

**Epigallocatechin Gallate in the Regulation of Insulin Secretion**

**Julia Kathryn Yuskavage**

**Thesis submitted to the faculty of the Virginia Polytechnic Institute and State  
University In partial fulfillment of the requirements for the degree of**

**Master of Science**

**In**

**Human Nutrition, Foods & Exercises**

**Dr. Dongmin Liu, Chair**

**Dr. William E. Barbeau**

**Dr. Frank C. Gwazdauskas**

**April 30, 2008**

**Blacksburg, Virginia**

**Copyright 2008, Julia Yuskavage**

## Epigallocatechin Gallate in the Regulation of Insulin Secretion

Julia Kathryn Yuskavage

### ABSTRACT

In both Type 1 diabetes (T1D) and Type 2 diabetes (T2D), inadequate  $\beta$ -cell mass and  $\beta$ -cell dysfunction lead to impaired insulin secretion, and ultimately worsen glycemic control. Green tea has drawn wide attention due to its possible health-promoting properties, including enhancement of  $\beta$ -cell function. We assessed the acute and relative long-term effects of epigallocatechin gallate (EGCG) on insulin secretion and synthesis from clonal  $\beta$ -cells (INS1E cells), rat islets, and human islets, using 0.1, 1, or 5  $\mu$ M. We determined if EGCG decreased blood glucose in healthy rats acutely, using 50 or 150 mg/kg body weight (BW), and after 12 days of supplementation in drinking water, using 0.1% and 0.5%. In the *in vitro* studies, EGCG significantly potentiated glucose-stimulated insulin secretion (GSIS) in rat islets (at 0.1, 1, and 5  $\mu$ M) and human islets (at 1  $\mu$ M), and elevated insulin content within INS1E cells (at 0.1, 1, and 5  $\mu$ M) and human islets (at 1  $\mu$ M), ( $P < 0.05$ ). Nutritional supplementation of EGCG (0.5% in drinking water) for 12 days in healthy rats significantly increased insulin synthesis, compared to that of controls, from  $0.2 \pm 0.02$  to  $1.4 \pm 0.2$  ng/mg protein, without alteration of insulin secretion in isolated islets ( $P < 0.05$ ). These findings demonstrate that EGCG may play a role in the regulation of pancreatic  $\beta$ -cell function, thereby contributing to an anti-diabetic effect of this agent.

Keywords: EGCG; green tea; catechin; diabetes; pancreatic  $\beta$ -cell; islets; insulin.

## **ACKNOWLEDGEMENTS**

I offer many thanks to Dr. Dongmin Liu for his endless guidance and mentorship throughout my time in HNFEE. My gratitude extends to Dr. William Barbeau and Dr. Frank Gwazdauskas, for offering suggestions and advice from the beginning to the end of my research.

I am also thankful to Wen Zhang, Zhuo Fu, Wei Zhen, Hongwei Si, Yanling Zhang, and Kathy Reynolds for their continuous help during my studies. Not only did my lab mates teach me a great deal about biological research, but they also became my good friends during my time in Blacksburg.

## TABLE OF CONTENTS

Abstract .....	ii
Acknowledgement.....	iii
Table of Contents.....	iv
List of Tables.....	vii
List of Figures.....	viii
Glossary of Terms.....	ix
Introduction.....	1
Review of Literature.....	4
Tea.....	4
Green tea.....	4
Other teas.....	5
Chemistry of catechins.....	6
Absorption.....	8
Metabolism .....	9
Other food sources.....	11

Pancreatic $\beta$ -cells and diabetes.....	12
Insulin secretion.....	12
Diabetes mellitus.....	14
EGCG and diabetes.....	15
<i>In vitro</i> studies.....	15
Animal studies.....	17
Human studies.....	21
Summary .....	23
Significance of study.....	24
Materials and Methods.....	25
Reagents and materials.....	25
Cell and islet culture.....	25
GSIS assay.....	26
Animal study.....	28
<i>In vitro</i> free radical scavenging activity assay.....	29
Viability assay.....	29
Statistics analysis.....	30

Results.....	31
Discussion.....	39
References.....	47

## LIST OF TABLES

<b>Table.1.</b> Major food sources of catechins.....	12
--	----

## LIST OF FIGURES

<b>Fig.1.</b> Structures of flavonoids .....	7
<b>Fig.2.</b> Structures of green tea catechins .....	7
<b>Fig.3.</b> Biphasic insulin secretion.....	14
<b>Fig.4.</b> Effects of EGCG on rapid insulin secretion in INS1E cells.....	31
<b>Fig.5.</b> Effects of long-term EGCG exposure on insulin content in INS1E cells.....	32
<b>Fig.6.</b> Effects of EGCG on rapid insulin secretion in rat islets.....	33
<b>Fig.7.</b> Effects of EGCG on rapid insulin secretion in human islets.....	34
<b>Fig.8.</b> Effects of long-term EGCG exposure on insulin secretion and content in human islets.....	35
<b>Fig.9.</b> Effects of EGCG supplementation on glucose tolerance, insulin secretion and synthesis in rats.....	36
<b>Fig.10.</b> Free radical scavenging activity of EGCG.....	37
<b>Fig.11.</b> Effects of EGCG on human islet viability.....	38



## GLOSSARY OF TERMS

BW: Body weight

cAMP: Cyclic adenosine monophosphate

CREB: CRE-binding protein

EC: Epicatechin

ECG: Epicatechin gallate

EGC: Epigallocatechin

EGCG: Epigallocatechin gallate

ELISA: Enzyme-Linked ImmunoSorbent Assay

FBS: Fetal bovine serum

GSIS: Glucose stimulated insulin secretion

HBSS: Hank's buffered salt solution

HI: Heat inactivated

IP: Intraperitoneal

INS1E: Clonal rat pancreatic  $\beta$ -cell

K<sub>ATP</sub>: ATP-dependent potassium channels

OGTT: Oral glucose tolerance test

PKA: Protein kinase A

SD: Sprague-Dawley

STZ: Streptozotocin

T1D: Type 1 Diabetes

T2D: Type 2 Diabetes

WK: week

## INTRODUCTION

Almost 21 million people in the United States suffer from diabetes, and nearly twice as many currently exhibit pre-diabetes (1, 2). While the availability of novel drugs, techniques and surgical intervention have improved the survival rate of individuals with diabetes and diabetic complications, the prevalence of diabetes still increases in Americans. Each year hereafter, it is estimated that 2-4 million new cases of diabetes will be diagnosed (2). The medical costs associated with this disease in 2002 surged to \$132 billion in the United States, which include drug therapy and surgical treatment (1). In both Type 1 (T1D) and Type 2 (T2D) diabetes, inadequate  $\beta$ -cell mass and  $\beta$ -cell dysfunction lead to impaired insulin secretion, and ultimately worsen glycemic control (3). Thus, the search for novel and cost-effective preventative agents that enhance  $\beta$ -cell function is imperative to decrease morbidity and related complications from diabetes.

Derived from the *Camellia sinensis* plant, green tea was traditionally used in ancient Chinese medicine as a folk remedy for multiple health problems, including poor blood flow, joint pain, infections, depression, and unclear urine (4, 5). Legend suggests that tea originated when a gust of wind carried tea leaves into Chinese Emperor Shen Nung's cup of boiling water, dating prior to 3,000 B.C. Years ago, tea time was associated with more than just consuming food and beverage; it was part of a calm atmosphere and a calm sense of being (6). However, as time passed and as tea was introduced to new cultures, tea emerged as a health-promoting mainstream beverage (6, 7). The unique processing of green tea gives it medicinal properties, which have been investigated within the past 20 years (8). While it is unclear exactly how green tea extracts exert potential beneficial effects, it was found that green tea catechins, a kind of

flavonol, may be attributable to some of the health benefits of green tea. There are four major catechins, including epigallocatechin gallate (EGCG), epicatechin (EC), epigallocatechin (EGC), and epicatechin gallate (ECG) (9). In addition, green tea also contains gallic acid, gallic acid gallate, gallic acid catechin, and catechin, which are in smaller quantities and not widely utilized in research (6). Recently, EGCG, the most abundant catechin in green tea which accounts for >50% of the total catechin content, has drawn wide attention due to its possible health-promoting properties (10). EGCG has not only been studied for its role in diabetes, but also in alleviating and/or preventing cancers, cardiovascular disease, inflammation, renal hypertension, bacterial infections, dental caries, and neurological disorders, although some of these reports remain controversial and the mechanisms of these effects are unclear (6).

Recent studies suggest that EGCG may play a key role in improving health, especially diabetes mellitus. *Ex vivo* and *in vitro* studies showed that EGCG increased expression of genes involved in glycolysis and down-regulated genes involved in gluconeogenesis (11), while EGCG protected islets from apoptosis as well, indicating benefits for T1D (12, 13). Animal studies using various doses of green tea or EGCG demonstrated anti-diabetic potential of this agent in both healthy and diabetic rodents (8, 11, 14-16). However, human studies provided inconsistent results (17-21). Therefore, results regarding the beneficial effects of green tea/EGCG on human diabetes remain inconclusive.

Although emerging evidence suggests that green tea exerts anti-diabetic effects, *in vitro* studies involving the effects of EGCG on  $\beta$ -cell function are lacking. Additionally, available previous *in vitro* studies utilized pharmacological doses of green tea which were

not attainable through tea drinking, and/or methods of administration (such as injection) that are not practical for human use (22, 23). Furthermore, differences in animal models, dosage of EGCG/green tea, and lengths of study in *in vivo* studies may contribute to different results.

Type 2 diabetes develops primarily due to insulin resistance and insulin-producing pancreatic  $\beta$ -cell dysfunction, leading to insufficient insulin secretion. Efforts are therefore required on many fronts to address this major public health problem. Among these, a search for novel, cost-effective agents that have protective effects on islet  $\beta$ -cell function, including insulin secretion, is extremely important to decrease the burden of morbidity from diabetes and related complications, and thus promote the health of the American people. In the present study, we determined the effects of physiologically relevant doses of EGCG on both *in vitro* and *in vivo* insulin secretion and synthesis from  $\beta$ -cells.

## REVIEW OF LITERATURE

### TEA

#### Green tea

Green tea comparatively contains more total catechins content than any other tea. Unlike other teas that are fermented, green tea is produced via a brief and moderate heating process, in which the leaves are withered, steamed/pan-fired, rolled/shaped, and dried. The enzyme responsible for oxidizing catechins, polyphenol oxidase, exists in a separate layer from the catechins. Extensive rolling, crushing, or chopping of the tea leaves disturbs the separated leaf layers and allows for oxidation of catechins to occur, resulting in mostly dimers and polymers, such as those found in black tea. Due to the brief processing step that is used to produce green tea, polyphenol oxidase is left inactivated and the catechins (monomers) are preserved (5, 24, 25). Thus, green tea retains greater catechin content, which accounts for bitterness of green tea (26).

In addition to the bitter component caffeine, green tea contains very small amounts of other common methylxanthines, theobromine (0.1%) and theophylline (0.02%), as well as the amino acid theanine (4-6%) which is an N-methylated derivative of glutamine and is unique to tea (26). Other components of green tea include quercetin and kaempferol (5-10%), 2 flavonols that are closely related to the catechins, but have a higher level of oxidation on ring C, theogallin (2-3%) which is a condensation product of gallic (0.5%) and quinic (2%) acids, negligible levels of carotenoids that are the precursors of the volatile fraction (0.2%) (responsible for aroma), and mineral content (6-

8%) which is relatively rich in aluminum and manganese. Contrary to common thought, tannic acid (pentagalloylglucose) is not found in green tea, but trigalloylglucose is (25).

### **Other teas**

Along with green tea, several other popular teas have been studied for possible health-promoting benefits. Black tea is processed via rigorous fermentation (withered, rolled and cut, fully fermented, and dried), thus contains lower amounts of catechin monomers compared to green tea (0-70 mg/237 ml cup vs. 30-130 mg/237 ml cup) (27). A longer fermentation process and exposure of tea leaves to polyphenol oxidase, during the production of black tea, causes further conversion of catechin monomers into dimers and polymers, such as theaflavins and thearubigins (28).

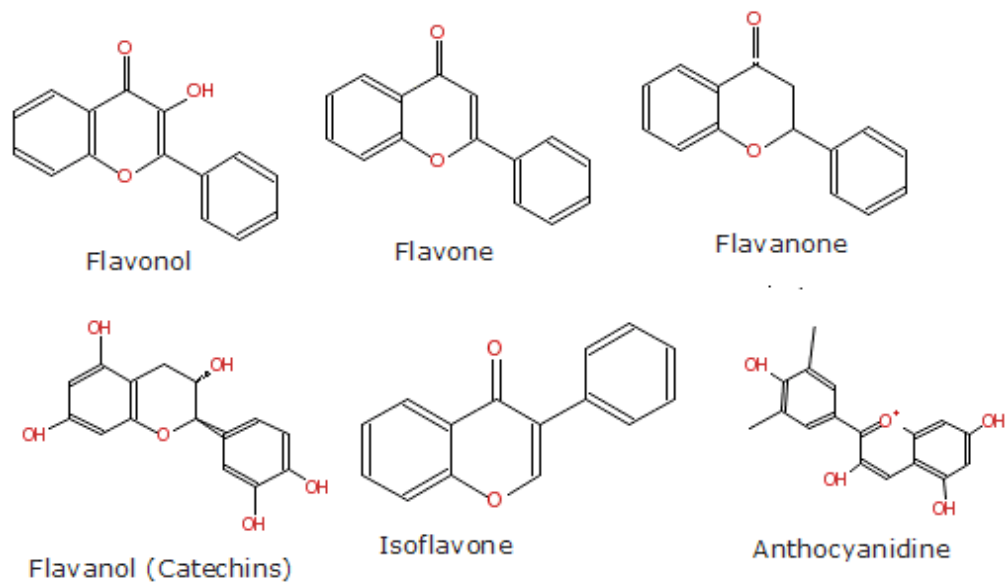
Oolong tea is a partially oxidized beverage that retains a catechin content greater than black tea but less than that of green tea, due to a shorter fermentation process that is in between that of black and green tea (29). Even with lower catechin monomer content, oolong tea reduced fasting blood glucose levels in T2D adults (30).

White tea retains the highest amount of catechin monomer content due to the use of immature tea leaves and steaming process involving little oxidation followed by a brief drying process. Although green tea contains a high amount of catechin monomers, it is less than white tea due to usage of more mature tea leaves, plus an additional processing step of withering before steaming (31).

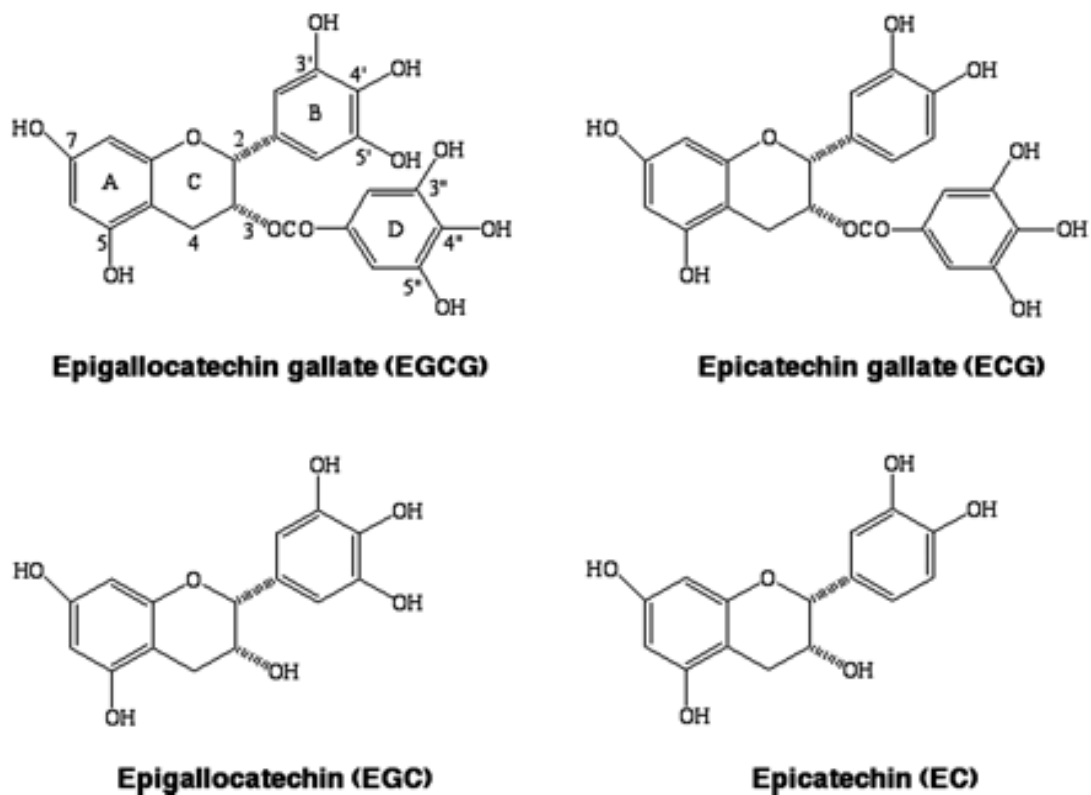
## Chemistry of catechins

Recently, EGCG, the most abundant catechin in green tea which represents more than half of the total catechin content, has drawn wide attention due to its possible health-promoting properties (10). The beneficial effects of EGCG may be attributable to its specific chemical structure. As shown in Fig. 1, flavonoids share a common structure, consisting of 2 aromatic rings connected by a chain of 3 carbon atoms that form an oxygenated heterocycle. They are divided into several subclasses, including the flavanols (catechins) (32). As shown in Fig. 2, the chemical structure of EGCG consists of a phenolic ring (A) integrated with an oxygenated heterocycle (C) that connects to one phenolic ring (B) at C-2 and another phenolic ring (D) at C-3. EC is similar to EGC, with both compounds consisting of 2 hydroxyl groups at C-3' and C-4' on ring B and a hydroxyl group at C-3 on ring C. However, EGC is differentiated by the addition of one more hydroxyl group at C-5 on ring B ECG differs from EC in having a gallate moiety esterified at C-3 of ring C; EGCG differs from EGC in the same manner (25, 33-35).





**Fig.1.** Structures of flavonoids (36). Used with permission of Parul Lakhanpal.



**Fig.2.** Structures of green tea catechins (33). Used with permission of Mary E. Waltner-Law.

## Absorption

Flavanols such as EGCG are absorbed through small intestinal mucosa, where extensive metabolism occurs. After absorption, catechin molecules are conjugated by methylation, sulfation, and/or glucuronidation in the liver (37). Both free and conjugated catechins are found in the bloodstream after respective metabolism has occurred (38).

Due to the rapid metabolizing effects on EGCG, its bioavailability is very low compared to that of other tea catechins. In 8 healthy subjects who received a single oral dose of green tea (EGCG, EGC, EC, 20 mg tea solids/kg body weight [BW]), plasma EGCG level was lowest among these ingested catechins (39). In agreement with this finding, Nakagawa et al. (40) found only 0.2-2% of ingested EGCG detected in circulating blood. EGCG may be hydrolyzed at the gallate moiety by bacterial esterases, which may account for the low bioavailability of EGCG (41). The bioavailability of EGC is more variable, with 3-13% of the free form found in human plasma, and larger amounts of the glucuronidated and sulfated forms (42). Regarding ECG and EGC, it was shown that peak plasma concentrations were 3.1  $\mu\text{M}$  and 5.0  $\mu\text{M}$ , respectively, while the concentration of EGCG was 1.3  $\mu\text{M}$ , after high-dose human consumption of 1.5 mM of the respective catechin in water (43). Given the low absorption rate of catechins, plasma levels following *regular* tea consumption are generally less than 1  $\mu\text{M}$  (44). In agreement with this finding, Lee et al. (39) found that 1 dose (equivalent to ~2 cups of tea) of green tea extract consumption in humans resulted in a mean peak plasma EGCG level of 0.17  $\mu\text{M}$  (39). However, serum EGCG level in humans consuming 6 cups (200 ml/cup) of green tea (200 mg catechins/cup) can reach 1  $\mu\text{M}$  (42).

Species differences were found regarding bioavailability profiles of EGCG and should be carefully considered when extrapolating animal studies to human relevance. For example, Kim et al. (45) found that 0.6% green tea polyphenols given to rats in drinking water for 28 days resulted in lower plasma EGCG levels and higher EGC and EC levels, suggesting relatively poor absorption of EGCG in rats. When mice were given the same treatment, plasma EGCG levels were higher than those of EGC and EC levels. It was thought that differences were due to species, and not gender differences. No difference in plasma EGCG concentration between men and women was observed (45, 46). However, further research in this area is warranted.

It was reported that EGCG had a half life of about 2-3.4 hrs in blood (47), but other studies reported a half life of 5 hrs (39, 48). The discrepancy of these results may be due to the differences in experimental conditions, ages of subjects and doses of catechins. Nevertheless, EGCG has a relatively short half-life in blood circulation. After 24 hrs of tea consumption, EGCG returns to baseline levels, while EGC and ECG remain elevated in plasma (43), demonstrating the rapid metabolizing effects on EGCG. Therefore, consumption of a cup (containing ~100 mg polyphenols) or more of green tea every few hours may be needed to maintain plasma EGCG concentration. However, it is presently unknown if EGCG can be stored within target tissues.

## **Metabolism**

Catechins are largely metabolized by the time they are distributed to tissues, thus caution must be taken when interpreting *in vitro* results. Research suggests that there may be differences in the abilities of individuals to metabolize catechins and their

conjugates (49). This may be due to functional polymorphisms in metabolic enzymes, as well as dietary, environmental, and behavioral factors (smoking and alcohol usage) which can influence glucuronidation of catechins (37). In addition, certain catechin metabolites may have different biological functions compared to original compounds. For example, results indicated that only catechin metabolites were able to inhibit monocyte adhesion to human aortic endothelial cells, whereas unconjugated catechin had no such effect (50).

Catechin metabolites may be excreted through different pathways. In a recent study using healthy beagles, EC and EGC were excreted in urine as the conjugated forms, whereas EGCG and ECG were largely absent in urine (51), which may be due to absorbed unconjugated EGCG being excreted through bile (52). While it is not clear how catechin metabolites are excreted in other animal species, a human trial found that, in agreement with Mata-Bilbao et al. (51), EGC and EC, but not EGCG, were partly recovered in urine (as well as catechin metabolites), further suggesting that EGCG may be excreted along with bile (53). In the beagles, EGCG and ECG were found unconjugated in plasma, possibly due to the presence of a gallate moiety at C-3 of ring C, and the hydroxyl group at C-5' on EGCG (51). EGCG has a lower glucuronidation rate than other catechins, and the hydroxyl group on EGCG may facilitate the access to active sites of metabolic enzymes (54). EGCG from tea was methylated into 4',4''-di-*O*-methyl-EGCG, and the concentration of this metabolite in red wine was ~15% that of EGCG in human plasma (55, 56).

High doses of EGCG (800 mg) administered to subjects resulted in higher systemic availability of EGCG, most likely due to saturation of metabolic enzymes and

conjugation pathways, which could allow for disproportionately higher unconjugated EGCG in human plasma (49).

### **Other food sources**

As shown in Table. 1, catechins are abundantly supplied in many fruits and vegetables, among other commonly consumed food products. Catechin and EC are the major flavanols in fruits, whereas EGC and EGCG are mainly found in legumes (especially beans), grapes, and tea (57, 58). Green tea is perhaps the most well-known source of catechins, although catechins exist in lesser amounts in other teas, such as oolong and black tea, due to longer fermentation processes (27, 30). The average catechin intake is ~18-50 mg/day, which can be elevated by drinking green tea, and by consuming chocolate, apples, pears, grapes, and red wine (57, 59). A typical cup of green tea may contain 1 g of tea leaves in 100 ml of boiling water, containing 250-350 mg of dry materials that are comprised of 30-42% catechins and 3-6% caffeine (34). While polyphenolic compounds in green tea including flavanols and flavonoids account for about 30% dry weight of green tea leaves, catechins themselves account for more than 80% of total flavonoids in green tea (5). The remaining 20% of total flavonoids are oxidized catechin polymers, similar to those found in black tea, due to oxidation during the withering process (5). Older green tea leaves contain more EGCG and total catechins, but less caffeine, than young tea leaves. Along with age of tea leaves, species, cultivating location, season of harvest, plucking position, climate, and horticulture practices affect tea composition (29). For example, tea leaves exposed to sunlight contain more flavonols because their biosynthesis is stimulated by sunlight (60).

Additionally, actual catechin content from commercial green teas range from 9-48% of label claims, and the actual values are much lower than the claims (61).

**Table.1.** Major food sources of catechins (32)

<b>Food sources</b>	<b>Catechins, mg/100 g food</b>
Chocolate	46-61
Beans	35-55
Apricot	10-25
Cherry	5-22
Grape	3-17.5
Peach	5-14
Apple	10-43
Raspberry	2-48
Strawberry	2-50
Blackberry	9-11
Green tea	10-80
Black tea	6-50
Red wine	8-30
Cider	4

Modified from Manach, C., Scalbert, A., Morand, C., Rémésy, C. & Jiménez, L. 2004. Polyphenols: food sources and bioavailability.

Am J Clin Nutr 79, 727-47. Used with permission of Claudine Manach.

## **PANCREATIC $\beta$ -CELLS AND DIABETES**

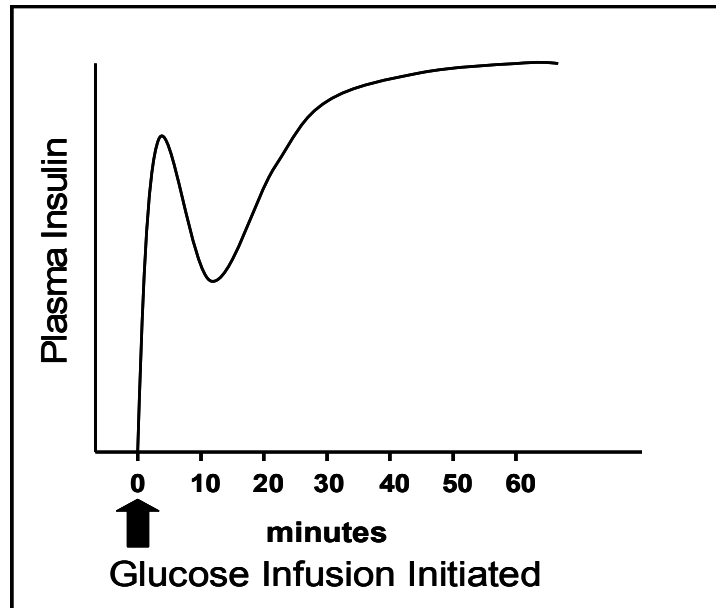
### **Insulin secretion**

The pancreas contains islet cells embedded within endocrine tissue. In the islets, 65-80% of the cells are  $\beta$ -cells, which secrete insulin in response to glucose. The islets also contain  $\alpha$ -cells, which secrete glucagon;  $\delta$ -cells, which secrete somatostatin; and F-cells, which secrete pancreatic polypeptide (62, 63). After insulin is produced, it is released from the  $\beta$ -cell via exocytosis, providing stimulation by an agonist occurs. As the intracellular ATP/ADP ratio increases due to the glycolysis and tricarboxylic acid

cycle, ATP-dependent potassium ( $K_{ATP}$ ) channels close, thus less  $K^+$  exits the  $\beta$ -cells. This process subsequently causes cell depolarization, leading to the opening of voltage-dependent  $Ca^{2+}$  channels, causing  $Ca^{2+}$  influx into the cells. This ultimately causes exocytosis of insulin into the bloodstream, which is called the first phase insulin response of the biphasic pattern (Fig. 3). The first phase insulin response subsides within the first 10 min of eating, and is mediated by the  $K_{ATP}$ -dependent mechanism (64). As glucose tolerance becomes progressively impaired, the first phase insulin response is drastically decreased, resulting in hyperglycemia. Due to this physiological response, the second phase insulin response is elevated as a compensatory measure (2).

This second phase insulin response occurs when glucose levels begin to rise (following tapering of the first phase insulin response) again slowly and progressively for up to several hours, with insulin pulses occurring in 5-15 min intervals (64). In addition to the  $K_{ATP}$ -dependent mechanism, a  $K_{ATP}$  channel-independent pathway plays a role in the second phase insulin response, although the mechanism by which it augments the response to increased  $Ca^{2+}$  is still unclear (65). While the definitive mechanism of the second phase insulin secretion is unknown, several proposed mechanisms include involvement of cyclic adenosine monophosphate (cAMP), protein kinase A (PKA), protein kinase C, phospholipase C, amino acids, glyceraldehydes, phospholipase A2, nitric oxide, cyclic guanosine monophosphate, and phosphatidylinositol 3-kinase (64, 65). Whereas the rate-determining step in the first phase insulin response is the rate of signal transduction between sensing the rise in  $Ca^{2+}$  and subsequent exocytosis of releasable insulin granules, the rate-determining step in the second phase insulin response is the

conversion of available, releasable insulin granules to the point at which they are immediately available for release via exocytosis (65).



**Fig.3.** Biphasic insulin secretion (66). Used with permission of R. Bowen.

### **Diabetes mellitus**

The 2 major forms of diabetes mellitus are T1D and T2D. T2D is characterized by 4 primary metabolic changes; obesity, impaired insulin action, dysfunction of insulin secretion, and increased endogenous glucose output from the liver, whereas T1D is characterized primarily by little or no insulin production due to  $\beta$ -cell destruction (67, 68). Healthy  $\beta$ -cells adapt to changes in both glucose and insulin concentration; that is, a decrease in insulin action is accompanied by upregulation of insulin secretion. However, in both T1D and T2D, inadequate  $\beta$ -cell mass and  $\beta$ -cell dysfunction lead to impaired insulin secretion, and ultimately worsen glycemic control (3).



In T1D, progressive  $\beta$ -cell failure is caused by an autoimmune disease. Inflammatory reactions cause mononuclear cells to invade and destroy pancreatic islets.  $\beta$ -cell mass markedly deteriorates, by 70-80% at the time of diagnosis, which may be secondary to  $\beta$ -cell apoptosis (69). Inflammation plays a role in the pathophysiology of T2D when obesity is present; excess adipose tissue secretes proinflammatory cytokines, which are linked to insulin resistance in T2D (70). At the time of T2D onset, insulin secretion is definitively defective and continues to decline with disease progression, and is paralleled with worsening insulin sensitivity (71, 72). In the insulin resistant, hyperglycemic individual, the body initially compensates for decreased insulin action by increasing  $\beta$ -cell mass and function to maintain normal glucose range. The failure of this compensation ultimately leads to the development of T2D (3). Patients with T2D display accelerated endogenous glucose production, which is responsible for about 80% of all glucose entering the plasma (67, 73).

## **EGCG AND DIABETES**

### ***In vitro* studies**

Wolfram et al. (11) found that in rat H411E hepatoma cells, incubation of EGCG dose-dependently down-regulated glucose-6-phosphatase, a gene involved in gluconeogenesis, and fatty acid synthase, a gene involved in fatty acid synthesis (11). These results suggest that EGCG may improve glucose metabolism via changes in gene expression and promotion of fat oxidation in mice and humans. However, 100 and 50  $\mu$ M EGCG used in the above *in vitro* studies are far higher than the physiologically attainable concentrations, which are about 1  $\mu$ M, following drinking tea (11).

Zhang et al. (12) tested the effects of green tea extract on the survival rates of isolated human islets. Islets cultured in the presence of various concentrations of green tea extract (0-500 µg/ml extract) were significantly higher in number compared to the control. However, green tea did not significantly improve islet viability, purity, or morphology. These results indicate that physiologically attainable concentrations of green tea extract or EGCG may possess the ability to protect islets in individuals with T1D (12). In agreement, Hara et al. (13) found that EGCG decreased apoptosis of isolated islets from F344 rats in a dose-dependent manner (0-360 µM), and inhibited the decline of insulin function due to hypoxia and re-oxygenation that occurs during isolation (13). This anti-apoptotic effect may be useful for preserving viable and functional islets for transplantation, which is still the most important and effective approach for T1D treatment.

Hyperglycemia induces oxidative stress, which is thought to contribute to diabetic complications (74). EGCG can scavenge free radicals and inhibit apoptosis in human keratinocytes exposed to ultraviolet light by inhibiting transcriptional factor NFκB nuclear translocation and interleukin-6 secretion (75). In contrast, administration of EGCG at 100 µM in mouse adipocyte culture stimulated intracellular reactive oxygen species release, which in turn activated AMP-activated kinase that led to cell apoptosis (76). It was recently shown that at nanomolar concentrations, EGCG acted as a pro-oxidant in isolated rat β-cells (74). EGCG (200 µg/ml) significantly reduced expression of interleukin-1β and interferon-γ in RINm5F cells, a rat β-cell line, which may be beneficial to T1D, seeing as EGCG attenuated inflammatory molecules (77). Nevertheless, the doses of green tea or EGCG used in most of these studies capable of

achieving either an anti-oxidant or a pro-oxidant effect are far beyond those physiologically achievable through dietary consumption (37, 75, 76, 78). It is not clear whether existing oxidative status and concentration of green tea given determine how green tea affects oxidation.

Ahmad et al. (22) found that 1 mM EC significantly increased insulin secretion in 1 month old isolated rat islets, compared to 12 month old rat islets, when stimulated with 2 and 20 mM glucose. It is important to note that 1 mM EC used is 1000 times the physiologically attainable concentration in humans via tea drinking. Additionally, EC significantly stimulated conversion of proinsulin (the precursor to insulin made in  $\beta$ -cells) to insulin in 1 month old islets, compared to 12 month old islets (22). However, it is unknown if EGCG mediates rapid insulin secretion in the islets.

While there are some studies showing the effects of EGCG on  $\beta$ -cells, *in vitro* studies regarding the insulin-potentiating effects of green tea or EGCG on  $\beta$ -cells are deficient.

### **Animal studies**

Wu et al. (14) fed standard rat chow and either water or 0.5% green tea to Sprague-Dawley (SD) rats. After 12 weeks (wk), an oral glucose tolerance test (OGTT) revealed that blood glucose and plasma insulin levels were significantly lower in green tea-treated rats compared to controls, whereas body weights and food intakes did not differ between treatment and control rats, suggesting that this green tea effect was not due to alterations of these traits. Additionally, in green tea-treated rats, plasma free fatty acids and triglycerides significantly decreased; isolated adipocytes displayed significantly

increased glucose uptake in green tea-fed rats, suggesting that green tea may increase insulin sensitivity, at least in fat tissues (14). However, it was not precisely understood how green tea exerted such hypoglycemic effects. In addition, it was unclear which component of green tea primarily contributed to this beneficial effect.

Cytokines produced from immune cells have been implicated in the development of diabetes, and some cytokines are elevated in STZ-induced diabetic animals (77, 79). In a recent study, Vinson and Zhang (8) examined the effects of 1.25% green and black teas given to STZ-induced diabetic rats in water for 3 months. Both tea groups displayed significantly lower blood glucose; the green tea group displayed significantly lower plasma triglycerides. The hypoglycemic effect in both tea groups slowed the progression of diabetic cataracts (8). The STZ-induced diabetes model is a widely used human T1D model caused by selectively destroying the islet  $\beta$ -cells, suggesting that green tea or EGCG may prevent diabetes and resultant complications in animals by directly targeting  $\beta$ -cells; however, this remains to be determined.

There is increasing evidence demonstrating that oxidative stress and reactive oxygen species play a potential role in the initiation of diabetes (74, 77, 79). Green tea extracts exhibited anti-oxidant activity, which may alleviate diabetes, although the results are controversial (75, 77, 80). Research has suggested that catechins prevent inflammatory reactions and toxicity, and can scavenge free radicals in male, streptozotocin (STZ)-induced diabetic SD rats (80). Dietary supplementation (0.25% and 0.5%) of green tea catechins for 4 wk in STZ rats dose-dependently inhibited the production of superoxide in the kidney and activity of polymorphonuclear leukocyte 5'-

lipoygenase, and thereby leukotriene B<sub>4</sub> production, indicating reduced inflammation (80). These data demonstrate a protective effect of green tea on renal oxidative damage.

Igarashi et al. (15) found that T2D Goto-Kakizaki rats that were fed 0.2% Polyphenon E (a green tea extract containing 65% EGCG) in their diet for 49 days had significantly lower blood glucose levels than control rats as determined by a glucose tolerance test, but not serum insulin levels. However, similar results were not observed during a second OGTT after 74 days of treatment. It was thought that due to the age of the rats (18 wk old) and progression of disease, by the second OGTT insulin sensitivity may have been too impaired to respond well to treatment. Consistent with these findings, Wolfram et al. (11) found that EGCG dose-dependently (0.25%, 0.5%, 1%) improved oral glucose tolerance in 14 wk old obese diabetic mice that had received dietary EGCG supplementation for 6 wks. After 6 wk of treatment, an intraperitoneal (ip) insulin tolerance test was done, and mice that had been given 1% EGCG displayed blood glucose levels that did not rebound as well as those of control mice did (within 1-3 hrs), suggesting that long-term EGCG consumption decreased endogenous glucose production. Additionally, plasma insulin concentrations significantly increased in 1% EGCG-treated mice, but food intake was not altered, suggesting that EGCG supplementation did not improve glucose tolerance by altering food intake. Due to the high purity of the specific EGCG extract utilized (>94% EGCG), it was unlikely that caffeine played a role in enhanced glucose-stimulated insulin secretion (GSIS) by EGCG (11). Wolfram et al. found that EGCG significantly increased expression of glucokinase, a gene involved in glycolysis, in the liver tissue of obese diabetic mice fed 1% EGCG- supplemented diet (11). Increases in glycolysis genes may account for the significant reduction of blood

glucose in these mice, and suggests that EGCG may alleviate hyperglycemia in diabetic animals by improving glucose metabolism. Consistently, a study using STZ-induced diabetic rats found that dietary supplementation of 0.5% green tea prevented hyperglycemia and increased plasma insulin (16).

Potenza et al. (81) found that, in 9 wk old spontaneously hypertensive rats, 200 mg/kg/day EGCG for 3 wk improved insulin sensitivity, increased fasting plasma insulin concentrations and normalized blood glucose levels with parallel reductions in body weight and food intake. In addition, EGCG reduced systolic blood pressure, improved cardiac function, and increased plasma adiponectin levels in spontaneously hypertensive rats, indicating that EGCG can alleviate multiple metabolic syndromes (81). Consistent with the above study in which EGCG altered food intake, Kao et al. (23) found that both SD rats and obese Zucker rats consumed ~50% less food compared to controls (determined not to be leptin-receptor dependent), after ip injection of >98% pure EGCG (85 and 92 mg/kg BW, respectively), for 7 days. In SD rats, blood glucose and plasma insulin significantly decreased after ip injection, but not after oral administration, of EGCG for 7 days, which may have been due to the low bioavailability of EGCG (38, 39). Blood glucose and plasma insulin decreased significantly in obese Zucker rats after 4 days of ip injection (23). Longer-term (>7 days) oral ingestion of EGCG may exert beneficial effects. Although blood glucose and plasma insulin decreased in both lean and obese rats due to injection of EGCG, it is important to note that ip injection is different from the method of green tea or EGCG consumption in humans (ie, tea drinking). Therefore, differences in methods of administration must be considered when extrapolating results to human relevance (23).

## **Human studies**

Several cohort studies revealed that in Europe and America, where coffee is a large caffeine source, higher intake of coffee is associated with decreased diabetes risk (82-84). In Japan, the major source of caffeine is green tea (85). Iso et al. (18) conducted a study in which over 17,000 healthy Japanese subjects (male and female) completed a questionnaire regarding tea intake and the relationship to T2D, following 5 years of a baseline questionnaire. Higher consumption of either green tea (6+ cups/day), or coffee consumption (3+ cups/day) was correlated with decreased risk for diabetes, but there was no decreased diabetes risk from drinking black or oolong teas, which have higher caffeine concentrations than green tea (18). While caffeine may have an anti-diabetic effect via the mechanisms that include increased basal energy expenditure (86), fat oxidation, muscle glycogen mobilization (87), and increased lipolysis from peripheral tissue (86), these results suggest that caffeine is not the primary component in green tea that lowers the risk of diabetes. Indeed, studies found that highly purified green tea extracts improved diabetic symptoms in obese diabetic mice, which acted through a pathway independent of caffeine (11). However, the major component in tea extract that exerted such a beneficial effect is not clear.

Hase et al. (17) found that in healthy Japanese subjects, long-term consumption of an EGCG-concentrated green tea supplement (300 mg EGCG) for 12 wk significantly reduced blood glucose and plasma insulin levels. However, subjects who consumed a lower dose green tea supplement did not display lower blood glucose, but lower plasma

insulin levels. The dose of EGCG utilized is more than the amount found in a typical cup of green tea (150-200 mg), but with continual green tea consumption throughout a day, the same effects may occur (17). In contrast to the above long-term study, healthy, Japanese subjects consumed 1.5 g green tea extract in water prior to an oral glucose load as part of a short-term study. Tea extract significantly decreased blood glucose levels. The results indicated that an acute, higher dose of green tea may control postprandial hyperglycemia in healthy individuals, thus potentially reducing the risk for diabetes (21).

Whereas the above studies demonstrated a potential anti-diabetic effect of green tea, other studies revealed that green tea and/or EGCG had no significant effect on human diabetes. In a long-term study performed by Mackenzie et al. (19), T2D adults who consumed green tea extract (375 or 750 mg) did not exhibit a hypoglycemic effect (19). The presence of theaflavins in the extract may have displaced important glucose-lowering effects from green tea catechins. Further, the subjects displayed relatively good baseline blood glucose levels; therefore, the glucose-lowering effect from green tea catechins may not have been as strong as it would have been in subjects with poor baseline blood glucose levels. Consistent with these findings, Ryu et al. (20) found no changes in blood glucose levels, insulin resistance, or markers of inflammation after T2D patients consumed green tea (9 g green tea in water) for 4 wk. Therefore, results have been shown to either support or negate the claim that green tea or EGCG acts as an anti-diabetic agent in humans.

Although green tea catechins increase insulin secretion both *in vitro* and *in vivo* (8, 11, 14, 15, 22, 81), some results from these studies reflect a pharmacological effect of green tea, and some involved directly injecting catechins into the bloodstream.



## SUMMARY

While it is unclear how green tea exerts beneficial effects on human health, some recent studies suggest that EGCG may play a role in improving health, especially diabetes mellitus. Although *in vitro* studies regarding EGCG and insulin secretion are lacking, in rat and human  $\beta$ -cells, EGCG protected cells against apoptosis (12, 13), reduced expression of inflammatory molecules (77), and down-regulated genes involved in gluconeogenesis (11). Animal studies have shown that both healthy and diabetic rodents given various concentrations of green tea in various forms displayed improved glucose metabolism and improved insulin profiles (8, 11, 14, 15, 81). However, variability in animal models utilized, dosage, preparation of green tea or EGCG, length of study, and method of administration differed among studies. Human studies have provided data that support, as well as negate, the acclaimed anti-diabetic effects of green tea (17-21). Due to these results, as well as a lack of mechanistic studies, the beneficial effects of green tea/EGCG on diabetes remain inconclusive.

In the present study, I tested the hypotheses that 1) EGCG, at physiologically attainable concentrations via tea drinking ( $\leq 1 \mu\text{M}$ ), induces insulin secretion and synthesis from  $\beta$ -cells; 2) physiologically attainable doses of EGCG (50 mg/kg BW or 150 mg/kg BW) via gavage decrease blood glucose in healthy SD rats; 3) EGCG administration (0.1% or 0.5% in drinking water) for 12 days decreases blood glucose, increases insulin secretion, and increases insulin synthesis in healthy SD rats.

## **SIGNIFICANCE OF STUDY**

About 21 million Americans suffer from diabetes (1), and each year hereafter, it is estimated that 2-4 million new cases of diabetes will be diagnosed (2). In both T1D and T2D, inadequate  $\beta$ -cell mass, along with  $\beta$ -cell dysfunction, lead to impaired insulin secretion, and ultimately worsen glycemic control (3). Thus, safe and cost-effective compounds that negate these changes could be useful in both preventing and alleviating diabetes. This study was designed to evaluate the effects of physiologically relevant doses of EGCG on insulin secretion by using clonal  $\beta$ -cells (INS1E), and rat and human pancreatic islets. In addition, we assessed both rapid (acute EGCG administration) and relative chronic (12 day EGCG administration) effectiveness of physiologically relevant low and high doses of EGCG on glucose levels, insulin secretion, and insulin *synthesis* (which has not yet been reported) in healthy rats. The long term goal of this research is to identify low cost and effective nutritional compounds that could be used to prevent and treat diabetes.

## MATERIALS AND METHODS

**Reagent and materials.** RPMI-1640 media (RPMI) was purchased from Sigma-Aldrich (St. Louis, MO), CMRL-1066 media (CMRL) was from Mediatech, Inc. (Herndon, VA), heat-inactivated (HI) fetal bovine serum (FBS) was obtained from HyClone (Logan, UT) and medium supplements from Invitrogen (Carlsbad, CA). EGCG (95% pure) for *in vitro* studies was purchased from Sigma-Aldrich. Stock solutions of EGCG at 20 mM were dissolved in sterilized water and stored at -80 °C before use. Sunphenon<sup>®</sup> EGCG (>90% pure, <1% caffeine) for *in vivo* studies was purchased from Taiyo International, Inc. (Minneapolis, MN). Ultrasensitive rat insulin enzyme-linked immunosorbent assay (ELISA) kits were obtained from Mercodia, (Winston-Salem, NC). All other chemicals were from Sigma-Aldrich. Glucose was dissolved in sterile water and stored at -80 °C.

**Cell and islet culture.** INS1E cells (a kind gift from Dr. Pierre Maechler, University of Geneva, Switzerland) were cultured in RPMI-1640 medium (11.1 mM glucose, 10% HI FBS, 1 mM sodium pyruvate, 10 mM HEPES, 2 mM L-glutamine, 50 µM β-mercaptoethanol, and 100 units/ml penicillin/streptomycin) and maintained at 37°C (88). Medium was changed every 2-3 days until cells were approximately 70% confluent. Human islets were isolated from cadaver organ donors in the Islet Cell Resource Centers at Southern California Resource Center & Southern California Islet Consortium at National Medical Center (Duarte, CA), Washington University (St. Louis, MO), the University of Minnesota (Minneapolis, MN), the University of Miami (Miami, FL), University of Illinois at Chicago (Chicago IL), University of Pennsylvania (Philadelphia,

PA), University of Alabama (Tuscaloosa, AL), and Joslin Diabetes Center. Human islets were maintained in CMRL containing 10% HI FBS at 37°C. Rat islets were isolated as previously described (89). Briefly, pancreases were disrupted by injection of collagenase (0.5 ml in Hank's Buffered Salt Solution [HBSS]) into the common bile duct after occlusion of the distal end, close to the duodenum (collagenase P, Roche, Indianapolis, IN). Digestion was performed in a water bath at 37 °C for 20 min, and was halted with addition of cold HBSS. The digested tissues were washed twice by centrifugation (HBSS, 290 x g, 2 min, 4°C). Islets were then separated by centrifugation (HBSS/1.083 g/ml, 290 x g, 20 min, 4°C) and cultured in RPMI containing 10 % HI FBS and 11.1 mM glucose in humidified 5 % CO<sub>2</sub> at 37 °C (89).

**GSIS assay.** For determining the effect of EGCG on rapid insulin secretion, INS1E cells were cultured in a 24-well plate in RPMI containing 11.1 mM glucose and 10% HI FBS at 37°C for 48 hrs, then replaced with RPMI containing 5.5 mM glucose and 5% HI FBS for 96 hrs. Cells were washed with Krebs-Ringer bicarbonate buffer (KRBB; 129 mM NaCl, 4.8 mM/KCl, 1.2 mM MgSO<sub>4</sub>, 1.2 mM KH<sub>2</sub>PO<sub>4</sub>, 2.5 mM CaCl<sub>2</sub>, 5 mM NaHCO<sub>3</sub>, 0.1% BSA, and 10 mM HEPES, pH 7.4) and incubated in RPMI containing 1 mM glucose and 1% HI FBS at 37°C for 30 min. Cells were washed again with KRBB and adapted to KRBB containing 3 mM glucose at 37°C for 30 min, followed by stimulation with 0.1, 1 or 5 μM EGCG in either 3 or 20 mM glucose at 37°C for 30 min. Supernatants were collected and centrifuged (16,100 x g [13,200 rpm], 2 min). Cells were exposed to 100 μl/well of lysis buffer (20 mM Tris, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton x-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerolphosphate, and 1 mM Na<sub>3</sub>VO<sub>4</sub>), and lysates were sonicated and centrifuged

(16,100 x g [13,200 rpm], 2 min) for measuring protein content. For determining the chronic effect of EGCG on insulin secretion and content, cells were incubated with 0.1, 1 or 5  $\mu$ M EGCG in RPMI containing 5.5 mM glucose and 5% HI FBS at 37°C for 48 hrs. Supernatants were collected for measuring insulin. After this procedure, islets were lysed as described above and protein concentrations were determined using a Bio-Rad kit. Insulin levels were normalized to protein concentrations from the same sample. Insulin in the supernatants and lysates was measured by ELISA.

To determine the effect of EGCG on insulin secretion and content from isolated rat islets from the animal study, islets were centrifuged (197 x g [1000 rpm], 1 min) in RPMI containing 11.1 mM glucose and 10% HI FBS, washed with KRBB, and adapted to KRBB containing 3 mM glucose at 37°C for 30 min. Then, the islets from each group were washed with KRBB, seeded in a 24-well plate (~200 islets/well), and stimulated with either 3 or 20 mM glucose at 37°C for 40 min. Supernatants were collected and centrifuged (2,300 x g [5000 rpm], 5 min). For determining the effect of EGCG on rapid insulin secretion, rat islets were maintained overnight in RPMI containing 11.1 mM glucose and 10 % HI FBS at 37°C, centrifuged (197 x g [1000 rpm], 1 min), washed with KRBB, and adapted in KRBB containing 3 mM glucose at 37°C for 30 min. Then, the islets were washed with KRBB, seeded in a 24-well plate (~100 islets/well), and stimulated with KRBB containing either 3 or 16.7 mM glucose  $\pm$  0.1, 1 or 5  $\mu$ M EGCG at 37°C for 40 min. Supernatants were collected for measuring insulin. After this procedure, islets were lysed as described above and protein concentrations were determined using a Bio-Rad kit. Insulin levels were normalized to protein concentrations from the same sample. Insulin in the supernatants and lysates was measured by ELISA.

Human islets were maintained overnight in CRML, adapted to RMPI containing 11.1 mM glucose and 10% HI FBS, seeded in a 24-well plate (~200 islets/well), and incubated at 37°C for 48 hrs, to allow islets to attach to the plate. For assessing the effect of EGCG on rapid insulin secretion, islets were centrifuged (197 x g [1000 rpm], 1 min) and adapted to KRBB containing 3 mM glucose at 37°C for 30 min. Then, islets were stimulated with either 3 or 16.7 mM glucose in the presence or absence of 0.1, 1 or 5 μM EGCG at 37°C for 40 min. Supernatants were collected and centrifuged (2,300 x g [5000 rpm], 5 min). To determine the chronic effect of EGCG on insulin secretion and content, human islets were incubated in the presence or absence 1 μM EGCG in RPMI containing 5.5 mM glucose and 5% HI FBS for 48 hrs. At the end, islets were adapted to KRBB containing 3 mM glucose for 30 min and then stimulated with either 3 or 16.7 mM glucose for 30 min. Supernatants were collected for measuring insulin. After this procedure, islets were lysed as described above and protein concentrations were determined using a Bio-Rad kit. Insulin levels were normalized to protein concentrations from the same sample. Insulin in the supernatants and lysates was measured by ELISA.

**Animal study.** Five-wk old, male SD rats were purchased from Harlan (Indianapolis, IN). Animals were housed in a room maintained on a 12 hr light/dark cycle under constant temperature (22–25° C) with *ad libitum* access to food and water. The protocol of this study was approved by the Institutional Animal Care and Use Committee at Virginia Polytechnic Institute and State University. After an initial acclimation period, rats were fed Teklad 2018 diet containing 18% protein and 5% fat, and given plain drinking water for 2 wk. Rats were randomly divided into 3 groups (low dose EGCG [LD], high dose EGCG [HD], and control) with 8 rats per group. Before EGCG was

administered, blood was drawn from a tail puncture of the lateral tail vein, and baseline blood glucose levels were measured using glucometers (Kroger, Cincinnati, OH) in rats fasted overnight. To determine whether acute administration of EGCG had an effect on glucose tolerance of animals, a bolus of glucose solution (2 g/kg BW) with or without EGCG (LD: 50 mg/kg BW, HD: 150 mg/kg BW) was administered via gavage feeding. Blood glucose levels were measured at 0, 15, 30, 60 and 120 min after glucose administration. To assess relative long-term effects of EGCG on blood glucose, after the initial OGTT, rats in treatment groups were given EGCG (LD: 0.1%, HD: 0.5%) in drinking water, and control rats received plain drinking water for 12 days. Body weight, food intake, and fluid intake were recorded. After 12 days, an OGTT was performed to evaluate glucose tolerance and islet function. Following glucose injection as above, blood glucose levels were measured at 0, 15, 30, 60 and 120 min. After this procedure, animals were euthanized for isolation of pancreatic islets for measuring GSIS and insulin content.

***In vitro* free radical scavenging activity assay.** Oxygen radical absorbance activity (ORAC) assay was conducted to measure the peroxy radical scavenging activity of EGCG, with Trolox as the antioxidant standard (90). Peroxy radicals were generated from 2, 2'-azobis (2-amino-propane) dihydrochloride 75 mM phosphate buffer (pH 7.4). Fluorescence was monitored by a plate reader; reactions of peroxy radicals with fluorescein resulted in loss of its fluorescence (Perkin-Elmer, Turku, Finland).

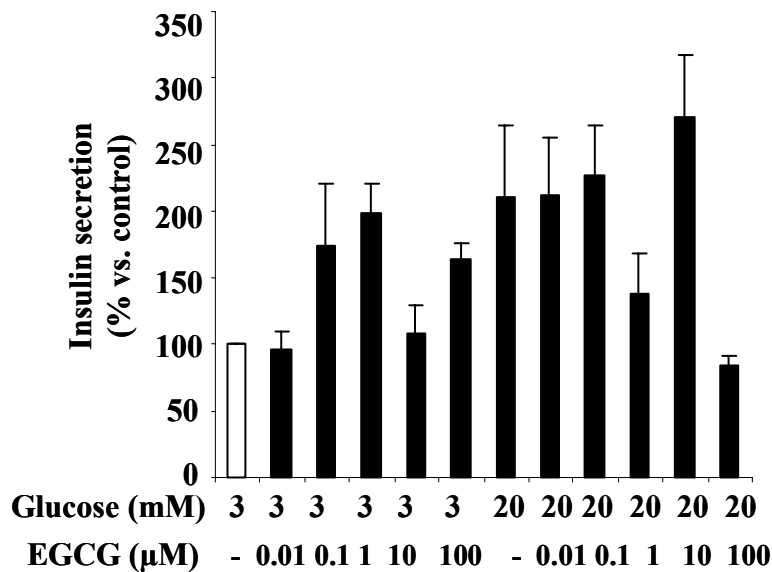
**Viability assay.** Human islets (~200 islets/well) were cultured in RPMI containing 5.5 mM glucose and 5% HI FBS in the presence or absence of 1  $\mu$ M EGCG for 48 hrs. The number of viable islet cells was assessed using a CellTiter 96 aqueous assay kit.

**Statistical Analysis.** Data were analyzed using one-way analysis of variance ( $Y_{ij} = \mu + \alpha_i + \epsilon_{ij}$ ) in JMP (statistical analysis software produced by the makers of SAS, Cary, NC) and expressed as means  $\pm$  standard error (SE). Treatment differences were subjected to Tukey's-HSD test. Statistical significance was determined at  $P < 0.05$ .



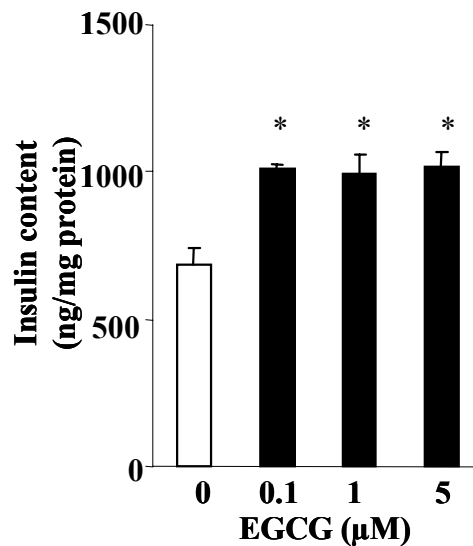
## RESULTS

**Effects of EGCG on rapid insulin secretion in INS1E cells.** We examined whether EGCG stimulated rapid GSIS from INS1E cells, a stable rat  $\beta$ - cell line. As shown in Fig. 4, EGCG did not significantly induce GSIS in INS1E cells. Average concentration of insulin secretion at 3 mM glucose was  $10.9 \pm 3.5$  ng/mg protein (n=3).



**Fig.4. Effects of EGCG on rapid insulin secretion in INS1E cells.** INS1E cells were incubated in KRBB with various concentrations of EGCG in the presence of 3 or 20 mM glucose at 37°C for 30 min. Insulin secreted into supernatants was measured by ELISA. Values were expressed as mean  $\pm$  SE (n=3).

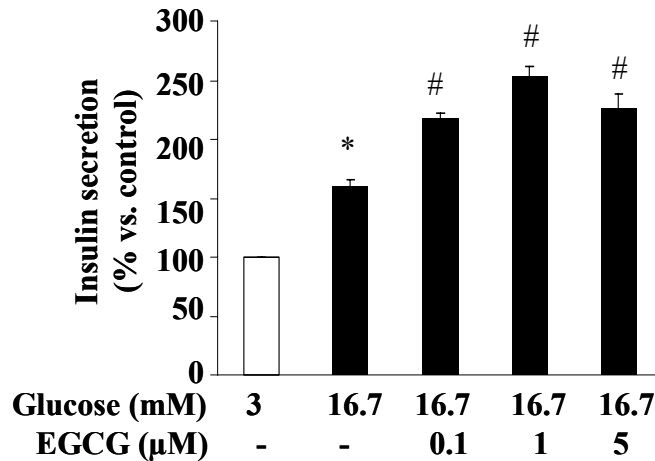
**Effects of long-term EGCG exposure on insulin content in INS1E cells.** We examined whether EGCG stimulated increased insulin synthesis in INS1E cells after long-term incubation. As shown in Fig. 5, the addition of 0.1, 1, and 5  $\mu$ M EGCG significantly increased insulin synthesis by 327.5%, 312.6%, and 337.8%, respectively (n=5).



**Fig.5. Effects of long-term EGCG exposure on insulin content in INS1E cells.** Cells were incubated in RPMI (5.5 mM glucose, 5% FBS) with various concentrations of EGCG. 48 hrs later, cells were lysated with lysis buffer and insulin was measured by ELISA. Values were expressed as mean  $\pm$  SE (n=5). \*,  $p < 0.05$  vs. control.

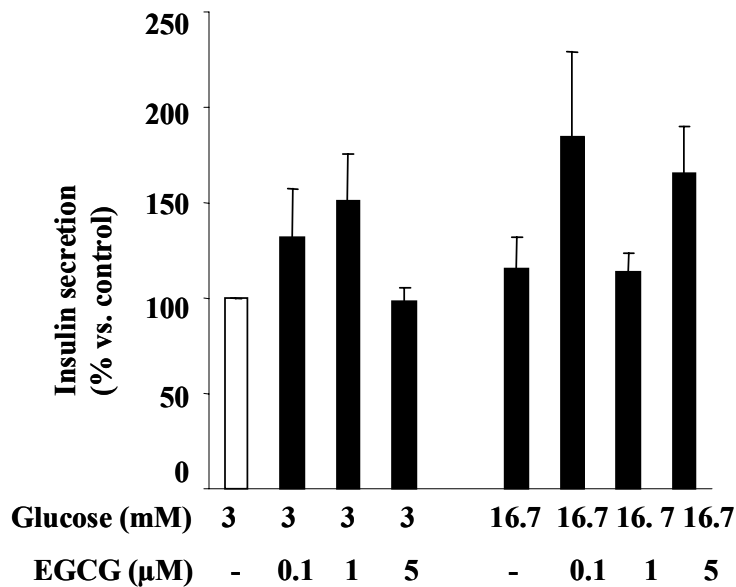
**Effects of EGCG on rapid insulin secretion in rat islets.** We next evaluated whether EGCG stimulated rapid GSIS from rat islets. As shown in Fig. 6, incubation of the islets with 16.7 mM glucose significantly increased insulin secretion by 59.8%, compared to 3

mM glucose ( $P < 0.05$ ), ( $n = 7$ ). Compared to 16.7 mM glucose, addition of 0.1, 1 and 5  $\mu\text{M}$  EGCG for 40 min significantly increased GSIS by 58.2%, 93.2%, and 66.5%, respectively ( $P < 0.05$ ), (Fig. 6).



**Fig.6. Effects of EGCG on rapid insulin secretion in rat islets.** Islets (100 islets/well) were incubated in KRBB with various concentrations of EGCG in the presence or absence of 3 mM or 16.7 mM glucose for 40 min at 37°C. Insulin secreted into the supernatants was measured by ELISA. Values were expressed as mean  $\pm$  SE ( $n = 7$ ). \*,  $p < 0.05$  vs. 3 mM glucose-treated islets; #,  $p < 0.05$  vs. 16.7 mM glucose alone-treated islets.

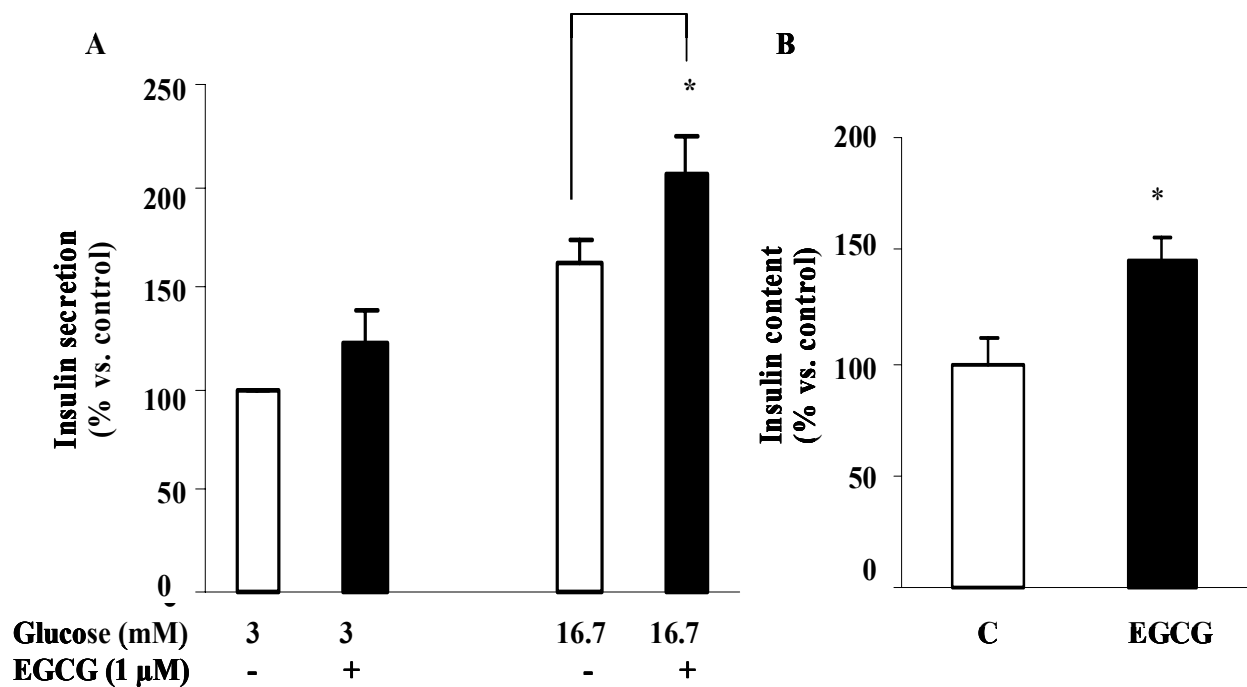
**Effects of EGCG on rapid insulin secretion in human islets.** To determine whether EGCG stimulated rapid GSIS from human islets, islets were incubated with EGCG in the presence of basal or high glucose for 40 min. EGCG (0.1, 1 or 5  $\mu\text{M}$ ) had no significant effect on GSIS from human islets at basal or high glucose (Fig. 7). Average concentration of insulin secretion at 3 mM glucose was  $25.2 \pm 5.1$  ng/mg protein ( $n = 7$ ).



**Fig.7. Effects of EGCG on rapid insulin secretion in human islets.** Islets (200/well) were incubated in KRBB with various concentrations of EGCG in the presence or absence of 3 mM or 16.7 mM glucose for 40 min at 37°C. Insulin secreted into the supernatants was measured by ELISA. Values were expressed as mean  $\pm$  SE (n=7).

### **Effects of long-term EGCG exposure on insulin secretion and content in human**

**islets.** We determined whether EGCG stimulated GSIS and insulin synthesis from human islets, after long-term incubation. As shown in Fig. 8A, islets significantly increased GSIS by 45.1% at 16.7 mM glucose when incubated with 1  $\mu$ M EGCG, compared to 16.7 mM glucose alone ( $P<0.05$ ). However, no significant increase occurred at 3 mM glucose with addition of 1  $\mu$ M EGCG, compared to 3 mM glucose alone. As shown in Fig. 8B, insulin synthesis significantly increased, by 52%, with the addition of 1  $\mu$ M EGCG, compared to the control ( $P<0.05$ ), (n=5).



**Fig.8. Effects of long-term EGCG exposure on insulin secretion and content in human islets.** Islets (200/well) were cultured in RPMI (5.5 mM glucose, 5% FBS) for 48 hrs in the presence or absence of 1 μM EGCG. Then, islets were incubated in KRBB (3 mM glucose) for 30 min before stimulation with 3 or 16.7 mM glucose in KRBB for 30 min at 37° C. Insulin secreted into the supernatants (A) and within the islets (B) was measured by ELISA. Values were expressed mean ± SE (n=5). \*,  $p < 0.05$ .

**Effects of EGCG supplementation on glucose tolerance, insulin secretion and**

**synthesis in rats.** After acute EGCG administration at both LD (50 mg/kg BW) and HD

(150 mg/kg BW), we did not find any significantly lower blood glucose levels between

groups (Fig. 9A). After administering LD (0.1%) and HD (0.5%) EGCG

supplementation in drinking water for 12 days to healthy rats, we did not find any

significantly lower blood glucose levels between groups (Fig. 9B), (n=8). We evaluated,

*in vitro*, the interactions between 3 or 20 mM glucose and isolated islets from healthy rats

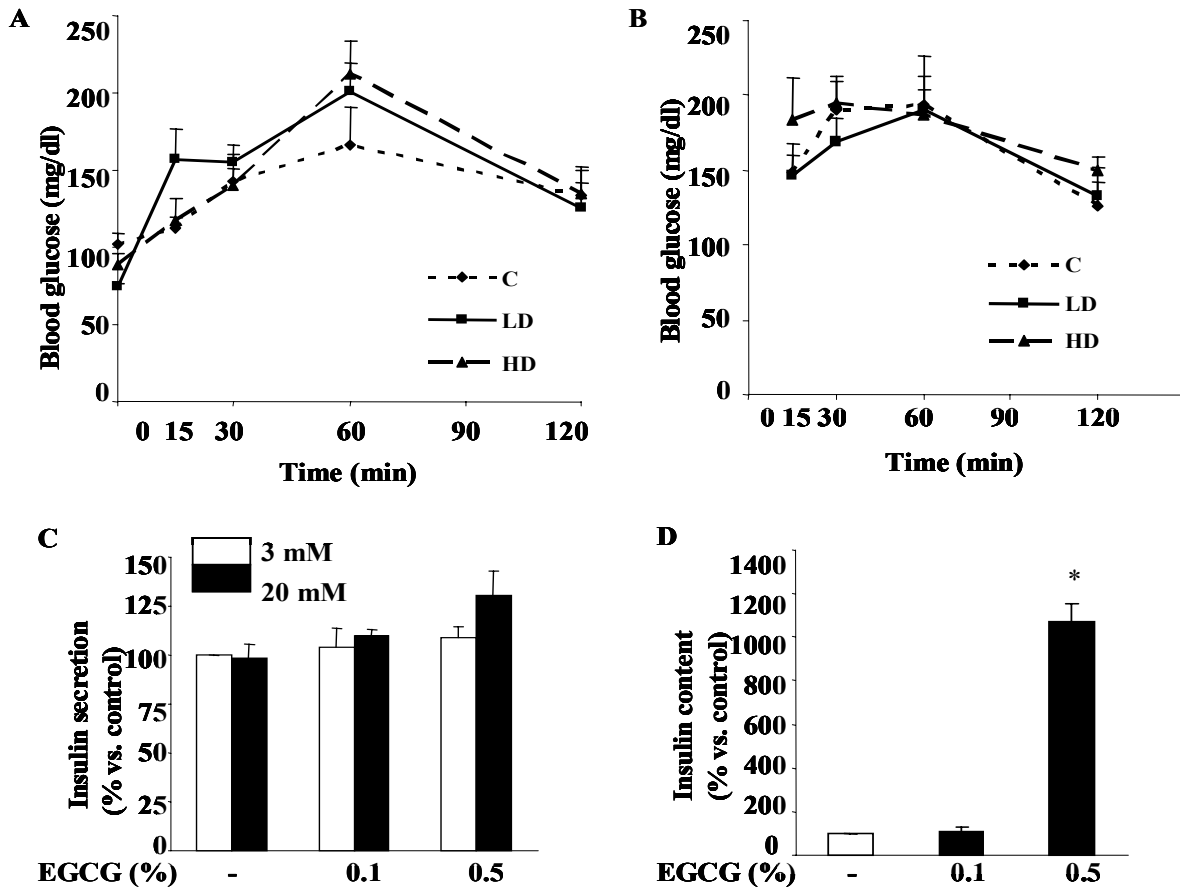
that consumed LD (0.1%) or HD (0.5%) EGCG in drinking water for 12 days. No

increases in insulin secretion occurred in islets from rats given 0.1% or 0.5% EGCG,

when stimulated with 3 mM or 20 mM glucose (Fig. 9C). Average concentration of

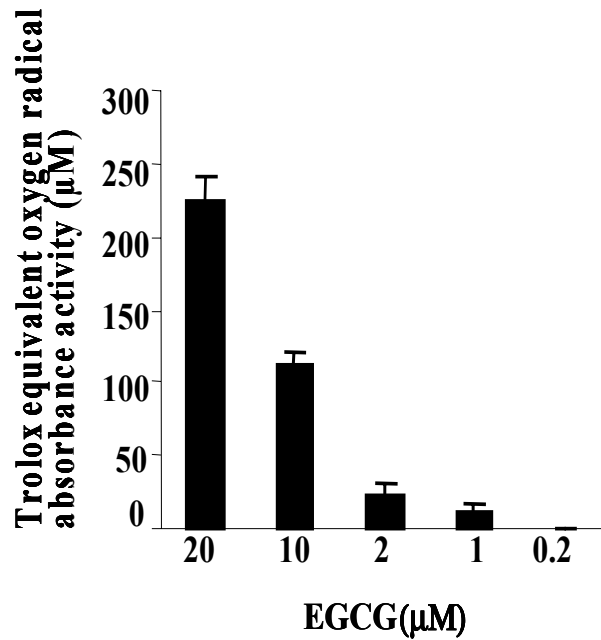
insulin secretion from the control group was  $8.2 \pm 0.4$  ng/mg protein, while insulin

secretion from the 0.5% group was  $9.9 \pm 0.8$  ng/mg protein. The same samples were used to obtain insulin concentration from inside rat islets; that is, insulin synthesis. As shown in Fig. 9D, 0.5% EGCG supplementation significantly increased insulin synthesis ( $P < 0.05$ ) inside rat islets by 978.2% compared to the control group, but no such effect occurred with 0.1% supplementation. Average concentration of insulin content from the control group was  $0.2 \pm 0.02$  ng/mg protein, while insulin content from the 0.5% group was  $1.4 \pm 0.2$  ng/mg protein ( $n=4$ ).



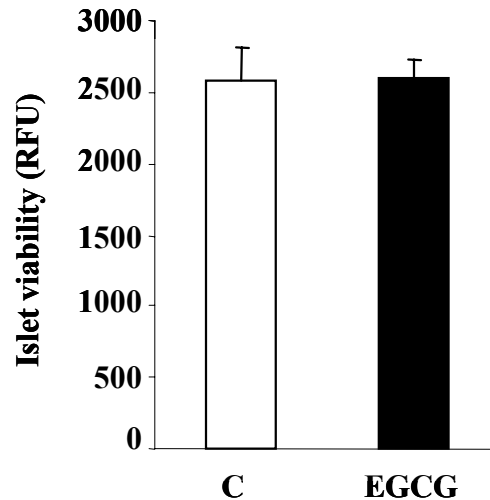
**Fig.9. Effects of EGCG supplementation on glucose tolerance, insulin secretion and synthesis in rats.** (A) Fasting blood glucose levels were measured at 0,15, 30, 60 and 120 min following acute EGCG administration via gavage (control, LD [50 mg/kg BW EGCG], or HD [150 mg/kg BW EGCG]) ( $n=8$ ). (B) Fasting blood glucose levels were measured at 0, 15, 30, 60 and 120 min following 12 days of EGCG supplementation of 0 (control), 0.1% (LD), or 0.5% (HD) ( $n=8$ ). (C, D) Islets (200/well) were incubated in KRBB with 3 or 20 mM glucose for 40 min at 37°C. Insulin secreted into the supernatants (C) and within the islets (D) was measured by ELISA. Values were expressed as mean  $\pm$  SE ( $n=4$ ). \*,  $p < 0.05$  vs. control at 3 mM glucose.

**Free radical scavenging activity of EGCG.** Our data showed that at  $<2 \mu\text{M}$ , EGCG did not induce significant free radical scavenging activity (Fig. 10). However, at pharmacological doses ( $10+ \mu\text{M}$ ) EGCG exerted free radical scavenging activity ( $n=2$ ).



**Fig.10. Free radical scavenging activity of EGCG.** Oxygen radical absorbance activity assay was conducted to measure the peroxy radical scavenging activity of EGCG with Trolox as the antioxidant standard. Peroxy radicals were generated from 2, 2'-azobis (2-amino-propane) dihydrochloride 75 mM phosphate buffer (pH 7.4). Fluorescence was monitored by a plate reader. Values were expressed as mean  $\pm$  SE ( $n=2$ ).

**Effects of EGCG on human islet viability.** Results showed that 1  $\mu$ M EGCG had no effect on the islet viability (Fig. 11). This indicated that at the dose used in the insulin secretion study, chronic EGCG exposure improves insulin secretion and synthesis via a mechanism that is likely not related to the islet viability (n=3).



**Fig.11. Effects of EGCG on human islet viability.** Human islets (200 islets/well) were cultured in RPMI (5.5 mM glucose, 5% FBS) in the presence or absence of 1 $\mu$ M EGCG for 48 hrs. The number of viable islet cells was assessed using a CellTiter 96 aqueous assay kit. Values were expressed as mean  $\pm$  SE (n=3).



## DISCUSSION

EGCG or green tea is a widely used dietary supplement and beverage. Recent studies show that green tea, and specifically EGCG, may exert beneficial effects on many diseases including diabetes mellitus. While the results from human studies evaluating the effectiveness of EGCG on diabetes are inconsistent, an array of animal studies has shown that EGCG can improve insulin sensitivity and glucose metabolism (8, 11, 14, 15, 81). However, how EGCG exerts anti-diabetic effects is still not clear. In the present study, we found that physiologically relevant doses of EGCG significantly increased rapid insulin secretion in rat islets (Fig. 6) and long-term insulin secretion in human islets (Fig. 8A), although it did not significantly increase rapid or long-term insulin secretion in INS1E cells (Fig. 4). However, chronic exposure of INS1E cells (Fig. 5), and rat and human islets (Fig. 9D, Fig. 8B) to EGCG significantly increased insulin synthesis, with 0.1  $\mu$ M already inducing a maximal effect. The results observed in INS1E cells and human islets were confirmed with the animal study, suggesting that physiologically relevant concentrations of EGCG may have anti-diabetic implications by directly acting on  $\beta$ -cells to induce insulin synthesis. This EGCG effect on  $\beta$ -cells is not dependent on its potential effects on antioxidant status or cell viability; rather, it is through a mechanism that has yet to be determined.

The reported plasma concentration of EGCG in both humans and rodents through dietary supplementation is usually less than 1  $\mu$ M (37, 42, 44). To consider the potential biological relevance of the observed effects of EGCG on  $\beta$ -cell insulin secretion, we used EGCG concentrations in the present study that are comparable to the physiologically achievable levels through dietary means. In our animal study, 0.1% and 0.5% of EGCG

in drinking water equate to approximately 27.5 mg and 137.5 mg per day, respectively, considering the amount of tea that the rats consumed per day (approximately 60 ml per day). The concentration of EGCG given and the amount of EGCG consumed per day overlaps with those amounts used in both animal and human studies and the concentrations of EGCG found in green tea beverages. Therefore, 0.1% was undoubtedly within the physiological range, while 0.5% was close. Additionally, the amounts of EGCG used in the gavage part of the animal study (50 mg/kg BW and 150 mg/kg BW) were well within the physiological range.

We observed a significant increase in insulin content within INS1E cells when cells were stimulated with 0.1, 1, and 5  $\mu$ M EGCG for 48 hrs (Fig. 5). INS1E is a widely used  $\beta$ -cell line (91), and these cells display dose-dependent and rigorous insulin secretion between passages 40 and 100 in response to up to 20 mM glucose (88). These results were confirmed by the human islet study. We observed significantly increased insulin synthesis in human islets that were treated with 1  $\mu$ M EGCG for 48 hrs (Fig. 8B), and increased insulin secretion in response to stimulation of 16.7 mM glucose for 30 min (Fig. 8A). This indicated a beneficial effect of long-term EGCG exposure, and may suggest that continual consumption of green tea is more useful than a single, periodic dose. We also found that rat islets significantly increased insulin secretion when stimulated with 0.1, 1, and 5  $\mu$ M EGCG for 30 min in the presence of 16.7 mM glucose, compared to glucose alone (Fig. 6). This may be beneficial since *in vitro* conditions represented hyperglycemia, and 0.1  $\mu$ M is surely achievable through dietary means. Furthermore, 1  $\mu$ M EGCG may be achievable with continual consumption of green tea throughout the day.

To confirm if the *in vitro* findings that EGCG induces insulin secretion and synthesis also occur *in vivo*, we administered 0.1% and 0.5% EGCG in drinking water to healthy rats for 12 days. We found that 0.5% EGCG supplementation significantly increased insulin synthesis in isolated rat islets (Fig. 9D). To our knowledge, this effect has not been previously reported. The islets isolated from rats given 0.5% EGCG in drinking water for 12 days showed no increase in insulin secretion compared to controls (Fig. 9C). We initially gavaged EGCG (50 mg/kg BW or 150 mg/kg BW) to the rats, but this did not result in lower blood glucose levels (Fig. 9A). Although we did not observe decreased blood glucose levels after 12 days (Fig. 9B) of 0.1% or 0.5% EGCG in drinking water either, Wu et al. (14) found that 0.5% green tea in water decreased blood glucose and plasma insulin levels in SD rats when administered for 12 wk (14). In accordance with these results, Wolfram et al. (11) found that 0.5% EGCG supplementation in diet for 10 wk improved oral glucose tolerance in T2D rats. These studies differed from ours in that green tea was used instead of EGCG by Wu et al., and a more purified EGCG extract was used by Wolfram et al. The studies were much longer than ours (12 wk vs. 12 days). Thus, our study did not exert similar benefits in a short amount of time. It may be because 12 days was not long enough to observe any effects, or the fact that the rats were already healthy, thus leaving little room for any effects of EGCG on blood glucose.

Oxidative stress and reactive oxygen species played a potential role in the modulation of insulin secretion and the initiation of diabetes (74, 77, 79), and EGCG exhibited antioxidant activity (75, 77, 80). However, the antioxidant effect of EGCG is achieved only at concentrations over 10  $\mu$ M (Fig. 10), suggesting that EGCG is not a

physiologically effective antioxidant. This is because the achievable level of total plasma EGCG in both humans and rodents through dietary supplementation is usually no more than 1  $\mu\text{M}$  (37). Our study showed that EGCG exerted free radical scavenging activity at pharmacological doses (10 and 20  $\mu\text{M}$ ), which supports these reports. However, physiological relevant doses of EGCG (<2  $\mu\text{M}$ ) that were used in our studies did not exert free radical scavenging activity. Therefore, it is unlikely that the insulinotropic effect of EGCG is due to its potential antioxidant activity. However, these results have not been brought to light in human studies, and there are many differences between physiological actions between cell systems in rodents and humans. As some studies showed that EGCG has an effect on cell apoptosis (12, 13), we considered the possibility that enhanced insulin synthesis by EGCG might be due to its effect on cell viability. However, our studies excluded this possibility based on the observation that exposure of EGCG to  $\beta$ -cells or islets for 48 hrs, the same duration for some insulin secretion studies, had no effect on cell viability (Fig. 11), suggesting that the increased insulin synthesis by EGCG was not due to a change in cell apoptosis.

While antioxidant capacity and viability promotion are unlikely causes of enhanced insulin secretion and synthesis by EGCG, a proposed mechanism involves the cAMP pathway (65). A recent study suggested that cAMP signaling is important in normal pancreatic islet cell function (92), and that EGCG may improve glucose metabolism via changes in gene expression and promotion of fat oxidation in mice and humans (11). Additionally, although not proven in  $\beta$ -cells, EGCG was at least partially necessary to mediate beneficial effects through PKA in bovine aortic endothelial cells. However, it is unclear whether or not EGCG can activate PKA in  $\beta$ -cells, and whether

this will lead to insulin secretion and/or synthesis (93). Intracellular cAMP is converted from ATP by adenylate cyclase, which subsequently activates cAMP-dependent protein kinase, PKA. PKA contains 2 regulatory and 2 catalytic subunits, and binding of cAMP to PKA regulatory subunits allows the catalytic subunits to separate from the complex and translocate to the nucleus where it phosphorylates cAMP response element-binding protein (CREB), a nuclear transcription factor, on a single serine. CREB then binds to cAMP-response element, recruits CREB-binding protein, and together they regulate cAMP-mediated gene transcription (94). Thus, cAMP affects rapid insulin secretion by positively regulating insulin gene expression, given that there are the cAMP-response element sites within the promoter region of insulin gene. Although it is known that cAMP plays an important role in GSIS both in clonal and primary  $\beta$ -cells (92), it is unknown if EGCG elevates cAMP levels, thereby mediating EGCG-induced rapid insulin secretion and/or synthesis in  $\beta$ -cells. Therefore, this pathway should be studied further.

As discussed above, some studies have utilized pharmacological doses of EGCG that are not attainable through drinking tea, or have administered the compound via injection, which is not practical for human use (22, 23). The rather low bioavailability of EGCG must be taken into account when extrapolating *in vitro* experiment results to human relevance. For example, most ingested EGCG does not enter the blood stream and reach target tissues; rather, it is excreted through bile. Therefore, a dosage of EGCG that produces results *in vitro* may not do the same *in vivo* (39). It may be most beneficial for humans to consume continuous green tea throughout the day for maximum, constant exposure to catechins, and it is not uncommon for certain populations to consume up to 20 cups of green tea per day (95). It was reported that at least 6 cups of green tea (200

ml/cup) must be consumed to achieve a human plasma concentration of EGCG of 1  $\mu$ M, and that a 200 ml cup of green tea would contain 200 mg catechins, including 88 mg EGCG (42). Of this amount, only 2% of ingested EGCG may be detected in plasma (40).

It is important to consider EGCG supplement safety, especially since some studies have administered EGCG at high doses. It was shown that is it safe for healthy individuals to consume green tea polyphenol products in amounts equivalent to the EGCG content in 16 cups of green tea (800 mg EGCG or polyphenon E) one time per day, for 4 wk. This amount resulted in >60% increase in the area under the curve of free EGCG, and it should not accumulate in the body due to the observed short half-life of EGCG, ranging from 2-5 hrs (39, 47, 48). However, subjects experienced undesirable gastrointestinal side-effects, as well as headaches and muscle pain (95). The safety of Teavigo, a highly concentrated EGCG extract, was also evaluated. In rats and guinea pigs, an oral dose of 2000 mg/kg BW EGCG was lethal to rats, but 200 mg/kg BW EGCG was not toxic. When dietary EGCG was given to rats for 13 wk, no toxicity occurred until 500 mg/kg/day was administered. This dosage, when given in 2 divided amounts, was not lethal to dogs, but was lethal when given in a single dose (96). Therefore, although some high-dose EGCG supplements have been shown safe in humans and animals when consumed for a specific length of time, long-term studies regarding the safety of chronic EGCG supplement usage should be performed.

Caffeine can lower insulin sensitivity in healthy humans, thus actually contributing to insulin resistance, when consumed in doses of moderate amounts (~200 mg/day) (97, 98). However, it is possible that due to an acquired tolerance to the effects of caffeine on insulin sensitivity, due to chronic caffeine consumption, insulin sensitivity

may begin to recover (97). Also, prolonged, increased insulin levels in response to caffeine ingestion did not result in lower blood glucose levels in healthy humans, thus contributing to insulin resistance (99). In diabetic humans, caffeine elevated not only insulin levels, but blood glucose levels, too (100). However, contradictory results were found *in vitro*; that is, caffeine increased insulin secretion from  $\beta$ -cells (101). Additionally, caffeine may have an anti-diabetic effect via the mechanisms that include increased basal energy expenditure (86), fat oxidation, muscle glycogen mobilization (87), and increased lipolysis from peripheral tissue (86). Therefore, results from *in vitro* and *in vivo* studies regarding effects of caffeine on insulin secretion are inconsistent. While EGCG used in both our *in vitro* and *in vivo* studies had purities of 95% and >90%, respectively, it may still contain caffeine, given that caffeine may be present as 3-6% in green tea (34). It is known that <1% caffeine was present in the EGCG compound used in the *in vivo* study. It is unknown if this amount of caffeine contributed to increased insulin secretion and synthesis. The EGCG compound used in the *in vitro* studies was 95% pure, leaving room for caffeine, which may or may not have affected results. Therefore, in green tea that has been utilized in many studies, caffeine should be studied for its effect alone on glucose and insulin metabolism, compared to EGCG and green tea.

In summary, we have reported for the first time, to our knowledge, that 0.5% EGCG supplementation in drinking water for 12 days significantly increases insulin synthesis in healthy rats, (Fig. 9D) indicating a key role for EGCG in the regulation of  $\beta$ -cell function. Proper insulin secretion and synthesis, both of which reflect healthy pancreatic  $\beta$ -cell function, are very important aspects for management of diabetes. Since physiological relevant doses of EGCG increased insulin secretion and synthesis in rat

islets (*in vitro* and *ex vivo*) and human islets, EGCG holds promise as a preventative agent or treatment for both T1D and T2D. Future studies may be aimed at determining the effectiveness of EGCG on insulin synthesis in humans, and the mechanism of action by which EGCG promotes insulin secretion and synthesis in animals.



## REFERENCES

1. **CDC** 2005 National Diabetes Fact Sheet: General information and national estimates on diabetes in the United States. Atlanta: U.S. Department of Health and Human Services
2. **Leahy JL** 2005 Pathogenesis of type 2 diabetes mellitus. *Arch Med Res* 36:197-209
3. **Stumvoll M, Goldstein BJ, van Haeften TW** 2005 Type 2 diabetes: principles of pathogenesis and therapy. *Lancet* 365:1333-1346
4. **Weisburger JH, Chung FL** 2002 Mechanisms of chronic disease causation by nutritional factors and tobacco products and their prevention by tea polyphenols. *Food Chem Toxicol* 40:1145-1154
5. **Balentine DA, Wiseman SA, Bouwens LC** 1997 The chemistry of tea flavonoids. *Crit Rev Food Sci Nutr* 37:693-704
6. **Dufresne CJ, Farnworth ER** 2001 A review of latest research findings on the health promotion properties of tea. *J Nutr Biochem* 12:404-421
7. **Sumpio BE, Cordova AC, Berke-Schlessel DW, Qin F, Chen QH** 2006 Green tea, the "Asian paradox," and cardiovascular disease. *J Am Coll Surg* 202:813-825
8. **Vinson JA, Zhang J** 2005 Black and green teas equally inhibit diabetic cataracts in a streptozotocin-induced rat model of diabetes. *J Agric Food Chem* 53:3710-3713
9. **Sano M, Tabata M, Suzuki M, Degawa M, Miyase T, Maeda-Yamamoto M** 2001 Simultaneous determination of twelve tea catechins by high-performance liquid chromatography with electrochemical detection. *Analyst* 126:816-820
10. **Bettuzzi S, Brausi M, Rizzi F, Castagnetti G, Peracchia G, Corti A** 2006 Chemoprevention of human prostate cancer by oral administration of green tea catechins in volunteers with high-grade prostate intraepithelial neoplasia: a preliminary report from a one-year proof-of-principle study. *Cancer Res* 66:1234-1240
11. **Wolfram S, Raederstorff D, Preller M, Wang Y, Teixeira SR, Riegger C, Weber P** 2006 Epigallocatechin gallate supplementation alleviates diabetes in rodents. *J Nutr* 136:2512-2518
12. **Zhang G, Matsumoto S, Hyon SH, Qualley SA, Upshaw L, Strong DM, Reems JA** 2004 Polyphenol, an extract of green tea, increases culture recovery rates of isolated islets from nonhuman primate pancreata and marginal grade human pancreata. *Cell Transplant* 13:145-152
13. **Hara Y, Fujino M, Takeuchi M, Li XK** 2007 Green-tea polyphenol (-)-epigallocatechin-3-gallate provides resistance to apoptosis in isolated islets. *J Hepatobiliary Pancreat Surg* 14:493-497
14. **Wu LY, Juan CC, Ho LT, Hsu YP, Hwang LS** 2004 Effect of green tea supplementation on insulin sensitivity in Sprague-Dawley rats. *J Agric Food Chem* 52:643-648
15. **Igarashi K, Honma K, Yoshinari O, Nanjo F, Hara Y** 2008 Effects of dietary catechins on glucose tolerance, blood pressure and oxidative status in gotokakizaki rats. *J Nutr Sci Vitaminol (Tokyo)* 53:496-500

16. **Islam MS, Choi H** 2007 Green tea, anti-diabetic or diabetogenic: a dose response study. *Biofactors* 29:45-53
17. **Hase T, Komine Y, Meguro S, Takeda Y, Takahashi H, Matsui Y, Inaoka S, Katsurag Y, Tokimitsu I, Shimasaki H, Itakura H** 2001 Anti-obesity Effects of Tea Catechins in Humans. *Journal of Oleo Science* 50:599-605
18. **Iso H, Date C, Wakai K, Fukui M, Tamakoshi A** 2006 The relationship between green tea and total caffeine intake and risk for self-reported type 2 diabetes among Japanese adults. *Ann Intern Med* 144:554-562
19. **Mackenzie T, Leary L, Brooks WB** 2007 The effect of an extract of green and black tea on glucose control in adults with type 2 diabetes mellitus: double-blind randomized study. *Metabolism* 56:1340-1344
20. **Ryu OH, Lee J, Lee KW, Kim HY, Seo JA, Kim SG, Kim NH, Baik SH, Choi DS, Choi KM** 2006 Effects of green tea consumption on inflammation, insulin resistance and pulse wave velocity in type 2 diabetes patients. *Diabetes Res Clin Pract* 71:356-358
21. **Tsuneki H, Ishizuka M, Terasawa M, Wu JB, Sasaoka T, Kimura I** 2004 Effect of green tea on blood glucose levels and serum proteomic patterns in diabetic (db/db) mice and on glucose metabolism in healthy humans. *BMC Pharmacol* 4:18
22. **Ahmad F, Khan MM, Rastogi AK, Chaubey M, Kidwai JR** 1991 Effect of (-)epicatechin on cAMP content, insulin release and conversion of proinsulin to insulin in immature and mature rat islets in vitro. *Indian J Exp Biol* 29:516-520
23. **Kao YH, Hiipakka RA, Liao S** 2000 Modulation of endocrine systems and food intake by green tea epigallocatechin gallate. *Endocrinology* 141:980-987
24. **Lakenbrink C, Lapczynski S, Maiwald B, Engelhardt UH** 2000 Flavonoids and other polyphenols in consumer brews of tea and other caffeinated beverages. *J Agric Food Chem* 48:2848-2852
25. **Graham HN** 1992 Green tea composition, consumption, and polyphenol chemistry. *Prev Med* 21:334-350
26. **Horie H, Mukai T, Kohata K.** 1997 Simultaneous determination of qualitatively important components in green tea infusions using capillary electrophoresis. *J Chromatogr A.* 758:332-335
27. **Balentine DA, Paetau-Robinson I** 2000 Tea as a source of dietary antioxidants with a potential role in prevention of chronic diseases. Lancaster: Technomic Publishing Co., Inc.
28. **Crespy V, Williamson G** 2004 A review of the health effects of green tea catechins in in vivo animal models. *J Nutr* 134:3431S-3440S
29. **Lin YS, Tsai YJ, Tsay JS, Lin JK** 2003 Factors affecting the levels of tea polyphenols and caffeine in tea leaves. *J Agric Food Chem* 51:1864-1873
30. **Hosoda K, Wang MF, Liao ML, Chuang CK, Iha M, Clevidence B, Yamamoto S** 2003 Antihyperglycemic effect of oolong tea in type 2 diabetes. *Diabetes Care* 26:1714-1718
31. **Santana-Rios G, Orner GA, Amantana A, Provost C, Wu SY, Dashwood RH** 2001 Potent antimutagenic activity of white tea in comparison with green tea in the Salmonella assay. *Mutat Res* 495:61-74

32. **Manach C, Scalbert A, Morand C, Remesy C, Jimenez L** 2004 Polyphenols: food sources and bioavailability. *Am J Clin Nutr* 79:727-747
33. **Waltner-Law ME, Wang XL, Law BK, Hall RK, Nawano M, Granner DK** 2002 Epigallocatechin gallate, a constituent of green tea, represses hepatic glucose production. *J Biol Chem* 277:34933-34940
34. **Khan N, Mukhtar H** 2007 Tea polyphenols for health promotion. *Life Sciences* 81:519-533
35. **Higdon JV, Frei B** 2003 Tea catechins and polyphenols: health effects, metabolism, and antioxidant functions. *Crit Rev Food Sci Nutr* 43:89-143
36. **Lakhanpal P, Rai DK** 2007 Quercetin: a versatile flavonoid. *Internet Journal of Medical Update* 2:2
37. **Scalbert A, Williamson G** 2000 Dietary intake and bioavailability of polyphenols. *J Nutr* 130:2073S-2085S
38. **Ohgashi H** 2000 Food Factors: Proceedings of the 2nd International Conference on Food Factors. IOS Press.
39. **Lee MJ, Maliakal P, Chen L, Meng X, Bondoc FY, Prabhu S, Lambert G, Mohr S, Yang CS** 2002 Pharmacokinetics of tea catechins after ingestion of green tea and (-)-epigallocatechin-3-gallate by humans: formation of different metabolites and individual variability. *Cancer Epidemiol Biomarkers Prev* 11:1025-1032
40. **Nakagawa K OS, Miyazawa T** 1997 Dose-Dependent Incorporation of Tea Catechins, (-)-Epigallocatechin-3-Gallate and (-)-Epigallocatechin, Into Human Plasma. *Bioscience Biotechnology and Biochemistry* 61:1981-1985
41. **Rice-Evans C, Packer L** 2003 Flavonoids in Health and Disease. CRC Press.
42. **Lee M, Wang Z, Li H, Chen L, Sun Y, Gobbo S, Balentine D, Yang C** 1995 Analysis of plasma and urinary tea polyphenols in human subjects. *Cancer epidemiology, biomarkers & prevention* 4:393-399
43. **Van Amelsvoort JM, Van Hof KH, Mathot JN, Mulder TP, Wiersma A, Tijburg LB** 2001 Plasma concentrations of individual tea catechins after a single oral dose in humans. *Xenobiotica* 31:891-901
44. **Kimura M, Umegaki K, Kasuya Y, Sugisawa A, Higuchi M** 2002 The relation between single/double or repeated tea catechin ingestions and plasma antioxidant activity in humans. *Eur J Clin Nutr* 56:1186-1193
45. **Kim S, Lee M-J, Hong J, Li C, Smith TJ, Yang G-Y, Seril DN, Yang CS** 2000 Plasma and Tissue Levels of Tea Catechins in Rats and Mice During Chronic Consumption of Green Tea Polyphenols. *Nutrition and Cancer* 37:41-48
46. **Yang CS, Chen L, Lee MJ, Balentine D, Kuo MC, Schantz SP** 1998 Blood and urine levels of tea catechins after ingestion of different amounts of green tea by human volunteers. *Cancer Epidemiol Biomarkers Prev* 7:351-354
47. **Ullmann U, Haller J, Decourt JP, Girault N, Girault J, Richard-Caudron AS, Pineau B, Weber P** 2003 A single ascending dose study of epigallocatechin gallate in healthy volunteers. *J Int Med Res* 31:88-101
48. **Yang CS, Kim S, Yang GY, Lee MJ, Liao J, Chung JY, Ho CT** 1999 Inhibition of carcinogenesis by tea: bioavailability of tea polyphenols and mechanisms of actions. *Proc Soc Exp Biol Med* 220:213-217

49. **Chow HH, Cai Y, Alberts DS, Hakim I, Dorr R, Shahi F, Crowell JA, Yang CS, Hara Y** 2001 Phase I pharmacokinetic study of tea polyphenols following single-dose administration of epigallocatechin gallate and polyphenon E. *Cancer Epidemiol Biomarkers Prev* 10:53-58
50. **Koga T, Meydani M** 2001 Effect of plasma metabolites of (+)-catechin and quercetin on monocyte adhesion to human aortic endothelial cells *Am J Clin Nutr* 73:941-948
51. **Mata-Bilbao MdL, Andrés-Lacueva C, Rouraa E, Jáureguia O, Escribano E, Torrea C, Lamuela-Raventosa RM** 2008 Absorption and pharmacokinetics of green tea catechins in beagles. *British Journal of Nutrition* 21:1-7
52. **Swezey RR, Aldridge DE, LeValley SE, Crowell JA, Hara Y, Green CE** 2003 Absorption, Tissue Distribution and Elimination of 4-[<sup>3</sup>H]-Epigallocatechin Gallate in Beagle Dogs. *Toxicology* 22:187-193
53. **Pietta PG, Simonetti P, Gardana C, Brusamolino A, Morazzoni P, Bombardelli E** 1998 Catechin metabolites after intake of green tea infusions. *Biofactors* 8:111-118
54. **Crespy V, Nancoz N, Oliveira M, Hau J, Courtet-compondu M-C, Williamson G** 2004 Glucuronidation of the Green Tea Catechins, (-)-epigallocatechin-3-gallate and (-)-epicatechin-3-gallate, by Rat Hepatic and Intestinal Microsomes. *Free Radical Research* 38:1025-1031
55. **Meng X, Sang S, Zhu N, Lu H, Sheng S, Lee M-J, Ho C-T, Yang CS** 2002 Identification and Characterization of Methylated and Ring-Fission Metabolites of Tea Catechins Formed in Humans, Mice, and Rats. *Chemical research in toxicology* 15:1042-1050
56. **Donovan JL, Bell JR, Kasim-Karakas S, German JB, Walzem RL, Hansen RJ, Waterhouse AL** 1999 Catechin Is Present as Metabolites in Human Plasma after Consumption of Red Wine. *Journal of Nutrition* 129:1662-1668
57. **Arts ICW, Putte Bvd, Hollman PCH** 2000 Catechin Contents of Foods Commonly Consumed in The Netherlands. 1. Fruits, Vegetables, Staple Foods, and Processed Foods *J Agric Food Chem* 48:1746-1751
58. **Arts ICW, Putte Bvd, Hollman PCH** 2000 Catechin Contents of Foods Commonly Consumed in The Netherlands. 2. Tea, Wine, Fruit Juices, and Chocolate Milk *J Agric Food Chem* 48:1752-1757
59. **Manach C, Williamson G, Morand C, Scalbert A, Rémésy C** 2005 Bioavailability and bioefficacy of polyphenols in humans. I. Review of 97 bioavailability studies *Am J Clin Nutr* 81:230S-242S
60. **Price SF, Breen PJ, Valladao M, Watson BT** 1995 Cluster Sun Exposure and Quercetin in Pinot noir Grapes and Wine *American Journal of Enology and Viticulture* 46:187-194
61. **Manning J, Roberts JC** 2003 Analysis of catechin content of commercial green tea products. *J Herb Pharmacother* 3:19-32
62. **Sussel L, Kalamaras J, Hartigan-O'Connor DJ, Meneses JJ, Pedersen RA, Rubenstein JL, German MS** 1998 Mice lacking the homeodomain transcription factor Nkx2.2 have diabetes due to arrested differentiation of pancreatic beta cells. *Development* 125:2213-2221

63. **Gersell DJ, Gingerich RL, Greider MH** 1979 Regional distribution and concentration of pancreatic polypeptide in the human and canine pancreas. *Diabetes* 28:11-15
64. **Nunemaker CS, Wasserman DH, McGuinness OP, Sweet IR, Teague JC, Satin LS** 2006 Insulin secretion in the conscious mouse is biphasic and pulsatile. *Am J Physiol Endocrinol Metab* 290:E523 - E529
65. **Straub SG, Sharp GW** 2002 Glucose-stimulated signaling pathways in biphasic insulin secretion. *Diabetes Metab Res Rev* 18:451-463
66. **Bowen R** 2006 *Insulin Synthesis and Secretion: Control of Insulin Secretion*. Colorado State University.
67. **Weyer C, Bogardus C, Mott DM, Pratley RE** 1999 The natural history of insulin secretory dysfunction and insulin resistance in the pathogenesis of type 2 diabetes mellitus. *J Clin Invest* 104:787-794
68. **Cnop M, Welsh N, Jonas JC, Jorns A, Lenzen S, Eizirik DL** 2005 Mechanisms of pancreatic beta-cell death in type 1 and type 2 diabetes: many differences, few similarities. *Diabetes* 54 Suppl 2:S97-107
69. **Kloppel G, Lohr M, Habich K, Oberholzer M, Heitz PU** 1985 Islet pathology and the pathogenesis of type 1 and type 2 diabetes mellitus revisited. *Surv Synth Pathol Res* 4:110-125
70. **Shoelson SE, Lee J, Goldfine AB** 2006 Inflammation and insulin resistance. *J Clin Invest* 116:1793-1801
71. **Williams G, Pickup JC** 2004 *Handbook of Diabetes*. Blackwell Publishing.
72. **McClenaghan NH** 2005 Determining the relationship between dietary carbohydrate intake and insulin resistance. *Nutrition Research Reviews* 18:222-240
73. **Meyer C, Stumvoll M, Nadkarni V, Dostou J, Mitrakou A, Gerich J** 1998 Abnormal renal and hepatic glucose metabolism in type 2 diabetes mellitus. *J Clin Invest* 102:619-624
74. **Yun SY, Kim SP, Song DK** 2006 Effects of (-)-epigallocatechin-3-gallate on pancreatic beta-cell damage in streptozotocin-induced diabetic rats. *Eur J Pharmacol* 541:115-121
75. **Xia J, Song X, Bi Z, Chu W, Wan Y** 2005 UV-induced NF-kappaB activation and expression of IL-6 is attenuated by (-)-epigallocatechin-3-gallate in cultured human keratinocytes in vitro. *Int J Mol Med* 16:943-950
76. **Hwang JT, Park IJ, Shin JI, Lee YK, Lee SK, Baik HW, Ha J, Park OJ** 2005 Genistein, EGCG, and capsaicin inhibit adipocyte differentiation process via activating AMP-activated protein kinase. *Biochem Biophys Res Commun* 338:694-699
77. **Han MK** 2003 Epigallocatechin gallate, a constituent of green tea, suppresses cytokine-induced pancreatic beta-cell damage. *Exp Mol Med* 35:136-139
78. **Qanungo S, Das M, Haldar S, Basu A** 2005 Epigallocatechin-3-gallate induces mitochondrial membrane depolarization and caspase-dependent apoptosis in pancreatic cancer cells. *Carcinogenesis* 26:958-967
79. **Spranger J, Kroke A, Mohlig M, Hoffmann K, Bergmann MM, Ristow M, Boeing H, Pfeiffer AF** 2003 Inflammatory cytokines and the risk to develop type 2 diabetes: results of the prospective population-based European Prospective

- Investigation into Cancer and Nutrition (EPIC)-Potsdam Study. *Diabetes* 52:812-817
80. **Choi JH, Chai YM, Joo GJ, Rhee IK, Lee IS, Kim KR, Choi MS, Rhee SJ** 2004 Effects of green tea catechin on polymorphonuclear leukocyte 5'-lipooxygenase activity, leukotriene B4 synthesis, and renal damage in diabetic rats. *Ann Nutr Metab* 48:151-155
  81. **Potenza MA, Marasciulo FL, Tarquinio M, Tiravanti E, Colantuono G, Federici A, Kim JA, Quon MJ, Montagnani M** 2007 EGCG, a green tea polyphenol, improves endothelial function and insulin sensitivity, reduces blood pressure, and protects against myocardial I/R injury in SHR. *Am J Physiol Endocrinol Metab* 292:E1378-1387
  82. **Rosengren A, Dotevall A, Wilhelmsen L, Thelle D, Johansson S** 2004 Coffee and incidence of diabetes in Swedish women: a prospective 18-year follow-up study. *J Intern Med* 255:89-95
  83. **Salazar-Martinez E, Willett WC, Ascherio A, Manson JE, Leitzmann MF, Stampfer MJ, Hu FB** 2004 Coffee consumption and risk for type 2 diabetes mellitus. *Ann Intern Med* 140:1-8
  84. **van Dam RM, Feskens EJ** 2002 Coffee consumption and risk of type 2 diabetes mellitus. *Lancet* 360:1477-1478
  85. **King H, Aubert RE, Herman WH** 1998 Global burden of diabetes, 1995-2025: prevalence, numerical estimates, and projections. *Diabetes Care* 21:1414-1431
  86. **Astrup A, Toubro S, Cannon S, Hein P, Breum L, Madsen J** 1990 Caffeine: a double-blind, placebo-controlled study of its thermogenic, metabolic, and cardiovascular effects in healthy volunteers. *Am J Clin Nutr* 51:759-767
  87. **Spriet LL, MacLean DA, Dyck DJ, Hultman E, Cederblad G, Graham TE** 1992 Caffeine ingestion and muscle metabolism during prolonged exercise in humans. *Am J Physiol* 262:E891-898
  88. **Merglen A, Theander S, Rubi B, Chaffard G, Wollheim CB, Maechler P** 2004 Glucose sensitivity and metabolism-secretion coupling studied during two-year continuous culture in INS-1E insulinoma cells. *Endocrinology* 145:667-678
  89. **Kinasiewicz A, Juszczak M, Pachecka J, Fiedor P** 2004 Pancreatic islets isolation using different protocols with in situ flushing and intraductal collagenase injection. *Physiol Res* 53:327-333
  90. **Zhou K, Hao J, Griffey C, Chung H, O'Keefe SF, Chen J, Hogan S** 2007 Antioxidant properties of Fusarium head blight-resistant and -susceptible soft red winter wheat grains grown in Virginia. *J Agric Food Chem* 55:3729-3736
  91. **Pipeleers DG, in't Veld PA, Van de Winkel M, Maes E, Schuit FC, Gepts W** 1985 A new in vitro model for the study of pancreatic A and B cells. *Endocrinology* 117:806-816
  92. **Dachicourt N, Serradas P, Giroix MH, Gangnerau MN, Portha B** 1996 Decreased glucose-induced cAMP and insulin release in islets of diabetic rats: reversal by IBMX, glucagon, GIP. *Am J Physiol* 271:E725-732
  93. **Lorenz M, Wessler S, Follmann E, Michaelis W, Dusterhoff T, Baumann G, Stangl K, Stangl V** 2004 A constituent of green tea, epigallocatechin-3-gallate, activates endothelial nitric oxide synthase by a phosphatidylinositol-3-OH-kinase-

- , cAMP-dependent protein kinase-, and Akt-dependent pathway and leads to endothelial-dependent vasorelaxation. *J Biol Chem* 279:6190-6195
94. **Bruce Alberts AJ, Julian Lewis, Martin Raff, Keith Roberts, Peter Walter** 2001 *Molecular Biology of the Cell* 4 ed. New York: Garland Science
  95. **Chow HH, Cai Y, Hakim IA, Crowell JA, Shahi F, Brooks CA, Dorr RT, Hara Y, Alberts DS** 2003 Pharmacokinetics and safety of green tea polyphenols after multiple-dose administration of epigallocatechin gallate and polyphenon E in healthy individuals. *Clin Cancer Res* 9:3312-3319
  96. **Isbrucker RA, Edwards JA, Wolz E, Davidovich A, Bausch J** 2006 Safety studies on epigallocatechin gallate (EGCG) preparations. Part 2: dermal, acute and short-term toxicity studies. *Food Chem Toxicol* 44:636-650
  97. **Keijzers GB, De Galan BE, Tack CJ, Smits P** 2002 Caffeine can decrease insulin sensitivity in humans. *Diabetes Care* 25:364-369
  98. **Pizziol A, Tikhonoff V, Paleari CD, Russo E, Mazza A, Ginocchio G, Onesto C, Pavan L, Casiglia E, Pessina AC** 1998 Effects of caffeine on glucose tolerance: a placebo-controlled study. *Eur J Clin Nutr* 52:846-849
  99. **Graham TE, Sathasivam P, Rowland M, Marko N, Greer F, Battram D** 2001 Caffeine ingestion elevates plasma insulin response in humans during an oral glucose tolerance test. *Can J Physiol Pharmacol* 79:559-565
  100. **Lane JD, Barkauskas CE, Surwit RS, Feinglos MN** 2004 Caffeine impairs glucose metabolism in type 2 diabetes. *Diabetes Care* 27:2047-2048
  101. **Islam MS, Larsson O, Nilsson T, Berggren PO** 1995 Effects of caffeine on cytoplasmic free Ca<sup>2+</sup> concentration in pancreatic beta-cells are mediated by interaction with ATP-sensitive K<sup>+</sup> channels and L-type voltage-gated Ca<sup>2+</sup> channels but not the ryanodine receptor. *Biochem J* 306 ( Pt 3):679-686