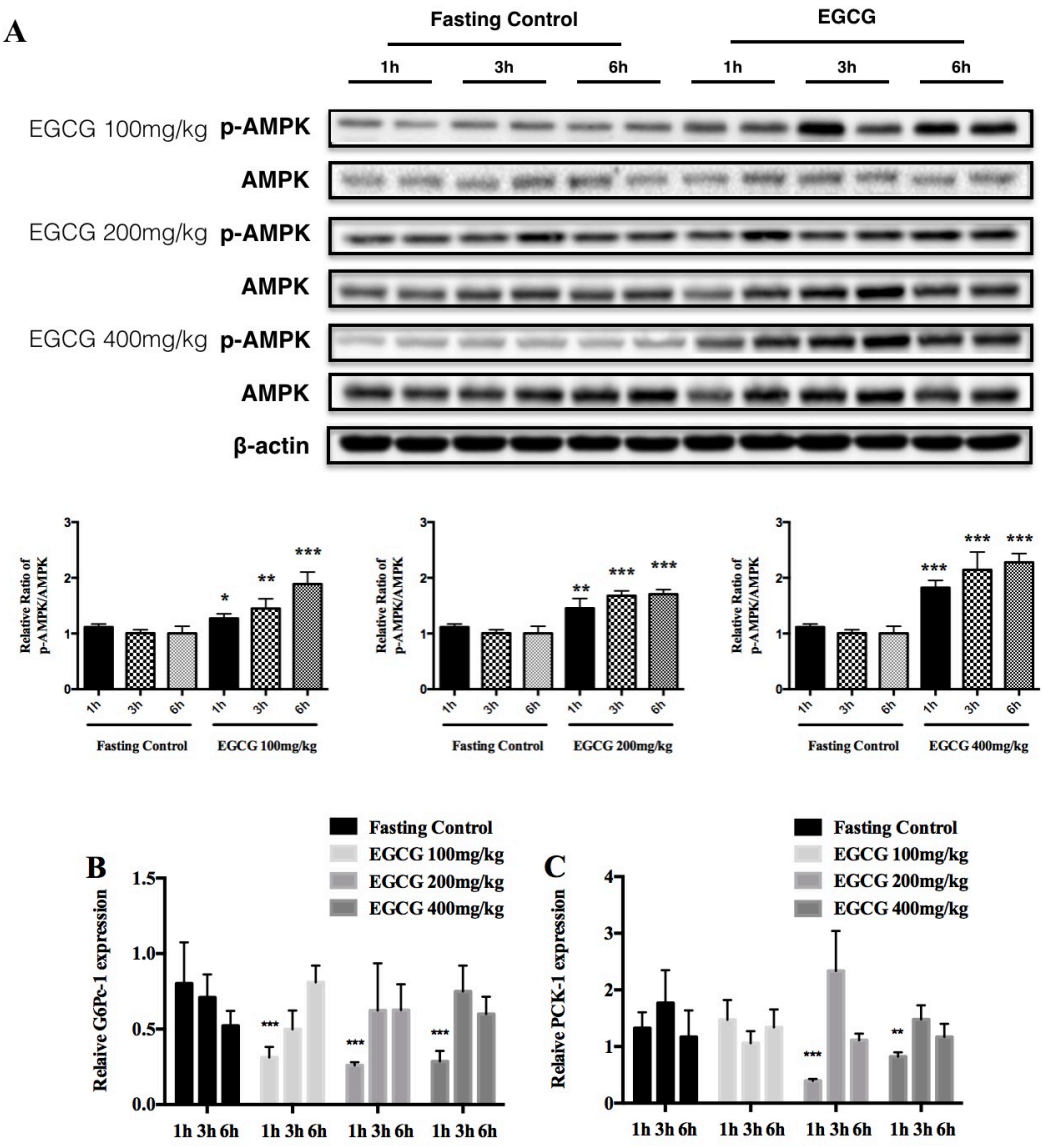
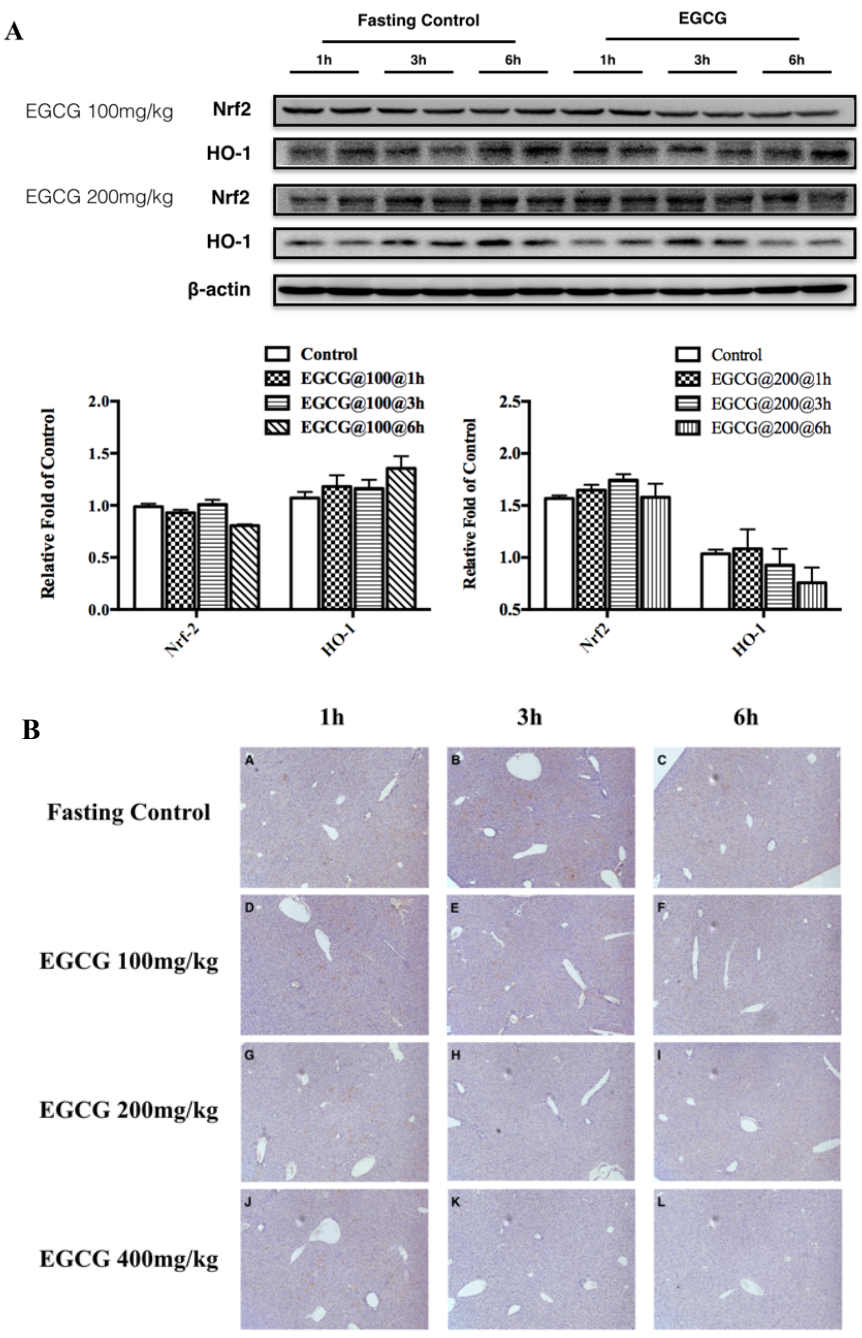


Figure 5. Activation of AMPK in C57BL/6J mice. After 12 hours fasting, female C57BL/6J mice (8-week old) were *i.g.* administered with water, or EGCG at 100, 200, or 400 mg/kg, and then sacrificed at 1, 3, and 6 hours. (A) Hepatic expression of AMPK and phosphorylated AMPK in mice. (B and C) Hepatic mRNA levels of G6Pc-1 and PCK-1, respectively. Data are presented as mean ± SEM (n=6). *, $p < 0.05$. **, $p < 0.01$. ***, $p < 0.001$.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Fasting Control
EGCG
1h
3h
6h
1h
3h
6h
EGCG 100mg/kg
Nrf2
HO-1
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Nrf2
HO-1
β-actin
Control
EGCG@100@1h
EGCG@100@3h
EGCG@100@6h
Relative Fold of Control
2.0
1.5
1.0
0.5
0.0
Nrf-2
HO-1
Control
EGCG@200@1h
EGCG@200@3h
EGCG@200@6h
Relative Fold of Control
2.5
2.0
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1.0
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Nrf2
HO-1
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Fasting Control
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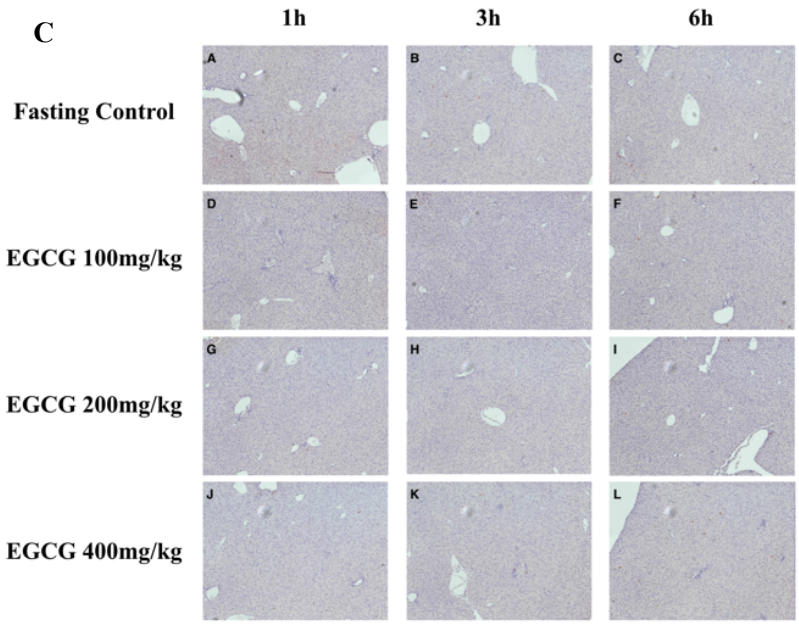
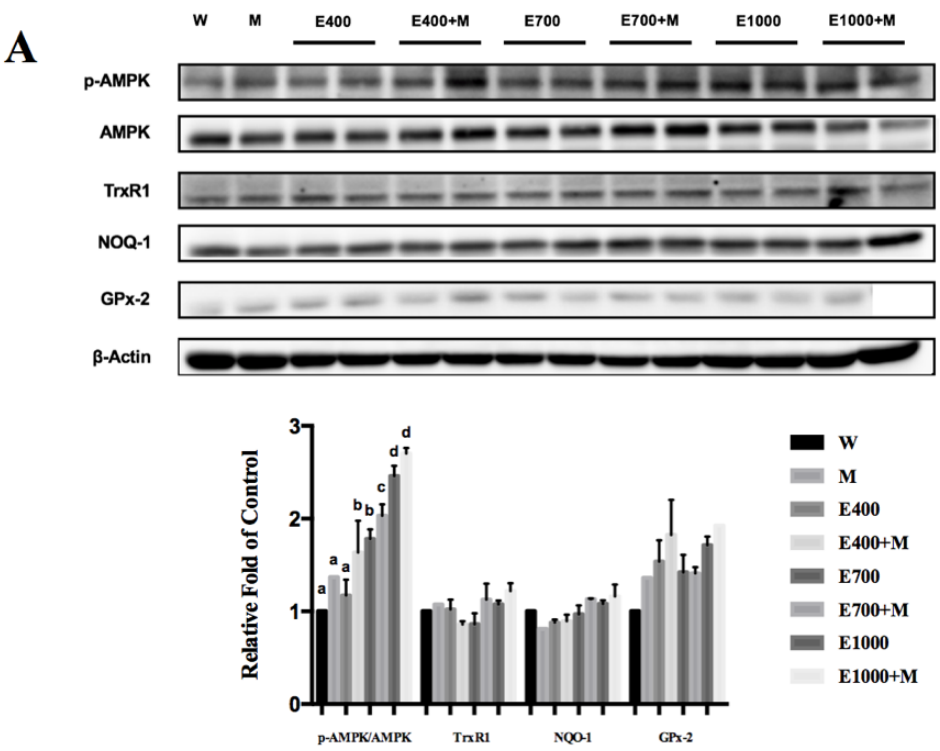


Figure 6. Expression of Nrf2 system and oxidative stress markers in C57BL/6J mice. After 12 hours fasting, female C57BL/6J mice (8-week old) were *i.g.* administered with water, or EGCG at 100 or 200 mg/kg, and sacrificed at 1, 3, and 6 hours. (A) Expression of Nrf2 and HO-1 in mouse liver. Data are presented as mean ± SEM (n=6). (B and C) IHC staining for 8-OHdG and γ-H2AX in mouse liver.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β-Actin
Relative Fold of Control
p-AMPK/AMPK
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E1000
E1000+M

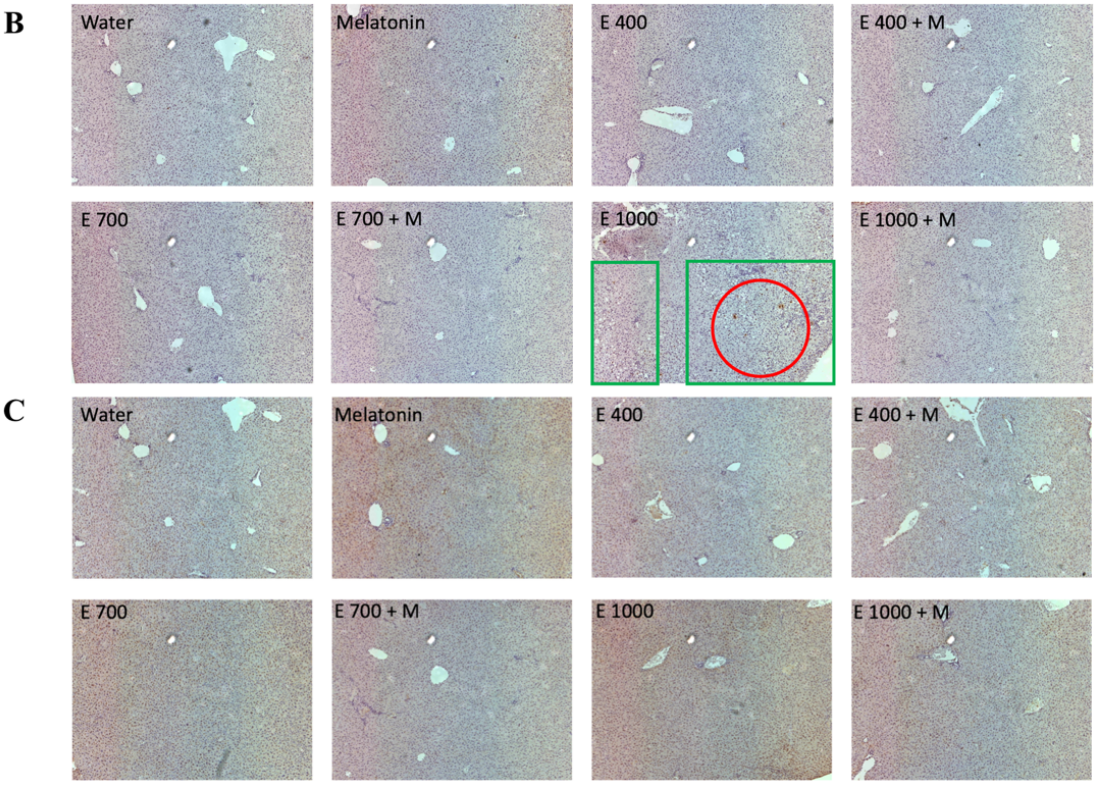
B
Water
Melatonin
E 400
E 400 + M
E 700
E 700 + M
E 1000
E 1000 + M
C
Water
Melatonin
E 400
E 400 + M
E 700
E 700 + M
E 1000
E 1000 + M

Figure 7. Influence of melatonin on EGCG activating AMPK in C57BL/6J mice. After a 12 hours starvation period, 8-week old C57BL/6J mice were giver either EGCG alone (700 or 1000mg/kg), or EGCG (700 or 1000mg/kg) combined with melatonin at 50 mg/kg (n=4 at each group). (A) Hepatic AMPK, p-AMPK, TrxR1, NQO-1, and GPx-2 levels in mice. Data are presented as mean ± SEM (n=4). (B) IHC staining for 8-OHdG and γ-H2AX in mouse liver.

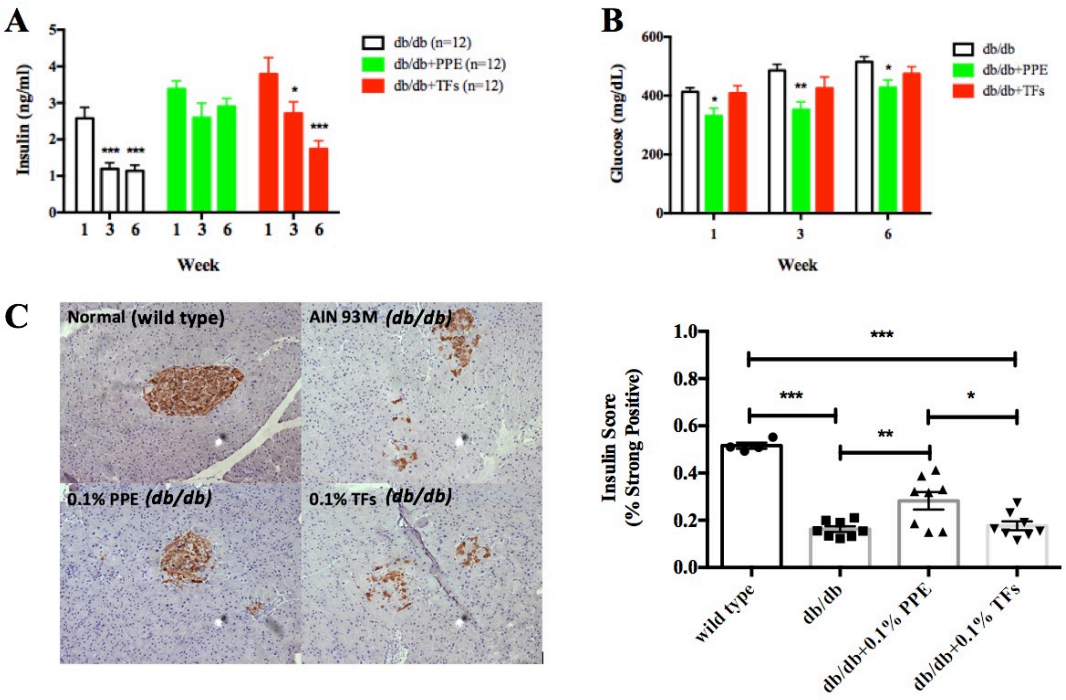

Figure 8. Protection on pancreatic β cells from damage in *db/db* mice. Eight-week old *db/db* mice were fed with AIN-93M, AIN-93M containing 0.1% PPE, and AIN-93M containing 0.1% TFs diet (n=12 each group), respectively, for 6 weeks. (A and B) Blood insulin and glucose level were monitored at each week (n=12). (C) IHC staining for insulin levels in pancreatic islets (left part) and insulin score (right part) in mice. Data are presented as mean ± SEM (n=10). *, $p < 0.05$. **, $p < 0.01$. ***, $p < 0.001$.

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