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Pharmacokinetic and Pharmacodynamic Interaction between Metformin and (−)-Epigallocatechin-3-gallate

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Abstract

(−)-Epigallocatechin-3-gallate (EGCG), a major flavonoid in green tea has multiple health benefits including chemoprevention, anti-inflammatory, anti-diabetic, and anti-obesity effects. In connection with these effects, EGCG can be a candidate to help the treatment of metabolic diseases. Metformin is a widely used anti-diabetic drug regulating cellular energy homeostasis via AMP-activated protein kinase (AMPK) activation. Therefore, the combination of metformin with EGCG may have additive or synergistic effects on treatment of type 2 diabetes. Nevertheless, there is no report for the pharmacokinetic and/or pharmacodynamic interaction of EGCG with metformin. Here, we evaluated the pharmacokinetic and pharmacodynamic interaction between metformin and EGCG in rats. Pharmacokinetics parameters of metformin were measured after oral administration of metformin in rats pre-treated with EGCG (10 mg/kg) or saline for 7 days. The results showed that there is no significant difference in pharmacokinetic parameters between saline control and EGCG-treated group. In addition, the hepatic AMPK activation by metformin in EGCG-treated rats was also similar to the control. The lack of additive effects of EGCG on AMPK activation or intracellular uptake of metformin was also evaluated in cells in the presence or absence of EGCG. Treatment of HepG2 cells with EGCG inhibited the metformin-induced AMPK activation. Combined results suggested that EGCG has no effect on the pharmacokinetics of metformin but may contribute to metformin action.

Keywords: (−)-Epigallocatechin-3-gallate, Metformin, Pharmacokinetics, AMP-activated protein kinase

Metformin is widely used for the treatment of hyperglycemia in patients with type 2 diabetes mellitus (T2DM). Metformin ameliorates hyperglycemia by reducing hepatic gluconeogenesis and by improving glucose utilization. The molecular mechanisms underlying antidiabetic effects of metformin appear to be related to the activation of AMP-activated protein kinase (AMPK), which suppresses glucagon-stimulated glucose production and causes an increase in glucose uptake in muscle and in hepatic cells. Metformin is a substrate of organic cation transporters (OCTs). It has been reported that the OCT1 mediated the intestinal absorption and hepatic distribution of metformin in rats and mice[1]. OCT1 also is required for the antidiabetic efficacy of metformin[2].

Green tea is one of the most widely consumed beverages worldwide. (−)-Epigallocatechin-3-gallate (EGCG), the major polyphenolic flavonoid in green tea, accounts for 50-80% of the tea catechins[3]. Among the many beneficial effects of EGCG[4-6], it lowers the incidence of streptozotocin-induced diabetes and reduces body weight, body fat, and blood levels of glucose in *fa*/*fa* obese rats[7,8]. In addition, EGCG protects pancreatic cells, enhances insulin activity, represses hepatic glucose production, reduces food uptake and absorption, stimulates thermogenesis and lipid excretion, and modulates insulin-leptin endocrine systems[7,9-13]. Epidemiologically, it has been suggested that green tea consumption prevents T2DM[14]. It was also reported that EGCG possesses pronounced anti-

diabetic efficacy in preclinical models of T2DM and suppress hepatic gluconeogenesis via AMPK activation[15]. Thus, the combination of metformin and EGCG may have an additive or synergistic effect. Based on these backgrounds, we evaluated the pharmacokinetic and pharmacodynamic interaction between EGCG and metformin in the present study.

Effect of EGCG Pretreatment on the Oral Pharmacokinetics of Metformin

The plasma concentration-time curves of metformin after oral administration at a dose of 100 mg/kg, with or without EGCG pretreatment, are shown in Figure 1. The estimated pharmacokinetic parameters are summarized in Table 1. The mean parameters of metformin pharmacokinetics were very similar between control and EGCG-treated group. There were no statistically significant differences in the plasma pharmacokinetic parameters of *AUC*, C_{max}, T_{max}, $T_{1/2}$, oral CL, and V_d for metformin (Table 1).

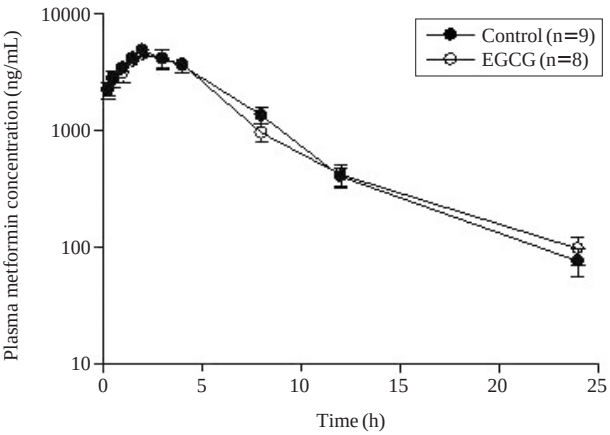

Figure 1. The plasma concentration-time curves of metformin after oral administration (100 mg/kg) in rats receiving EGCG (10 mg/kg, n=8) or saline (control group, n=9) for 7 days.

Effect of EGCG on AMPK Activation and OCT1 Expression *in vivo*

To assess the role of EGCG in metformin action *in vivo*, we examined the effects of metformin on AMPK activation in EGCG-treated rat liver tissues. Rats were intraperitoneally dosed with EGCG or vehicle (saline) for 7 days. One hour after intravenous administration of metformin, liver tissue was obtained for measurement of AMPK activation. After metformin treatment, phosphorylation of AMPK and ACC were not changed

Table 1. Pharmacokinetic parameters of metformin in rat plasma after metformin (100 mg/kg) oral administration with and without treatment of EGCG.

Parameter	Group	
	Control (n=9)	EGCG (n=8)
AUC (μg/h/mL)	28.95±5.60	27.28±4.33
C_{max} (μg/mL)	5.79±1.65	5.23±0.99
T_{max} (h)	2.67±0.97	2.50±0.76
$T_{1/2}$ (h)	3.57±0.81	3.81±0.81
Oral CL (mL/h)	3.56±0.64	3.74±0.56
V_d (L/kg)	18.35±5.61	20.40±4.03

Data are expressed as the mean±S.D. of 8 or 9 rats.
AUC: area under the plasma concentration-time curve, C_{max}: maximal peak plasma concentration, T_{max}: time to reach peak concentration, $T_{1/2}$: half-life, CL: clearance, V_d: volume of distribution.

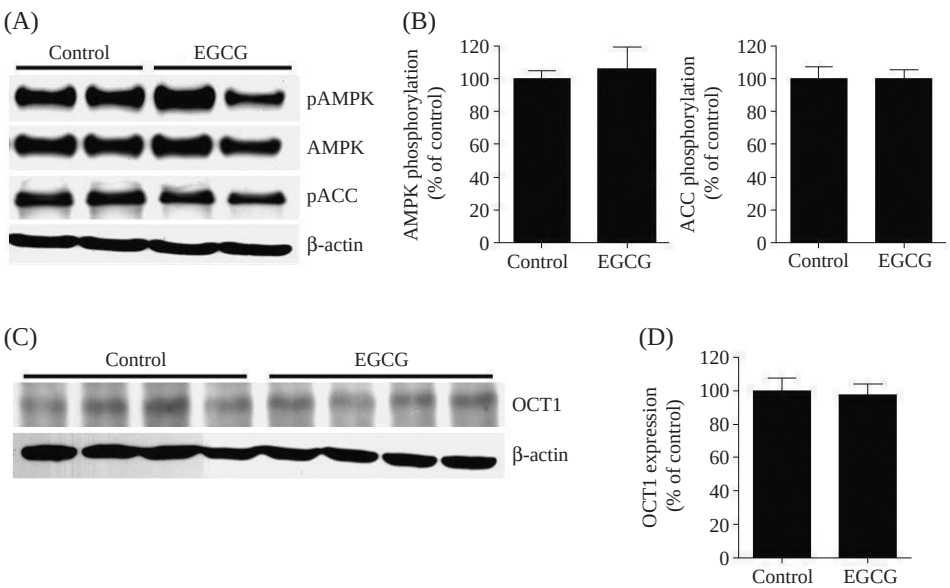

Figure 2. Effect of EGCG on the AMPK activation and OCT1 expression in rat liver. Rats were given EGCG (10 mg/kg, n=4) or saline (control group, n=4) for 7 days. Rats were sacrificed 1 h after i.v. administration of metformin (100 mg/kg). (A) and (C): Representative blot of phospho-AMPK (Thr^{172}), AMPK, phospho-ACC (Ser^{79}), OCT1 expression in rats treated with metformin and EGCG or saline. (B) and (D): Quantitation of phospho-AMPK (Thr^{172}), AMPK, phospho-ACC (Ser^{79}), OCT1 expression presented in (A) and (C).

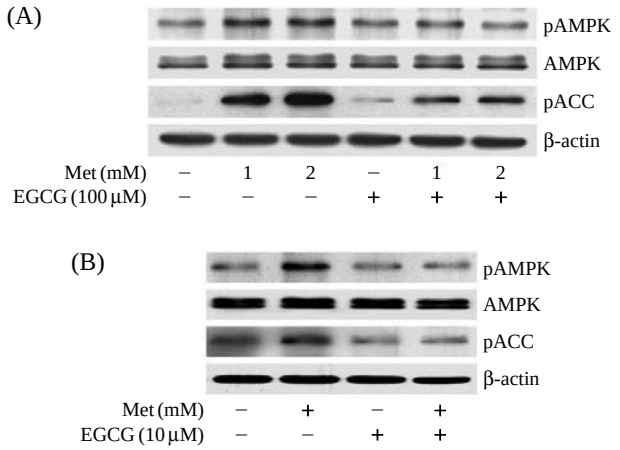

Figure 3. Effect of EGCG on the metformin-induced AMPK activation in HepG2 cells. Cells were treated with metformin (1 and 2 mM), EGCG (10 and 100 μM), or metformin plus EGCG for 6 h. The phosphorylation of AMPK and ACC were assessed by western blot analysis.

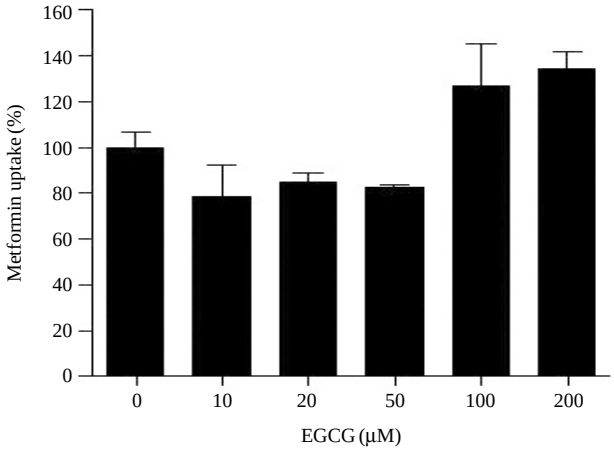

Figure 4. Effect of EGCG on the metformin uptake in HT-29 cells. Cells were pretreated with EGCG (10-200 μM) for 30 min and then treated with metformin (1 mM) for 1 h. Data represent the mean ± S.D. (n=3).

significantly in EGCG-treated rat liver (Figure 2A and B). In addition, OCT1 expressions in EGCG-treated rat were also similar to those of the control (Figure 2C and D).

Effect of EGCG on Metformin-stimulated AMPK and ACC Phosphorylation in HepG2 Cells

To understand the role of EGCG in metformin's pharmacological effects, we examined the effect of EGCG on metformin-induced AMPK activation in HepG2 cells. As shown in Figure 3A, metformin (1 mM and 2 mM) stimulated phosphorylation of AMPK and ACC. EGCG (100 μM) alone slightly increase the phosphorylation of AMPK and ACC. However, EGCG inhibited the activation of AMPK by metformin. Furthermore, we examined the effect of EGCG at lower concentration on AMPK activation. As shown in Figure 3B, EGCG did not stimulate the phosphorylation of AMPK and ACC at 10 μM. However, metformin-induced the phosphorylation of AMPK and ACC was also blocked by ECCG at 10 μM.

Effect of EGCG Pretreatment on the Metformin Uptake in HT-29 Cells

We investigated the effect of EGCG on metformin uptake by human HT-29 intestinal tumor cells. The expression of OCT1 mRNA in the HT-29cells was confirmed by RT-PCR (data not shown). Triplicate experiments showed that pretreatment of EGCG (10-50 μM) slightly decreased the metformin uptake in HT-29 cells. However, the uptake of metformin was slightly increased by the treatment of 100 and 200 μM EGCG (Figure 4). The increase or decrease of metformin uptake into the cells by EGCG did not reach the statistical significance due to the limited number of group (n=3).

Discussion

The green tea flavonoid EGCG is one of several compounds that have been reported to have glucose-lowering properties in mammals[7]. The increasing popularity of EGCG and the prevalence of T2DM worldwide suggest that there is a strong possibility of the combination of diabetes medications with dietary supplements such as EGCG. Therefore, this study was designed to determine whether the combination of EGCG with metformin could alter pharmacokinetic parameters and therefore affect on the efficacy of metformin.

Contrary to our expectation, our results show that it appears to be no important pharmacokinetic interaction between EGCG and metformin, although the possibility that EGCG may affect the pharmacokinetics of metformin in human still exists. EGCG are rapidly converted to metabolites through glucuronidation, sulfation, methylation, and ring fission[16], thus, the low bioavailability of EGCG may result in the lack of interaction with metformin in our study.

Metformin, a known AMPK activator, shows its pharmacological effect in the liver. In the present study, the *in vitro* pharmacodynamic property of metformin was confirmed by the AMPK activation in human HepG2 hepatocytes. Our results show that EGCG at 100 μM slightly activates AMPK compared

with the initial level, but does not activate at 10 μM. Moreover, EGCG has an inhibitory effect on the metformin induced-AMPK activation in HepG2 cells (Figure 3). It was reported previously that EGCG activates AMPK[15,19,20], and the activation of AMPK is associated with the inhibition of adipose differentiation and the apoptosis in colon cancer cells[17,18]. One of the disparities between our study and the previous studies[15,19,20] is the different experimental conditions such as the high glucose condition and different cell culture model. Our results showed that metformin-induced activation of AMPK and ACC in livers of EGCG-treated rat was similar to those of control rat (Figure 2A and B). Furthermore, we did not observe a significant difference in the expression of OCT1 in the liver between control and EGCG-treated rat (Figure 2C and D). OCT1 plays the key role in metformin action[2]. Because the EGCG treatment was no effect on OCT1 expression, the effect of metformin on AMPK activation may also similar in the liver. Another possible explanation is that EGCG was rapidly metabolized and therefore may not produce a significant effect *in vivo* in rats.

The absorption of metformin by intestinal epithelium may also contribute the metformin action. HT-29 cells express OCT1 that may be responsible for metformin uptake. Metformin uptake was increased by 100 and 200 μM of EGCG (Figure 4), although we only found a trend without statistical significance. EGCG (2 mM) increased the uptake of model organic cation[21]. On the other hand, in lower concentrations, EGCG decreased the metformin uptake in HT-29 cells. The inhibition of metformin-induced AMPK activation by EGCG (10 μM) in HepG2 cells may be a consequence of the decrease of metformin uptake. However, our results showed that 100 μM of EGCG also significantly inhibited activation of AMPK by metformin (Figure 3A), although EGCG (100 μM) increased the uptake of metformin (Figure 4). Therefore, the inhibitory effect of EGCG on the metformin-induced AMPK activation is not related to the extent of uptake of metformin via OCT1. Therefore, EGCG appears to inhibit the metformin-induced AMPK activation through other mechanisms distinct from metformin uptake. Further clinical studies are necessary to determine the effect of EGCG on metformin efficacy in healthy individuals or in patients with T2DM.

In conclusion, this study suggested that EGCG does not affect on the pharmacokinetics of metformin and hepatic AMPK activation by metformin *in vivo*. Although, EGCG inhibits the metformin-induced AMPK activation and modulates the intracellular accumulation of metformin *in vitro*, further clinical studies are necessary to conclude the pharmacodynamic interaction between EGCG and metformin.

Materials & Methods

Chemicals and Cell Culture

EGCG and metformin were purchased from Sigma (St Louis, MO, USA). Primary antibodies against phospho-AMPK (Thr^{172}), AMPK, phospho-ACC (Ser^{79}) (Cell signaling Technology, Beverly, MA, USA), OCT1 (Santa Cruz Biotechnology, California, USA), and β-actin (Sigma, St Louis, MO, USA) were purchased commercially. Cell culture media and reagents were from Gibco-BRL (Grand Island, NY, USA). All other chemicals and solvents were of the highest grade or HPLC grade commercially available. The intestinal epithelial cell line HT-29 and human hepatoma cell line HepG2 were obtained from the American Type Culture Collection (Manassas, VA, USA) and grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and penicillin/streptomycin at 37°C in an atmosphere of 5% CO_2.

Measurement of Cellular Metformin Uptake

For metformin uptake study, HT-29 cells were seeded in 6-well plates at a density of 1×10^6 cells/well. After attachment, cells were pre-incubated for 30 min with EGCG and further exposed to metformin (1 mM) for 1 h. At the end of incubation, the medium was removed and the cells were washed twice in ice-cold PBS. The cells were lysed in PBS by sonication. Then, intracellular levels of metformin were determined by HPLC analysis of cell lysate.

Animals and Treatment

Male Sprague-Dawley rats (Japan SLC Inc., Shizuoka, Japan) weighting 250-380 g were used in this study. The animals were housed individually in a temperature (22 ± 2°C) - and humidity (30-40%) - controlled room under a 12 h light/dark cycle with free access to food and water. After acclimation for 1 week, the animals were randomly divided into two groups. For EGCG-treated group (n=8), rats were administered intraperitoneally with EGCG (10 mg/kg) for 7 days. EGCG dissolved in saline and freshly prepared daily. Control rats (n=9) received saline of same volume with EGCG group. Rats were fasted overnight before the oral administration of metformin. Blood samples were collected from tail vein at 0.25, 0.5, 1, 1.5, 2, 3, 4, 8, 12, and 24 h after administration of a single dose of metformin (100 mg/kg). Metformin at doses of 100 mg/kg was also administered intravenously via the tail vein of rats treated with EGCG (n=4) or saline (n=4)

for 7 days, and blood samples were collected by cardiac puncture at 1 h after intravenous administration of metformin. Blood samples were centrifuged at 3,000 rpm for 10 min at 4˚C. Plasma was stored at −70˚C until HPLC analysis. Liver tissues were removed quickly, frozen in liquid nitrogen and kept at −70˚C. This study was approved by the Animal Care and Handling Committee of Inha University (Incheon, South Korea).

HPLC analysis of Metformin

The concentrations of metformin in the plasma and cell lysate were determined by HPLC. Fifty microliters of samples were deproteinized with 100 μL of acetonitrile. After vortex-mixing and centrifugation at 16,000 × g for 10 min, the supernatant was injected into a HPLC system (Shiseido Nanospace SI-2 HPLC; Tokyo, Japan). Metformin was separated using a Shodex ODP2 HP-2D column (150 mm × 2.0 mm i.d.) maintained at 35˚C. The mobile phase was comprised of 10 mM KH_2PO_4 : 10 mM K_2HPO_4 (1 : 1) and acetonitrile at the ratio of 47.8 : 52.2 (v/v). The flow rate of the mobile phase was 0.2 mL/min. The mobile phase was filtered through a Millipore 0.45 μm filter and degassed prior to use. The UV detector was set at 235 nm and connected to an S-MicroChrom chromatographic data system (Scientific Software, Shiseido, Tokyo, Japan). The sensitivity for the detection of metfromin (limit of quantification) was 50 ng/mL and there was linearity in range between 50-25,000 ng/mL.

Determination of Pharmacokinetic Parameters

The pharmacokinetic parameters were calculated using the BA Calc 2007 program[22]. The plasma concentration-time curves were analyzed using Prims® Version 4.0 (GraphPad Software Inc., San Diego, CA, USA).

Western Blot Analysis

For the preparation of whole cell lysates, cells were washed with ice-cold PBS and then scraped into ice-cold lysis buffer (10 mM sodium phosphate with pH 7.2, 1% Triton X-100, 1 mM NaF, 150 mM NaCl, 1 mM EDTA, 1 mM Na_3VO_4, 0.1% SDS, and protease inhibitors). Frozen liver tissues were homogenized in lysis buffer. Total protein extracts were obtained by centrifugation at 1,000 × g for 10 min after sonication. Protein contents were quantified using the BCA microprotein assay kit (Pierce, Rockford, IL, USA). Fifteen micrograms of total protein were separated by 10% SDS-PAGE. After electrophoresis, proteins were transferred electronically to PVDF membranes (Immobilon-P, Millipore). The membrane were blocked with 5% bovine serum albumin in Tris-buffered saline (TBS) containing 0.1% Tween 20 (TBS-T) for 1 h at room temperature and incubated with specific primary antibodies overnight at 4˚C. Unbound primary antibody was removed by washing with TBS-T and the membrane was incubated for 2 h at room temperature with the appropriate secondary antibodies conjugated with horseradish peroxidase (1 : 5,000-10,000). After washing, the membrane were briefly incubated with a Picosignal ECL system (Pierce, Rockford, IL, USA) and exposed to an X-ray film. Results were analyzed using Bio-Profil Bio1D software (Vilber Lourmat, Paris, France).

Statistical Analysis

Statistical analysis was performed using Prism version 4.0. Data are presented as means ± standard deviations (S.D.). Two-tailed Mann-Whitney *U* test was used to test a significant difference.

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