Type 2 diabetes is characterized by two main features: peripheral insulin resistance and beta-cell dysfunction. Both hereditary and environmental factors, such as obesity and prolonged hyperglycemia, may trigger or exaggerate human type 2 diabetes. Hyperglycemia causes both beta-cell damage and peripheral insulin resistance via multiple mechanisms, collectively referred to as glucotoxicity (1). In MODY-2 diabetes, functional defects in glucokinase genes restrict hepatic glucose uptake, bringing about prolongation of postprandial hyperglycemia (2), and eventually result in beta-cell overload. Hence, efforts to minimize postprandial hyperglycemia, as well as fasting blood glucose control, are of importance for the prevention and treatment of type 2 diabetes.

Green tea is one of the most popular traditional teas in Asian countries and it is currently consumed worldwide as a dietary supplement or beverage. Water-soluble green tea extract (GTE) contains various catechins, of which epigallocatechin-3-gallate (EGCG) is the most abundant (3, 4). With regard to type 2 diabetes, the effects of GTE or EGCG in in vitro and in vivo studies were intensively investigated. However, the debate over whether GTE or EGCG is actually applicable for the prevention or treatment of human obesity and type 2 diabetes has not been settled (5-7). Daily consumption of green tea by diabetic humans over a period of several months was recently revealed to be ineffective in alleviating blood glucose levels, HbA1C levels, insulin resistance and inflammation markers (6).

Interestingly, it has been demonstrated that orally-applied GTE may inhibit glucose (8-10) and cholesterol (11) absorption from the alimentary tract. This is thought to be one of the underlying mechanisms by which GTE influences type 2 diabetes and obesity. Gallated catechins (GC), including EGCG and epicatechin-3-gallate (ECG), appear to be responsible for the inhibitory effects, mainly through the inhibition of Na-glucose co-transporters (SGLT1) in the intestinal epithelia (9) and mixed micelle formation in the intestinal lumen (11). The amount of GC that needs to be ingested to exert the luminal effect appears to be endurable in humans (9, 12), probably due to the lower oral bioavailability of these molecules. Nevertheless, a certain proportion of the ingested catechins is inevitably absorbed into the blood and acts at other sites within the body. Therefore, the effects of orally-ingested GTE on glucose and lipid metabolism are the combination of the effects in the alimentary tract and in the circulation. Some reports demonstrated that oral application of GTE in rats (13) and humans (14) decreased blood glucose levels during an oral glucose tolerance test (OGTT). However, these results could be attributed to the luminal effect of GC (7) because the high glucose was applied immediately after the catechin ingestion. This prompted us to evaluate the effects of
GTEx and EGCG in the circulation as well as in the alimentary tract.

MATERIALS AND METHODS

Materials

Dulbecco's modified Eagle's medium (DMEM), phosphate-buffered saline (PBS), fetal bovine serum (FBS) and fetal calf serum (FCS) were purchased from Gibco (Carlsbad, CA). RPMI-1640 medium was purchased from Welgene (Daegu, Korea). Polyethylene glycol (PEG; Novasyn TG hydroxy resin) was delivered from Novabiochem (Darmstadt, Germany) for animal study. PEG for human study was kindly gifted from Taegoon (Seoul, Korea). Green tea leaves (BOSUNG SEIJKAK) were purchased from Bosung green tea Co. (Jeonnam, Korea). Epigallocatechin-3-gallate (EGCG), epicatechin-3-gallate (ECG), epigallocatechin (EGC) and epicatechin (EC) were purchased from Sigma-Aldrich Co. (St. Louis, MO). All other chemicals were obtained from Sigma-Aldrich.

Cell culture

Rat L6 myoblasts were cultured in low-glucose DMEM with 10% (v/v) FBS until they reached 80% confluence. To induce differentiation, cells were further cultured in DMEM (24.9 mM glucose) containing 2% FBS for 7 days. Cell viability was assessed by the trypan blue viability test. Myogenic differentiation to myotube status was evaluated both morphologically and biochemically. Mouse 3T3-L1 preadipocytes were grown to confluence at 37°C in 35-mm culture dishes in DMEM containing 10% FCS with no added insulin and growth hormone. At two days post-confluence (day 0), differentiation was induced with methylisobutylxanthine (0.5 mM), dexamethasone (0.5 µM), and insulin (5 µg/ml) in DMEM containing 10% FBS. On day 2, methylisobutylxanthine and dexamethasone were removed and insulin treatment was continued for 2 additional days. On day 4 and thereafter, DMEM (without insulin supplementation) plus 10% FBS was replaced every 2 days. Cells (2 x 10^6 cells/dish) were used for experiments on day 8. Cell differentiation was evaluated by the Oil red-O staining. Human hepatocellular carcinoma HepG2 cell line was maintained in DMEM containing 10% FBS at 37°C. Rat insulin-secreting INS-1 cells were grown in RPMI-1640 media supplemented with 10% FBS, 10 mM N-(2-Hydroxyethyl)piperezine-N-(2-ethanesulfonic acid) (HEPES), 1 mM sodium pyruvate, 50 mM 2-mercaptoethanol, 100 IU/ml penicillin, and 100 µg/ml streptomycin.

Preparation of GTE

For animal studies, 20 g of green tea leaves were added to 1000 ml of nanopure water. After being stirred for 5 min at 80°C, the tea leaves were removed by filtration using filter paper (Advantage 2 filter paper, Hyundai micro Co., Seoul, Korea) under reduced pressure. The extract was dried by lyophilization. (A total of 3 g of GTE was harvested, in which EGCG, EGC, EGC and EC were 100, 53, 56 and 31 mg/g GTE, respectively). An equal amount of the GTE solution was mixed with 2 g PEG bead for 5 min at room temperature. After filtration, the supernatant was lyophilized (EGCG, EGC, EGC and EC were 26, 14, 42 and 25 mg/g GTE, respectively) to obtain GC-deficient GTE (GTEx-GC). The results indicated that the resin pretreatment preferentially reduced GC from the GTE solution. For the human study, 30 g of green tea leaves were added to 500 ml of nanopure water. After being stirred for 3 min at 80°C, tea leaves were removed by filtration, remaining a 350 ml solution, which contained 500 mg of EGCG.

OGTT in humans

Healthy male volunteers aged 20-29 with no family history of diabetes and no insulin resistance judged by Homa index were randomly chosen and fasted overnight before the experiments began. First, individuals who exhibited fasting blood glucose level greater than 100 mg/dl were excluded. At 9:00 AM, each individual orally consumed 350 ml of water only (control), or 350 ml of GTE solution containing 125 mg, 250 mg or 500 mg of EGCG, immediately before or 1 h before ingestion of 150-ml water containing 75 g of glucose. Another group of subjects ingested PEG (110 mg/100 mg EGCG) with the GTE solution. After blood had been collected through an angiocatheter inserted into a forearm vein, blood glucose and plasma insulin levels were measured at the indicated times. Blood glucose levels were measured using the Glucocard test strip II (Arkray Inc., Kyoto, Japan). Plasma insulin levels were measured using an immunoradiometric kit (INSULIN MYRIA, Techno genetics, Sesto, Italy). Before the study began, its purpose and risks were carefully explained, and written informed consent was obtained from all participants. The protocol was approved by the IRB Keimyung University Ethics Committee, Daegu, Korea, regulating human research.

Intraperitoneal glucose tolerance test (IPGTT) and OGTT in animals

Sprague-Dawley rats were provided by Hyochang Science Co. (Seoul, Korea). After a 12 h fast, rats were anesthetized using pentobarbital sodium (40 mg/kg, i.p.; Nembutal, 50 mg/ml, Hanlim Pharmaceutical Co., Seoul, Korea) and then injected with one of the epicatechins: EGCG, EGC, ECG, and EC (each 10 mg/kg in 300 l PBS, i.v.). EC was dissolved in a minute amount of DMSO in advance before addition of PBS. In another experiment, rats were randomly divided into 3 groups: control, GTE and GTE-GC. The rats in the GTE group were injected with natural GTE (100 mg/kg in 300 µl PBS, i.v.; 10 mg/kg as EGCG) through the tail vein, while the control rats were injected with only 300 µl PBS and rats in the GTE-GC group were given an equal amount of GC-deficient GTE (100 mg/kg in 300 µl PBS, i.v.; 2.6 mg/kg as EGCG). Thirty minutes after the injection, glucose (2.0 g/kg in 600 µl distilled water, i.p.) was injected. For OGTT, the rats were ingested with 1 ml distilled water only (control), or 1 ml distilled water containing one of natural GTE (900 mg/kg), GC-deficient GTE (900 mg/kg), EGCG (90 mg/kg), and EGCG (90 mg/kg), natural GTE plus PEG (110 mg/100 mg EGCG) or PEG alone. Immediately after the ingestion, each rat was orally given 1 ml distilled water containing glucose (2 g/kg). For the assay of blood glucose levels, blood was drawn from the tail vein at the indicated times. All the experiments were approved by the Keimyung University institutional ethics committee, Daegu, Korea for supervising animal research.

Insulin tolerance test (ITT) in animals

Kir6.2 knock-out (k/o) mice were kindly provided by Prof. Susumu Seino at Kobe University in Japan. Following a 4 h fast, Kir6.2 k/o mice or normal rats were intravenously injected with PBS (100 µl for mouse, 300 µl for rat) only, or PBS containing EGCG (10 mg/kg), natural GTE (100 mg/kg) or GC-deficient GTE (100 mg/kg). Thirty minutes after the injection, the mice and rats were injected intraperitoneally with normal saline (300 µl for mouse, 600 µl for rat) containing insulin (1 IU/kg;
INSULIN LISPRO, Eli Lilly, IN). Blood samplings were done through the tail vein at the indicated times.

2-Deoxyglucose uptake assay

Briefly, after serum starvation for 30 min, the cells were washed with Krebs-Ringer phosphate-HEPES buffer (KRPH buffer: 10 mM phosphate buffer, pH 7.4; 1 mM MgSO4, 1 mM CaCl2, 136 mM NaCl, 4.7 mM KCl, and 10 mM HEPES, pH 7.6) and then incubated with or without 100 nM insulin for 20 min in KRPH buffer that contained EGCG (0, 0.1, 1.0 or 10 µM). Glucose transport was determined by the addition of 2-deoxy-[3H] glucose (0.1 mM, 0.5 µCi/ml; PerkinElmer Life and Analytical Science, Waltham, MA). After 10 min of incubation, the reaction was stopped by three quick washes with ice-cold PBS. The cells were then lysed in PBS containing 0.2 M NaOH, and glucose uptake was assessed by scintillation counting.

Western blot analysis

To determine the effect of EGCG on the activity of protein kinase B (PKB), the phosphorylation status of PKB was examined after exposure of L6, INS-1, HEPG2 and 3T3L-1 cells to media containing different concentrations of EGCG and insulin. As in the 2-deoxyglucose uptake assay, experimental cells were exposed to media without glucose for 30 min and then incubated with or without 100 nM insulin for 20 min in KRPH that contained EGCG (0, 0.1, 1.0 or 10 µM). After an additional 10 min of incubation with 5 mM glucose, the reaction was stopped by three quick washes with ice-cold PBS. Total cellular proteins were extracted in lysis buffer (10 mM Tris-Cl (pH 7.4), 130 mM NaCl, 5% (v/v) Triton X-100, 5 mM EDTA, 200 nM aprotinin, 20 mM leupeptin, 50 mM phenanthroline, 280 mM benzamidine-HCl) for 20 min at 4°C. Lysates were separated by SDS-PAGE and electrotransferred to an Immobilion-P membrane (Millipore, Billerica, MA). After probing with specific antibodies (Anti-phospho-PKB (Ser473) antibody (Cell Signaling Technology, Danvers, MA), and anti-β-actin antibody (Sigma)), the immunoreactive bands were visualized with horseradish peroxidase-conjugated secondary antibody (1:5000; Santa Cruz, CA) using enhanced chemiluminescence (Amersham Biosciences, Little Chalfont, UK).

Analysis of catechins from GTE with HPLC

HPLC analysis was conducted on a Waters Alliance 2695 liquid chromatograph equipped with a model 2487 dual absorbance detector (Waters Co., Milford, MA). A Waters symmetry C18 reversed-phase packing column (4.5 mm × 250 mm, 5 µm) was used at 25°C for separation throughout this study. Catechines were determined simultaneously at 235 nm. A gradient elution was performed by varying the proportion of solvent A (water-trifluoroacetic acid, 99.9:0.1 v/v) to solvent B (acetonitrile-trifluoroacetic acid, 99.9:0.1 v/v), with a flow rate of 1 ml/min. The mobile phase composition changed linearly from 9.5% to 14% solvent B in 10 min and then kept the same composition for 10 min, followed by a linear increase of solvent B to 27.5% within 15 min. The mobile phase composition was then brought back to the initial conditions over a period of 5 min for the next run. All the prepared solutions were filtered through 0.45 µm membranes (Sartorius, Maisemore, UK), and the mobile phase was degassed before injection into the HPLC.

Statistical analyses

The results are expressed as mean ±SEM. The SPSS (release 14.0) software package (SPSS Inc., Chicago, IL) was used for the statistical analyses. Area under the curve was calculated by using Microcal Origin software (version 7.0; Northampton, MA). Comparisons between two groups were performed with the Student's two-tailed t-test for paired or unpaired data. For comparisons of more than two groups, significance was tested by ANOVA followed by the Bonferroni test.
using an analysis of variance (ANOVA) with Bonferroni correction to deal with relatively small amounts of samples. Differences between groups were considered significant when \( P<0.05 \).

**RESULTS**

*Effect of GTE intake on blood glucose and plasma insulin levels during human OGTT*

The results of OGTT that had been performed with healthy male volunteers showed that the effects of GTE intake on glucose tolerance were changed depending on the time lag between GTE and glucose administration. Orally-administered GTE maintained blood glucose levels lower than the control during OGTT when glucose was taken immediately after GTE administration (Fig. 1A). This effect is the same with the previously reported results (14). The lowering effect of GTE on blood glucose levels during OGTT was reversed when it was administered 1 h earlier than the administration of glucose (Fig. 1C). The blood glucose level was significantly higher in the GTE group than in the control 60 min after glucose loading (\( P<0.01 \)).

![Fig. 2. Effects of circulating GC on glucose tolerance. (A) Changes in blood glucose levels during rat IPGTT, which was performed 30 min after each catechin injection. Rats were fasted for 12 h and then intravenously injected with PBS alone, or PBS containing EC in DMSO, ECG, EGC or EGCG (each 10 mg/kg). Area under the curve (AUC) was depicted in (B) as the percentage of the control value without any catechins. ANOVA with Bonferroni correction. * - \( P<0.05 \) and ** - \( P<0.01 \) compared to the control value (B) or that of the same time point (A), n=5-7 for each group.](image)

The 1 h interval between GTE and glucose intake was chosen because the blood concentrations of tea ingredients, especially catechins, were known to peak between 1-2 h after GTE ingestion (4, 15). Interestingly, plasma insulin levels were also significantly (\( P<0.05 \)) higher in the GTE group at the 60 min (Fig. 1D), implying that the higher blood glucose levels may induce a higher rate of insulin secretion.

*EGCG and ECG of tea catechins are critical for glucose intolerance*

To clarify which GTE components were responsible for the abnormal OGTT in humans, IPGTT was performed with rat (Fig. 2). Since epicatechins were studied intensively for its role in glucose tolerance, EGCG, ECG, EGC and EC were used for IPGTT. Each catechin was injected in rats 30 min before glucose administration. The amount of EGCG injected (10 mg/kg, i.v.) was chosen to achieve its blood concentrations around 1 µM at 30 min after injection (16, 17). Same amount of other catechins was used even though they had different pharmacokinetic profiles (10). EGCG and ECG caused more elevated blood glucose levels 30 min after glucose loading compared to control (\( P<0.01 \)) while EC and EGC had no effects. This result suggested that the effect of GTE on glucose intolerance was mainly due to the two GC, EGCG and ECG. Since the amount of EGCG in water-soluble GTE was about 2-3 times higher than ECG (18), further studies were performed with EGCG as a representative of GC.

*2-Deoxy-[\( ^3\)H]-glucose uptake into cells is hindered by EGCG*

The dose dependencies of basal and insulin-stimulated glucose uptake on EGCG were evaluated with metabolically-important cells including beta cells (Fig. 3). Both basal and insulin-stimulated glucose uptake were decreased by EGCG dose-dependently for all EGCG-pretreated cells. Also, in the tested cell lines up to 10 M EGCG, EGCG did not alter the basal and insulin-regulated expression of phospho-PKB (Fig. 4).

*Circulating EGCG acutely increases blood glucose levels and insulin resistance*

The possibility of association between EGCG-mediated glucose intolerance and ATP-sensitive potassium (K\(_{\text{ATP}}\)) channels, which modulate peripheral insulin resistance as well as beta-cell insulin secretion, was examined. Normal rats and Kir6.2 k/o mice were fasted for 4 h before EGCG injection. The significant elevation of blood glucose levels in both normal rats and Kir6.2 k/o mice were found 30 min after the EGCG injection (\( P<0.01 \); Fig. 5A, B). This result suggests that the EGCG-induced change in blood glucose level was not mediated through a mechanism involving K\(_{\text{ATP}}\) channels. When treated with insulin, the rate of blood glucose disappearance was delayed mildly but significantly in EGCG-pretreated rats and Kir6.2 k/o mice (\( P<0.01 \); Fig. 5C, D)

*Effect of GC-deficient GTE*

GTE injection with 4 h fasted rats caused significant elevation of blood glucose levels (\( P<0.05 \); Fig. 6A), which was similar finding to the injection with EGCG alone in Fig. 5A, while GC-deficient GTE injection did not (\( P=0.114 \)). ITT revealed remarkable insulin resistance in the GTE group (\( P<0.01 \) at the 20 min), whereas significant amelioration of insulin resistance was observed in the GTE-GC group (Fig. 6B). Rats in another experiment were fasted for 12 h, not 4 h, to minimize elevation of blood glucose level by GTE itself. Then they were injected
intravenously with PBS alone, or with PBS containing either natural GTE or GC-deficient GTE 30 min before glucose loading. As shown in Fig. 6C, blood glucose levels in the GTE-treated group during IPGTT were remarkably higher than those in the PBS control (P<0.01 at 10 and 20 min), while the GTE-GC group showed similar glucose levels to the control. The effects on glucose absorption into the circulation of GC within the gastrointestinal tract were evaluated by rat OGTT. It was performed by oral intake of GTE immediately followed by glucose ingestion (Fig. 6D). Expectedly, the blood glucose levels were maintained during OGTT at a lower level in the GTE-administered group than in the control group that ingested only PBS. The GC-deficient GTE- or EGCG-administered group appeared to inhibit glucose absorption less efficiently than in the GTE-treated group, indicating that GC is also critical for the effect of GTE on blockade of glucose absorption in the intestinal lumen.

**Effect of administration of PEG with GTE**

On the assumption that the resin PEG selectively inhibits the intestinal absorption of GC of GTE, natural GTE was ingested along with PEG. During rat OGTT immediately following the ingestion, blood glucose levels were significantly lower in the GTE+PEG group than in the control (P<0.05), comparable to the group with GTE alone (Fig. 7A). There was no difference between the control group and the PEG-alone-treated group in blood glucose levels, indicating that PEG itself did not affect glucose absorption or intestinal motility at the range of concentrations.
used. The time for human OGTT was extended to 3 h observing the effect of circulating GC on basal blood glucose levels (Fig. 7B). Although the blood glucose levels in the GTE group during the earlier period of OGTT appeared to be lower than in the control, they during the later period of OGTT were significantly higher than in the control, which was not observed in the GTE+PEG group. When human OGTT was conducted 1 h after GTE+PEG ingestion, blood glucose and plasma insulin levels were also normalized in contrast to the GTE group, which showed significant elevation of the glucose and insulin levels (Fig. 7C, D).
DISCUSSION

The present study has revealed that GC of green tea, such as EGCG and ECG, acutely reduces blood glucose levels mainly through its activities in the alimentary tract while increases the glucose level when GC is in the circulation (Fig. 8 for summary). Impaired glucose and fatty acid utilization was known to be associated with the development of insulin resistance and type 2 diabetes (19, 20). Although green tea polyphenols have been reported to inhibit intestinal sucrase and α-amylase activity as well, GC may not be the major inhibitor because the inhibitory effect of green tea was weaker than that of black tea which has smaller amount of GC (21). The intestinal glucose uptake is mediated by SGLT1, and GTE or GC has been found to be a competitive inhibitor of SGLT1 of human intestinal epithelial cells (9, 22). In the present study, the blood glucose uptake into metabolically-important cells was also inhibited in the presence of EGCG. The inhibitory effect of GC on cellular glucose transporters has been previously observed in mouse adipocytes (23), rat adipocytes (24) and human erythrocytes (7). Hence, one may speculate that GC might hinder the action of various glucose transporters in the body, including the Na-glucose co-transporters as well as the Na-independent glucose transporters in plasma membrane. Truly, GC was revealed to interact with various plasmalemmal proteins and lipid rafts (25). Therefore, GC in the alimentary tract may be helpful in controlling diabetes and obesity by decreasing glucose entry into the circulation (26), but it may be harmful in the circulation. These ambivalent effects of GC in the two compartments may confound attempts to evaluate their precise role in the blood glucose control. We further observed an acute insulin resistance either by circulating EGCG alone or more prominently by circulating GTE. The latter amplification may be due to the other constituents, such as ECG and gallic acid, which may exert additional effects, or may be due to the delayed catabolism of EGCG in the presence of other green tea components (16). Therefore, it is expected that the accumulation of glucose in the circulation caused by green tea catechins may induce insulin hypersecretion. It is not surprising that long-term beta-cell overload represents a detrimental consequence that leads to eventual beta-cell impairment.
In the present study, GTE was effective at blood EGCG concentrations of 1-2 µM, which is readily achievable by daily oral administration of green tea (27). Experiments that use rats are more likely to overlook this circulating effect, because rats have a much lower oral bioavailability of EGCG (15, 16). To clarify the effect of circulating GTE, we employed long-time OGTT in humans, who have the relatively higher oral bioavailability of EGCG than rats, and found that GTE hindered return of the glucose level to the control value. An in vivo report which examined the circulating EGCG effect within a concentration range applicable to human showed that relatively long-term intraperitoneal EGCG application led to weight loss in rats (17). However, they found significant deterioration in the sexual organs. It may be probably due to EGCG inhibition of glucose uptake in the androgen-producing Leidig cells (7). A recent investigation (7) revealed that 100 nM ECG competed with glucose for binding to glucose transporter type 1 in red blood cells, such that the reaction rate was half its maximal value. The same effect was attained with 1 µM EGCG. In this regard, it may be supposed that the decreasing effects on hepatic gluconeogenesis induced by EGCG might be partly due to the EGCG-mediated inhibition of hepatic glucose uptake (7, 28). In addition, the beneficial effects of EGCG in treatment of some cancers may be, at least partly, due to the glucose deprivation effect that EGCG may inflict on cancer cells which are more metabolically active and glycolytic-dependent than normal cells (29). Other polyphenols, such as quercetin and myricetin, seem to have relatively broader safety margins because they inhibit cellular glucose uptake around 10 µM (30).

The efforts to manage type 2 diabetes and obesity by natural green tea treatment appear to be cautious because systemically-absorbed GC actually blocks cellular glucose uptake and thereby increases blood glucose and insulin levels. Moderate green tea intake (1-2 cups per day) during a meal may be beneficial since the GC entering the circulation will be minimal, but its long-term effect may likely be limited to a certain category of the population (31). Many trials have measured the ability of natural products to inhibit intestinal glucose absorption. However, none of these trials has explored the use of non-absorbable, GC-derived inhibitors of glucose and lipid absorption. Maximizing the positive effect of GC in the intestinal lumen, while minimizing the negative effect in the circulation, would be recommended to control those metabolic diseases.

Acknowledgments: This work was supported by the Basic Research Program of Korea Science and the Engineering Foundation for Chronic Disease Research Center (Grant No. 2009-0091360).

Conflict of interests: None declared.

REFERENCES


Received: October 20, 2008
Accepted: November 6, 2009

Author’s address: Dae-Kyu Song, M.D., Ph.D., Associate Professor, Department of Physiology and Chronic Disease Research Center, Keimyung University School of Medicine, 194 Dongsan-Dong, Jung-Gu, Daegu, Korea; Phone: +82-53-250-7490; Fax: +82-53-252-1605; E-mail: dksong@kmu.ac.kr; dksong@dsme.or.kr