Effects of (−)-epigallocatechin-3-O-gallate on expression of gluconeogenesis-related genes in the mouse duodenum

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ABSTRACT
Green tea has been shown to have many beneficial health effects. We have previously reported that dietary (−)-epigallocatechin-3-O-gallate (EGCG), the major polyphenol in green tea, reduced gene expressions of gluconeogenic enzymes, glucose-6-phosphatase (G6Pase) and phosphoenolpyruvate carboxykinase (PEPCK), in the normal mouse liver. In the present study, we examined the effects of intragastrical administration of EGCG on the expression of gluconeogenesis-related genes in the mouse intestine. The results of experiments with the semi-quantitative reverse transcription-polymerase chain reaction indicated that EGCG at 0.6 mg/head caused a reduced expression of G6Pase, PEPCK, hepatocyte nuclear factor 1α (HNF1α), and HNF4α. Experiments using the quantitative real-time polymerase chain reaction confirmed these effects. We then examined the effects of EGCG using human colon carcinoma Caco-2 cells stimulated with dexamethasone and dibutyryl cAMP. The results were generally consistent with those from the experiments in vivo. The present findings suggest EGCG to contribute to the beneficial effects of green tea on diabetes, obesity, and cancer by modulating gene expression in the intestine.

Tea is manufactured from the leaves of the plant Camellia sinensis Kuntze (Theaceae) and has been regarded to possess anti-cancer, anti-obesity, anti-atherosclerotic, anti-diabetic, anti-bacterial, and antiviral effects (6, 12, 14, 31, 33). Many of the beneficial effects of green tea are related to the actions of (−)-epigallocatechin gallate (EGCG), a major component of green tea catechins (14, 27, 33) (Fig. 1). EGCG has been reported to repress glucose production in rat hepatoma H4IIE cells through down-regulation of the gene expression of gluconeogenic enzymes, glucose-6-phosphatase (G6Pase) and phosphoenolpyruvate carboxykinase (PEPCK) (30). The finding suggests a beneficial effect of green tea and EGCG on type 2 diabetes, since the disease is associated with enhanced glucose production in the postabsorptive state (30).

Besides the liver and kidney, these gluconeogenesis-related enzymes are present in the intestine, exhibiting a decreasing gradient of expression from the duodenum to the ileum. The significance of the intestinal expression of gluconeogenic enzymes in contribution to blood glucose levels has been reported (16, 17, 21, 22, 29), although some authors presented conflicting findings (20). Since PEPCK has also an important role in glyceroenogenesis (22, 28), any alteration to its expression would affect the homeostasis of triglycerides in the intestine and eventually in the whole body.

We have previously reported that the administration of EGCG caused a reduction in the gene expression of G6Pase and PEPCK in the mouse liver (13) in line with the findings in vitro (30). Wolfram et al. (32) observed similar effects of TEAVIGO, a
commercial product of EGCG, on gene expression in liver and adipose tissues of db/db mice. Since EGCG in tea beverages can be delivered directly to the gastrointestinal tract, it may affect the expression of gluconeogenesis-related genes in the gut, but this problem has not been studied so far. In the present study, we examined the effects of EGCG on gene expression in the mouse duodenum and in colonic carcinoma Caco-2 cells having some characteristics similar to intestinal epithelial cells (2, 5, 9). Since our previous results showed the EGCG-induced changes in the hepatic gene expression of a gluconeogenesis-related transcription factor, hepatocyte nuclear factor (HNF)4α, in association with those of G6Pase and PEPCK (34), and since HNF1α is also an important factor in the intestine (9), we examined here the expression of these four genes.

MATERIALS AND METHODS

Chemicals. Dexamethasone (Dex), dibutyryl-cAMP (dcAMP), and N-acetylcysteine (NAC) were obtained from Wako Pure Chemical Industries Ltd. (Osaka, Japan) and dissolved in dimethyl sulfoxide for use. TEAVIGO was kindly provided by DMS Nutrition Japan (Tokyo, Japan) and used as EGCG. (−)-Epicatechin (EC), (−)-epigallocatechin (EGC), and (−)-epicatechin gallate (ECG) were obtained from Funakoshi Chemicals (Tokyo, Japan). Antibodies against PEPCK (sc-74825), β-actin, and hepatocyte nuclear factor 4α (HNF4α, sc-6556), and horseradish peroxidase-conjugated bovine IgG against goat IgG (sc-2350) were from Santa Cruz Biotechnology, Inc. (California, USA), and HNF1α (GTX113850S) was from GeneTex, Inc. (California, USA).

Animal experimental design. Ethical approval for the study was obtained from the Committee for Animal Experimentation of the University of Shizuoka. Male Balb/c mice (7 weeks old, 22–25 g) were purchased from Shizuoka Laboratory Animal Center (Shizuoka, Japan). They were maintained with standard solid laboratory chow MF (Oriental Yeast Co. Ltd., Tokyo, Japan). After one week, the animals were divided into two groups of 5 to give the groups without significant differences in body weight: the control group and the group given EGCG at a dose of 0.6 mg/head. Animals received EGCG in water intragastrically at around 10 am and were killed after 4 h under ether-anesthesia to provide the duodenum samples. Separate experiments were done using different dosages of EGCG: 0.1 mg and 1.2 mg/head, while the control groups were treated with water. The samples were kept at −85°C in RNA later (Takara Bio Co. Ltd., Tokyo, Japan).

Cells and treatment. Human colonic carcinoma Caco-2 cells were obtained from Riken BRC Cell Bank (Ibaraki, Japan) and maintained in 10% fetal bovine serum in Dulbecco’s Minimum Essential Medium (DMEM) containing 4,500 mg/L glucose, 50 U/mL penicillin, 50 μg/mL streptomycin, and 2.5 μg/mL amphotericin B at 37°C under a 5% CO2 atmosphere. For stimulation, the cells were serum-starved for 24 h and then incubated with 0.1 mM dcAMP and 500 nM Dex in the serum-free DMEM containing 1,000 mg/L glucose for 4 h at 37°C as used for H4IIE cells (30). The highest concentration of EGCG was set at 50 μM on the basis of previous studies which examined the effects of EGCG at 0–100 μM on rat hepatoma H4IIE cells (30, 32).

Cell viability. Cell suspensions were prepared by trypsinization and a trypan blue dye exclusion assay was performed as reported previously (11).

Reverse transcription-polymerase chain reaction (RT-PCR) and quantitative real time PCR (Q-PCR). Total RNA was extracted from the duodenum and mRNA was prepared using a QIAamp RNA Blood Mini Kit (Qiagen Ltd., Tokyo, Japan) according to the manufacturer’s directions. To prevent possible contamination, samples were treated with deoxyribonuclease (RT-grade; Wako Pure Chemical Indus-
items Ltd.) as recommended by the manufacturer (1, 34). RT-PCR was performed as described previously (1, 34) using primers purchased from TaKaRa Bio., Tokyo, Japan (Table 1). Amplified DNA was subjected to electrophoresis in 2% agarose, stained with SYBR Green I (Molecular Probes Ltd., Oregon, USA), and imaged using a FluorImager (Molecular Dynamics Tokyo Ltd., Tokyo, Japan) as described previously (1). Expected sizes of amplified DNA were 139 bp, 133 bp, 97 bp, 109 bp, and 171 bp for G6Pase, PEPCK, HNF1α, HNF4α and β-actin, respectively. Q-PCR was performed using the Thermal Cycler Dice (TaKaRa Bio.) as described previously (1, 34). Primers were purchased from TaKaRa Bio. and are listed in Table 1.

**Western blotting.** Caco-2 cells were stimulated with dCAMP/Dex in the presence or absence of EGCG for 8 h. Cells were lysed in 50 mM Tris-HCl (pH 7.5) containing 150 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, and 0.1% sodium dodecyl sulfate and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Blotted PVDF membranes were probed with relevant antibodies and visualized with chemiluminescence as reported (19).

**Statistical analysis.** Data were analyzed by one-way ANOVA and presented as the mean ± standard error (SE) and *P* < 0.05 was considered significant.

**RESULTS**

*Effects of EGCG on gene expression in the mouse duodenum*

Among tea catechins, EGCG has been studied for health effects most extensively. In the present study, we first examined the effects of EGCG administered intragastrically to the mice. Animals were given EGCG (0.6 mg/head) and the duodenum was dissected after 4 h. The gene expression of G6Pase, PEPCK, HNF1α, and HNF4α was examined by RT-PCR (Fig. 2). The results of densitometric determination indicated that EGCG caused a reduction in gene expression of about 46, 70, 35, and 48% in average for G6Pase, PEPCK, HNF1α, and HNF4α, respectively, as compared with the vehicle-treated control. These suppressive effects of EGCG were confirmed by Q-PCR (Fig. 3A).

**Table 1** Primers used for RT-PCR and/or Q-PCR

<table>
<thead>
<tr>
<th>Mouse gene</th>
<th>Forward primer</th>
<th>Reverse primer</th>
</tr>
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<tbody>
<tr>
<td>G6Pase</td>
<td>5'-GTGCACTGGAACGTCTGTCG-3'</td>
<td>5'-TCCGGAGGCTGCGATTGTA-3'</td>
</tr>
<tr>
<td>PEPCK</td>
<td>5'-GTGGTTTGAAGGACCCGAGA-3'</td>
<td>5'-GAGCAAGGCTGCAATTTTG-3'</td>
</tr>
<tr>
<td>HNF1α</td>
<td>5'-CTGGAAACATCCACGTGGC-3'</td>
<td>5'-TTGGAACTCTGCTGATACAC-3'</td>
</tr>
<tr>
<td>HNF4α</td>
<td>5'-CCGGGCTGCAGAAGCAGTG-3'</td>
<td>5'-TGGCAGGACAGTCTGAGCCATC-3'</td>
</tr>
<tr>
<td>ß-Actin</td>
<td>5'-CATCCGTAAAGACCTCTATG-3'</td>
<td>5'-ATGGAGGCAACGCCATCCA-3'</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Human gene</th>
<th>Forward primer</th>
<th>Reverse primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>G6Pase</td>
<td>5'-AGGCCCAGCGCTTGGAGATCTAA-3'</td>
<td>5'-ATTAGCACATGCGCTGCGCATAC-3'</td>
</tr>
<tr>
<td>PEPCK</td>
<td>5'-GGCTATCTTTCTGGAGCGCGTG-3'</td>
<td>5'-GAGAGTTTGCGGAAGTGTAG-3'</td>
</tr>
<tr>
<td>HNF1α</td>
<td>5'-TTCGTAATTTGTACAGAAGCATC-3'</td>
<td>5'-ATGCCACACCAAGGCCATTCT-3'</td>
</tr>
<tr>
<td>HNF4α</td>
<td>5'-GTCCAAAGGACGTCTGCTCCCTA-3'</td>
<td>5'-ATGGACACCCGCTGCTCATC-3'</td>
</tr>
<tr>
<td>ß-Actin</td>
<td>5'-TGGACCACCCGACACATGAA-3'</td>
<td>5'-CTAAGTCATAGTCCGCTAGAAGCA-3'</td>
</tr>
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</table>
The effects on the gene expression of G6Pase and PEPCK in the animals given 0.1 and 1.2 mg/head of EGCG as determined by Q-PCR are shown in Figs. 3B and 3C, respectively. These findings indicated that the administration of 0.1 mg/head and 0.6 mg/head of EGCG caused the lowest expression of the G6Pase and PEPCK genes in the duodenum, respectively, among three experimental conditions different in the EGCG dose. The gene expression of HNF1α and HNF4α was the lowest in the group given 0.6 mg/head. It was noted that EGCG at 1.2 mg/head had little effects on the gene expression of G6Pase and HNF4α in the duodenum (Fig. 3C). In addition, EGCG up-regulated the HNF1α gene expression (Fig. 3C), but its biological significance was unclear at moment. A similar but different regulation of the gene expression of G6Pase and PEPCK has often been reported (3, 34).

**Effects of EGCG on gene expression in Caco-2 cells**

Caco-2 cells were stimulated with dcAMP/Dex for 4 h, since previous studies used dcAMP/Dex-stimulated H4IIE cells, a cell line of rat hepatoma (30, 34), and the gene expression of G6Pase and PEPCK was examined by Q-PCR. The stimulation caused about 3.5-fold and 10-fold increases in the gene expression of G6Pase and PEPCK, respectively (Fig. 4) as compared with the non-stimulated (basal) level. The up-regulation of the gene expression of these enzymes by dcAMP/Dex has been reported in the case of H4IIE cells and mouse primary hepatocytes (7, 30, 34).
When EGCG (50 μM) was included in the culture medium, the stimulated levels of gene expression of G6Pase and PEPCK were reduced to 50% and 40%, respectively, of the levels without EGCG (Fig. 4). Cell viability was found to be more than 90% in each case, indicating no cytotoxicity of EGCG even at the highest concentration studied (50 μM). Insulin repressed the up-regulated expression of these genes in Caco-2 cells (Fig. 4). Previous studies have shown that insulin repressed the gene expression of G6Pase and PEPCK in H4IIE cells (30, 34), and that of G6Pase in the duodenum of diabetic rats (21). Thus, EGCG had effects similar to insulin on colonic cancer and hepatoma cells, and the duodenum. For comparison, three other tea catechins (Fig. 1) were examined and the results showed that EC and ECG had no effect (Fig. 4). EGC repressed the gene expression of G6Pase, but not of PEPCK. The highest level of the activity of EGCG among catechins examined was in line with the previous result for H4IIE cells (30).

The dose-dependent effects of EGCG were found on the expression of these genes in dcAMP/Dex-stimulated cells (Fig. 5A). The suppressive effects of EGCG were demonstrated at the level of protein expression (Fig. 5B). Densitometric determination showed that the protein levels of PEPCK, HNF1α, and HNF4α in the cells treated with 50 μM EGCG were 0.44, 0.57, and 0.74 of those in the control cells, respectively.

Effects of NAC

Previous study demonstrated that anti-oxidant NAC attenuated EGCG’s effects on H4IIE cells (30). Similar experiments showed that NAC inhibited the effects of EGCG on Caco-2 cells as well (Fig. 6).

DISCUSSION

EGCG has been shown to decrease the mRNA expression of PEPCK in the liver and adipose tissue of db/db mice (32), and in the liver of normal mice (13). It has also been shown to alleviate diabetes in rodents (32). EGCG is contained in green tea, black tea, and oolong tea (12). Dry green tea leaves contain about 30% (w/w) catechins and a single cup of green tea may contain 30–130 mg of EGCG (12, 23). Thus, the gastrointestinal tract has direct contact with EGCG after the ingestion of tea (1), and a gene microarray analysis has indicated that EGCG affects the expression of many genes in the mouse intestine (25). However, no data have been reported for the effects of EGCG on the intestinal gene expression of gluconeogenic enzymes.

In the present study, we found that EGCG repressed the gene expression of two gluconeogenic enzymes, G6Pase and PEPCK, in the mouse duodenum (Figs. 2 and 3). EGCG also repressed the gene expression of HNF1α and HNF4α. Since these transcription factors act to enhance the expression of G6Pase and PEPCK genes by binding to the respective promoter region (4, 9, 15, 26), it is conceivable that the reduced expression of HNFs is related to EGCG’s suppressive effects on the gene expression of G6Pase and PEPCK. Our previous study suggested that insulin and EGCG suppressed the gene expression of G6Pase and PEPCK genes up-regulated by dcAMP/Dex by decreasing the expression of the HNF4α gene in H4IIE cells (34). However, the mechanism by which EGCG suppresses the gene expression of HNF1α and HNF4α remains elusive.

In the case of the highest dose of EGCG (1.2 mg/head), it appeared difficult to explain the changes in the gene expression of G6Pase, HNF1α and HNF4α on the basis of findings in the lower doses (Fig. 3). This dose corresponds to 48 mg/kg, the amount contained in about 20 cups of green tea for a person with a body weight of 50 kg, and may be too high for evaluation of its physiological effect. The changes in the expression of genes studied here were not found in the previous study using a gene microarray for animals given EGCG at 200 mg/kg (25). This is
probably because of a much higher dose used, since we found no change in the gene expression of G6Pase at the highest dose (1.2 mg/head or about 48 mg/kg) in the present investigation (Fig. 3C).

To confirm the in vivo findings, we examined the effects of EGCG using Caco-2 cells. These cells have been used for the intestinal expression of gluconeogenic enzymes, G6Pase and PEPCK, and that of related transcription factors, HNF1α and HNF4α (5, 9, 18, 24, 35). When stimulated with dCAMP/Dex, the gene expression of G6Pase and PEPCK was up-regulated (Fig. 4), suggesting the similarity in this modulation between intestinal and hepatic expression of these enzymes, since similar effects were found in hepatoma H4IIE cells and mouse primary hepatocytes (7, 30). The results from the experiments using Caco-2 cells were consistent in general with those from the animal experiments. EGCG suppressed dose-dependently the expression of four genes related to gluconeogenesis (Fig. 5A).

Fig. 5 Concentration-dependent effects of EGCG on the expression of gluconeogenesis-related genes in Caco-2 cells. The cells were stimulated with Dex/dcAMP in the absence (Control, 0) or presence of EGCG (10, 20, 50 μM) for 4 h. The expression of G6Pase, PEPCK, HNF1α, and HNF4α was determined by Q-PCR. The results were normalized using the level of β-actin, and are expressed as the mean ± SE relative to that for Control (100%) from 3 determinations. The data with a different mark (a–d) are significantly different at \( P < 0.05 \) (A). For Western blotting, Caco-2 cells were stimulated with Dex/dcAMP in the absence (Control, 0) or presence of EGCG (10, 20, 50 μM) for 8 h, and the protein expression of PEPCK, HNF1α, and HNF4α was determined using relevant antibodies (B). β-Actin was used as the internal reference protein.

Among various tea catechins (Fig. 1), EGCG showed the highest activity (Fig. 4), a similar finding to that reported for H4IIE cells (30). EGCG appears
to regulate the gluconeogenesis-related genes by modulating the redox state of the cell as proposed for H4IIE cells (30), since EGCG’s effects were attenuated by NAC (Fig. 6). However, the precise mechanism of EGCG’s action awaits future investigation.

Although the significance of the intestinal expression of PEPCK has not been established, we confirmed the gene expression of gluconeogenic enzymes and related transcription factors in the mouse duodenum. Assuming that glucose produced by intestinal gluconeogenesis contributes to the glucose in portal blood (8, 16), its suppression by EGCG may be beneficial for controlling diabetes. PEPCK is also important to regulate glyceroneogenesis in adipose and liver tissues (10, 28). Although the role of PEPCK in intestinal glyceroneogenesis has not been clearly defined (22), the suppression of PEPCK by EGCG may lead to less synthesis of triglycerides in the tissue. This might result in a change in the homoeostasis of fat metabolism, which should be examined in future studies. EGCG is also a candidate for a chemopreventive agent, since HNF4α was proposed as a target for the inhibition of colorectal cancer (24).

Intestinal G6Pase might be involved in the absorption of glucose from the small intestine (17), and its decreased expression caused by EGCG may contribute to the lower glucose absorption. This notion would provide another point of view to propose that EGCG may be beneficial for the prevention of diabetes and obesity.

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REFERENCES


