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Structure-Dependent Inhibitory Effects of Green Tea Catechins on Insulin Secretion from Pancreatic β-Cells

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The effects of green tea catechins on glucose-stimulated insulin secretion (GSIS) were investigated in the β-cell line INS-1D. Epigallocatechin gallate (EGCG) at 10 μM or gallocatechin gallate (GCG) at 30 μM caused significant inhibitory effects on GSIS, and each of these at 100 μM almost abolished it. In contrast, epicatechin (EC) or catechin (CA) had no effect on GSIS at concentrations up to 100 μM. We thus investigated the structure–activity relationship by using epigallocatechin (EGC) and gallocatechin (GC) containing a trihydroxyl group in the B-ring, and epicatechin gallate (ECG) and catechin gallate (CG) containing the gallate moiety. EGC, GC, and ECG caused an inhibition of GSIS, although significant effects were obtained only at 100 μM. At this concentration, EGC almost abolished GSIS, whereas GC and ECG partially inhibited it. In contrast, CG did not affect GSIS at concentrations up to 100 μM. EGCG also abolished the insulin secretion induced by tolbutamide, an ATP-sensitive K+ channel blocker, and partially inhibited that induced by 30 mM K+. Moreover, EGCG, but not EC, inhibited the oscillation of intracellular Ca2+ concentration induced by 11.1 mM glucose. These results suggest that some catechins at supraphysiological concentrations have inhibitory effects on GSIS, the potency of which depends on their structure; the order of potency was EGCG>EGC>GCG>GC≈ECG. The inhibitory effects seem to be mediated by the inhibition of voltage-dependent Ca2+ channels, which is caused, at least in part, by membrane hyperpolarization resulting from the activation of K+ channels.

Key words catechin; insulin secretion; β-cell

The health benefits of green tea has been studied extensively, especially those related to cardiovascular and metabolic health.1-3 These health benefits are attributed to green tea catechins and may be due to the presence of phenolic hydroxyl groups on the B-ring and/or the presence of the gallate group in the 3’ position.4 Structural, epigallocatechin (EGC) and gallocatechin (GC) contain the pyrogallol moiety; epicatechin-3-gallate (ECG) and catechin-3-gallate (CG) have the gallate moiety; and epicatechin gallate (EGCG) and gallocatechin gallate (GCG) have both moieties (Fig. 1). Among these catechins, EGCG, the most abundant species in green tea catechins, is primarily responsible for various beneficial biological effects.5,6 Several studies have investigated the effects of green tea catechins on insulin secretion from pancreatic β-cells. However, conflicting data were shown: Catechin (CA) augmented glucose-stimulated insulin secretion (GSIS) in the β-cell line MIN6,7 whereas EGCG decreased insulin secretion from isolated rat islets8 and plasma insulin level in rats.9 Moreover, it remains to be elucidated whether there is any relationship between the effects of catechins on insulin secretion and their chemical structure.

The present study thus investigated the effects of green tea catechins on insulin secretion from pancreatic β-cells. We show here that GCG, EGC, GC, and ECG as well as EGCG, but not epicatechin (EC), CA, or CG, inhibit GSIS in pancreatic β-cell line INS-1D, which seems to be mediated by a decrease in intracellular Ca2+ concentration ([Ca2+]i).

MATERIALS AND METHODS

Materials (-)-Catechin (CA), (-)-catechin gallate (CG), (-)-epicatechin (EC), (-)-epicatechin gallate (ECG), (-)-epigallocatechin gallate (EGCG), (-)-gallocatechin gallate (GCG), and β-glutamine, sodium pyruvate, and RPMI-1640 medium were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Bovine serum albumin (BSA), Fura-PE3 acetoxy-methyl ester (Fura-PE3/AM), 2-mercaptoethanol, poly-L-lysine solution, and trypsin were obtained from Sigma-Aldrich (St. Louis, MO, U.S.A.), and penicillin G potassium and streptomycin sulfate were obtained from Meiji Seika (Tokyo, Japan). Chemicals were dissolved in either purified water or dimethyl sulfoxide (DMSO) and stored at −20°C for later use. The stock solutions were dissolved in RPMI-1640 medium supplemented with 10 mM N-(2-hydroxyethyl)piperazine-N’-2-ethanesulfonic acid (HEPES), 1 mM sodium pyruvate, 50 μM 2-mercaptoethanol, 100 μg/mL streptomycin, 100 U/mL penicillin G, 0.3 mg/mL l-glutamine, and 10% fetal bovine serum (FBS), before each experiment.

Cell Culture INS-1D cells, a gift from Dr. C. Wollheim (University Medical Center, Geneva, Switzerland), were cultured at 5% CO2/95% air and 37°C in the supplemented RPMI-1640 medium containing 11.1 mM glucose according to the method by Asfari et al.8 The medium was replaced every third day.

Measurement of Insulin Secretion INS-1D cells at passages between 48 and 59 were preincubated in HEPES-Krebs buffer solution (HK solution: in mM; 129 NaCl, 4.7 KCl, 1.2 MgSO4, 1 CaCl2, 1.2 KH2PO4, 10 HEPES, 5 NaHCO3, 2.8
glucose; pH 7.4 with NaOH) with 0.1% BSA for 1 h at 37°C. The cells were then incubated for 1 h in the HK solution containing 2.8 or 11.1 mM glucose in the presence or absence of each catechin. The incubation media were collected and stored at −20°C for later assay. The amount of insulin in the media was measured using a radioimmunoassay kit (Eiken Chemical, Tokyo, Japan).

**Measurement of \[Ca^{2+}\]_i** \[Ca^{2+}\]_i was measured in Fura PE-3-loaded cells by dual-wavelength fluorometry. INS-1D cells were loaded with 4 μM Fura PE-3/AM for 2.5 h at 37°C, put in a chamber mounted on the stage of an inverted microscope (IX71, OLYMPUS, Tokyo, Japan) and continuously superfused with the HK solution at a flow rate of 1 mL/min. \[Ca^{2+}\]_i was measured using an AQUACOSMOS/RATIO system (Hamamatsu Photonics, Hamamatsu, Japan), with alternating excitation of cells at 340 and 380 nm. The resultant emission was monitored at 510 nm. Pairs of fluorescence images at 340 and 380 nm were captured every 10 s and were converted to the 340/380 ratio images. The 340/380 ratio was used to indicate the relative \[Ca^{2+}\]_i. Drugs were applied in the superfusing HK solution.

**Statistics** Data are shown as the mean±standard error of the mean (S.E.M.). Comparisons were made using Dunn’s multiple comparison test.

**RESULTS**

**Effects of Catechins on Insulin Secretion** The exposure of INS-1D cells to 11.1 mM glucose for 1 h increased insulin secretion (Fig. 2). GSIS was significantly reduced by 10 and 30 μM EGCG and abolished by 100 μM EGCG (Fig. 2A). Similarly, GSIS was significantly reduced and abolished by GCG at 30 and 100 μM, respectively (Fig. 2B). In contrast, EC or CA had no effect on the insulin secretion at concentrations up to 100 μM (Figs. 2C, D). EGCG and GCG contain an additional hydroxyl group at the 5’ position of the B ring and a gallate group at the 3 hydroxyl position of the C ring when compared with EC and CA, respectively (Fig. 1). Thus, the structure–activity relationship of the inhibitory effects of catechins on insulin secretion was investigated with EGC and GC containing the hydroxyl group, and ECG and CG containing the gallate group (Fig. 1). EGC, GC, and ECG showed a significant inhibitory effect on GSIS, although a higher concentration, i.e., 100 μM, was needed to induce the inhibitory effect compared with EGCG and GCG. At this concentration, EGC almost abolished GSIS, whereas GC and ECG partially inhibited it. In contrast, CG had no significant effect on it at concentrations up to 100 μM (Figs. 2E–H). The inhibitory effects of catechins on GSIS are thus likely to depend on their structure; the order of potency was EGCG > GCG > EGC > GC = ECG. EGCG at 100 μM also abolished the insulin secretion induced by tolbutamide (100 μM), an ATP-sensitive K+ (K_ATP) channel blocker, whereas it caused only a partial decrease in the insulin secretion induced by 30 mM KCl (Fig. 3).

**Effects of Catechins on \[Ca^{2+}\]_i of β-Cells** Since an increase in \[Ca^{2+}\]_i of β-cells has been well established as a trigger for insulin secretion,9 we investigated the effects of catechins on \[Ca^{2+}\]_i of INS-1D cells. INS-1D cells, levels of \[Ca^{2+}\]_i were low and stable at 2.8 mM glucose. Elevating the glucose concentration to 11.1 mM induced slow \[Ca^{2+}\]_i oscillations (Fig. 4). These \[Ca^{2+}\]_i oscillations were completely abolished by nicardipine (1 μM), a Ca2+ channel blocker (data not shown), implying that they were caused by Ca2+ influx through voltage-dependent Ca2+ channels. One hundred μM EGC largely decreased the amplitude of the \[Ca^{2+}\]_i oscillations (Fig. 4A). Similar inhibition of the \[Ca^{2+}\]_i oscillations was observed in the presence of GCG and ECG at 100 μM (data not shown). In contrast, the same concentration of EC had no effect on the oscillations (Fig. 4B).

**DISCUSSION**

The present study investigated whether green tea catechins have any effects on GSIS from pancreatic β-cells. INS-1D...
cells used in the present study retain many characteristics of native pancreatic β-cells, with the most important feature being the release of insulin in response to elevated glucose levels. The data shown here suggest that EGCG, GCG, EGC, GC, and ECG, at high concentrations, have inhibitory effects on GSIS, and that the potency of each catechin is dependent on its structure.

The effects of catechins on insulin secretion are controversial. In the β-cell line MIN6, CA at 20–200 µM significantly augmented insulin secretion induced by 25 mM glucose. In contrast, another research group reported that EGCG at 20 µM inhibited insulin secretion induced by the non-metabolized...
Fig. 3. Effects of EGCG on the Insulin Secretion Induced by Tolbutamide (A) and 30 mM KCl (B) in INS-1D Cells

The cells were incubated for 1 h in the HK solution containing 2.8 mM glucose with or without 100 µM tolbutamide (A) or 30 mM KCl (B) in the absence or presence of 100 µM EGCG. Each represents the mean±S.E.M. of 4 independent experiments. **p<0.01 compared with the control without EGCG.

Fig. 4. Effects of EGCG (100 µM; A) and EC (100 µM; B) on [Ca^{2+}]_{i} Oscillations Induced by 11.1 mM Glucose (G11.1 mM) in INS-1D Cells

Tracings in A and B are representative of 278 and 316 cells from 3 and 4 experiments, respectively.
leucine analog BCH in isolated rat islets.63 BCH stimulates glutamate dehydrogenase (GDH), which facilitates glutaminolysis and provides the ATP signal necessary for insulin secretion. This same study also demonstrated that EGCG inhibited GDH, implying that EGCG inhibits insulin secretion only at high GDH activity. In fact, EGCG showed no effect on insulin secretion induced by high glucose conditions where GDH is fully inhibited. The inhibition of GDH was also observed with ECG, but not with EGC or EC. Neither study is consistent with our current observation showing that CA had no effects on insulin secretion and that EGC as well as EGCG and ECG showed inhibitory effects on GSIS. Since EGCG inhibited insulin secretion and that EGC as well as EGCG and EC, but not with EGC or EC. Neither study is consistent with fully inhibiting. The inhibition of GDH was also observed with ECG, but not with EGC or EC. Neither study is consistent with fully inhibiting.

ECG, and that the gallate moiety, and EGCG is likely to inhibit Ca^{2+} influx through voltage-dependent Ca^{2+} channels by directly inhibiting the channels or by indirectly inhibiting them via membrane hyperpolarization.

EGCG has been shown to have dual effects on K_{ATP} channels; EGCG at 1 µM preferentially interacts with phosphatidylinositol polyphosphates (PIP)-binding sites of the channels, whereas it at 10 µM affects ATP binding in oocytes co-expressing the Kir6.2 and SUR1 subunits of K_{ATP} channels.23 Since K_{ATP} channels are activated by PIP and inhibited by ATP, uncoupling of K_{ATP} channels from PIP and ATP by EGCG might lead to the inhibition and activation of K_{ATP} channels, respectively. Results from this study also showed that glucose-induced [Ca^{2+}]_{i} elevation was reduced by 10 µM EGCG in rat β-cells.17 This is consistent with our present results. It is thus suggested that EGCG at high concentrations inhibits insulin secretion by inducing the uncoupling of K_{ATP} channels from ATP. We also showed that EGCG inhibited sulfonamide-induced insulin secretion. This finding could also be explained by the suggested mechanism of EGCG, since sulfonamides exert their insulinotropic effects by increasing ATP sensitivity of K_{ATP} channel Kir6.2 subunits.10 The involvement of K^{+} channels in the effect of EGCG is further supported by the present results that EGCG showed less potent inhibition of high K^{+}-induced insulin secretion. Under high K^{+} conditions, a decreased K^{+} gradient across the plasma membrane would diminish the membrane hyperpolarization induced by the opening of K^{+} channels. Although Jin et al. have shown that EGC or ECG at 10 µM has no effect on the ATP sensitivity of K_{ATP} channels,7 this finding does not contradict our present results; significant inhibitory effects of EGC and ECG were observed only at 100 µM in our present study.

Structurally, EGC and GC have three hydroxyl groups on the B-ring, ECG and CG have three hydroxyl groups in the gallate moiety, and EGCG and GCC have both (Fig. 1). Hydroxyl groups can form hydrogen bonds with polar amino acids; hence, the presence of three hydroxyl groups may contribute to the interaction of catechins with plasma membranes. The interaction of catechins with plasma membranes is considered to be necessary to exert their biological activity. In fact, EGCG and ECG have been shown to interact with lipid bilayers.11,12 The affinity to lipid bilayers may depend on their lipophilicity, a factor determined by the number of hydroxyl groups on the B-ring and the presence of the gallate moiety.13 The affinity is also influenced by the stereochemical structure of catechins; cis-type catechins have higher affinity than the corresponding trans-type catechins. As an example, the hydrophobic domain of CG, a trans-type catechin, is located in the center of the molecule, and the interaction of this domain to lipid bilayers is disturbed by hydrophilic A-ring, B-ring, and the gallate moiety.10 The lack of inhibitory effects of CG on insulin secretion observed in the present study may be due to its stereochemical feature. Thus, the affinity of catechins for plasma membranes appears to be critical for the occurrence of the effect.

Nakayama and colleagues have extensively investigated the affinity of green tea catechins for model biological membranes by using several different analytical methods. The order of affinity appears to be ECG > EGCG > EGC,13-15 These results appear to be closely correlated with the result of the partition coefficients (calculated by dividing the amount of each catechins in n-octanol by that in diluted phosphate-buffered saline). In contrast, a number of biological active properties of catechins may be attributed to their antioxidant properties involved in scavenging reactive oxygen species. The order of antioxidative effectiveness of catechins has been shown to be as follows: ECG > EGCG > EGC > EC,3,16 It appears that the gallate group in the 3’ position is important for antioxidant activity. In the present study, however, the order of the potency of epicatechins in the inhibition of GSIS was shown to be EGCG > EGC > ECG. The inhibitory effects of catechins on insulin secretion are therefore likely to be determined by factors other than the antioxidant activity. It is possible that catechins exert their inhibitory effects on insulin secretion by inducing the uncoupling of K_{ATP} channels from ATP as discussed previously in this paper. The phosphate tail of ATP has been shown to interact with positively charged amino acids, R50, K185, and R201, in the cytoplasmic domain of K_{ATP} channel Kir6.2 subunit.17 The release of a proton from the hydroxyl groups on the B-ring or the gallate moiety of catechins leaves a negatively charged oxygen. This negatively charged oxygen may form an ionic bond with a positively charged amino acid, thereby interfering with the interaction of ATP with K_{ATP} channels.

Although it is well established that EGCG is a major biological component of green tea catechins, a discrepancy exists regarding the actual plasma levels of EGCG after consumption. The average peak plasma concentration of EGCG was shown to reach approximately 1 μM with a single oral dose of 800mg EGCG in humans.63 In contrast, another group reported that an oral intake of 525mg EGCG, which is equivalent to drinking 0.5–1L of green tea, could lead to plasma levels of EGCG up to 4.4 μM.69 In either case, the plasma concentration of EGCG after oral consumption of green tea in vivo is less than the concentration at which EGCG inhibited GSIS in the present in vitro study. Thus, the inhibitory effects of the catechins on insulin secretion are likely to be produced only at supra-physiological concentrations. The harmful effects of the catechins including impaired GSIS may be a matter to be considered when EGCG is overused for health-promoting purposes or administered by parenteral routes.

Green tea is believed to have beneficial effects in preventing type 2 diabetes. Several in vitro studies have provided evidence supporting the anti-diabetic effect of green tea catechins. It has been shown that EGCG increases glucose transporter 4 translocation in rat skeletal muscle cells60 and suppresses gluconeogenesis in rat hepatoma cells.25 In addition, ECG has been shown to suppress glucose absorption in the rabbit small intestine.25
In conclusion, some green tea catechins showed inhibitory effects on GSIS in INS-1D β-cells at supra-physiological concentrations. The potency was dependent on their structure, the order of which was EGCG > GCG > EGC > GC = ECG. EGCG abolished the insulin secretion induced by tolbutamide, and caused a partial decrease in the insulin secretion induced by 30 mM KCl, implying the involvement of K+ channel opening in the inhibitory effects of EGCG. EGCG also decreased the amplitude of glucose-induced [Ca2+]i oscillations in INS-1D cells. The inhibitory effects of the catechins on GSIS are thus suggested to be caused by the inhibition of voltage-dependent Ca2+ channels, which is mediated, at least in part, by membrane hyperpolarization resulting from the opening of K+ channels.

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Conflict of Interest The authors declare no conflict of interest.

REFERENCES


