Effects of a catechin-free fraction derived from green tea on gene expression of gluconeogenic enzymes in rat hepatoma H4IIE cells and in the mouse liver

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ABSTRACT

Many biological activities of green tea have been attributed to a major constituent, (−)-epigallocatechin gallate (EGCG). We previously reported that EGCG and a catechin-rich green tea beverage modulated the gene expression of gluconeogenic enzymes, glucose-6-phosphatase (G6Pase) and phosphoenolpyruvate carboxykinase (PEPCK), in the mouse liver. However, it remains to be examined whether or not a constituent other than EGCG contributes to the change in gene expression of these enzymes. In this study, we separated the hot water infusion of green tea leaves (GT) into an ethanol-soluble fraction (GT-E) and an EGCG-free water-soluble fraction (GT-W), and examined their effects using rat hepatoma H4IIE cells. The inclusion of GT, GT-E, and GT-W in the culture medium reduced the gene expression of G6Pase and PEPCK. GT-W caused a decrease in expression of the transcription factor HNF4α. Reduced levels of PEPCK and HNF4α proteins were demonstrated in the cells treated with GT-W. GT-W showed an activity similar to insulin, but different from EGCG. Administration of GT-W to mice for 4 weeks reduced the hepatic expression of G6Pase, PEPCK, and HNF4α. These results suggest that green tea contains some component(s) with insulin-like activity distinguishable from EGCG and that drinking green tea may help to prevent diabetes.
were cultured in serum-free medium for 24 h and then incubated for 4 h in the presence of GT, GT-E or GT-W. The highest concentration of GT and GT-W was set at 0.5 mL per 20 mL of the culture medium, since a similar amount of green tea beverage was used in a previous study (14). In the case of GT-E, the culture medium (20 mL) received the amount equivalent to that contained in 0.5 mL of GT. When necessary, DMSO was added to make the final concentration equivalent in the culture medium. For the determination of cell viability, a cell suspension was prepared by trypsinization and a trypan blue dye exclusion assay was performed as reported (4). In some experiments, the serum-starved cells were stimulated with 500 nM Dex and 0.1 mM dcAMP in serum-free culture medium (12, 14) and the effects of inhibitors were examined as described previously (12).

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). After feeding on a standard laboratory diet (MF) for 1 week, the animals were divided into three groups of 5 without significant differences in body weight: the control group, the 0.2% GT-W group, and the 0.5% GT-W group. They were killed after 4 weeks under ether-anesthesia to provide the liver samples. The samples were kept at −85°C in RNAlater (Takara Bio Inc., Otsu, Japan).

Quantitative real-time PCR (Q-PCR). Total RNA extracted from the cells and the liver was used for preparation of mRNA using a QIAamp RNA Blood Mini Kit (Qiagen Ltd., Tokyo, Japan) according to the directions provided. To prevent possible contamination, samples were treated with deoxyribonuclease (RT-grade, Wako Pure Chemical Industries Ltd.) as recommended by the manufacturer (1, 14). Q-PCR was performed using a Thermal Cycler Dice (Takara Bio) as described previously (1, 14). Primers were purchased from Takara Bio and are listed in Table 1.

Western blotting. H4IIE cells were cultured with GT-W for 8 h. The 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 dodecylsulfate (SDS) and subjected to SDS-polyacrylamide gel electrophoresis. Blotted PVDF membranes were probed with relevant antibodies.
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Effects on gene expression of insulin-like growth factor binding protein 1 (IGFbp1)
The IGFbp1 gene has an insulin response sequence similar to that of PEPCK and insulin represses the gene expression of IGFbp1 (7, 10). To see if GT-W has insulin-like properties, we examined its effect on the gene expression of IGFbp1 (Fig. 4). The results indicated that GT-W showed an insulin-like activity in that it down-regulated the expression of this gene. In contrast, EGCG did not down-regulate the expression of this gene.

Effects of NAC (N-acetylcysteine)
Previous studies demonstrated that EGCG acted differently from insulin in that the anti-oxidant NAC attenuated EGCG’s effects on H4IIE cells but not insulin’s effects (12). When H4IIE cells were stimulated with Dex/dcAMP according to a method described previously (12), there were about 2- and 8-fold increases in the gene expression of G6Pase and PEPCK similar to findings reported previously (14) (Fig. 5). GT-W repressed the expression of these genes stimulated by Dex/dcAMP. NAC did not affect GT-W’s activity (Fig. 5), indicating that the change of the redox state in the cell was not related to the action mechanism of GT-W.

Effects of a phosphatidylinositol-3-phosphate kinase (PI3K) inhibitor
It has been shown that the actions of both insulin and EGCG are dependent on PI3K (12). To know if GT-W’s action is also dependent on PI3K, we examined the effects of the fraction on Dex/dcAMP-stimulated H4IIE cells according to a method described previously (12). The PI3K inhibitor LY294002 attenuated the effects of GT-W, suggesting that GT-W’s action is also dependent on PI3K (Fig. 6).

and visualized by chemiluminescence as reported (8).

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

RESULTS

Fractionation of GT
Extraction of the green tea leaves (2 g) with hot water (50 mL) yielded about 330 mg of dry powder of GT. GT was then extracted with warm ethanol to give GT-E (about 200 mg). The residues (130 mg) were used as GT-W. The results of TLC indicated that GT-E contained polyphenols corresponding to catechins including EGCG and that GT-W did not contain such low-molecular weight catechins (Fig. 1).

Effects of GT, GT-E, and GT-W on H4IIE cells
When H4IIE cells were incubated in the presence of GT (0.5 mL/20 mL), the results of Q-PCR showed that the gene expression of G6Pase and PEPCK decreased as compared with the untreated control (Fig. 2). GT-E and GT-W had similar effects (Fig. 2). The trypa blue exclusion assay indicated that these fractions did not affect cell viability under the conditions used (data not shown). The effects of GT-W on the gene expression of gluconeogenic enzymes were dose-dependent (Fig. 3A). In addition, dose-dependent down-regulation of the gene expression of the transcription factor HNF4α by GT-W was demonstrated. The suppressive effects of GT-W were also demonstrated at the level of protein expression of PEPCK and HNF4α (Fig. 3B).

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Primers used for Q-PCR</th>
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<tr>
<td>Rat gene</td>
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<tr>
<td>G6Pase</td>
<td>5’-AACGTCTGTCTGTCCTGGATCTAC-3’</td>
</tr>
<tr>
<td>PEPCK</td>
<td>5’-TGCCCCATCGAAGGACAAGTCA-3’</td>
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<tr>
<td>HNF4α</td>
<td>5’-TGAGCACCTGCTTGCTGGA-3’</td>
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<td>IGFbp1</td>
<td>5’-GAATGCCATTAGCAGCTACAGCAG-3’</td>
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<tr>
<td>β-Actin</td>
<td>5’-TGACAGGATGCAGAAGGAGA-3’</td>
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<tr>
<td>Mouse gene</td>
<td></td>
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<tr>
<td>G6Pase</td>
<td>5’-GTGCACTGGAACGTCTGTCTGTC-3’</td>
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<td>PGC1α</td>
<td>5’-CCGTAAAATCTGGGAGATGG-3’</td>
</tr>
<tr>
<td>β-Actin</td>
<td>5’-CATCCGAAGACCTATCTGCAAC-3’</td>
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gene expression of G6Pase and PEPCK was significantly lower in the mice treated with 0.5% GT-W than water-treated mice (Fig. 7). The gene expression of PEPCK in the mice treated with 0.2% GT-W was also significantly lower than that of the control. The gene expression of HNF4α was down-regulated significantly by treatment with GT-W (Fig. 7), suggesting that the transcription factor is intimately associated with the expression of gluconeogenic enzymes as described previously (14).

The results also showed that GT-W caused the reduction in the gene expression of peroxisome proliferator-activating receptor γ co-activator 1α (PGC1α) (Fig. 7). The level of PGC1α mRNA was shown to be elevated in the liver of experimental model mice with diabetes (15). Adenovirus-mediated overexpression of PGC1α resulted in the increased levels of mRNA of G6Pase and PEPCK in mouse primary
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Therefore, the reduced hepatic expression of the PGC1α gene in mice treated with GT-W could be correlated to the down-regulation of the gene expression of gluconeogenic enzymes (3, 15). In the case of H4IIE cells, correlation was not evaluated, since these cells do not express this gene (3).

DISCUSSION

Green tea has numerous beneficial effects on health and many of its biological activities have been attributed to a major constituent, EGCG (6, 9, 13). Our previous study showed that a fraction depleted of low-molecular weight catechins including EGCG from a catechin-rich green tea beverage affected the gene expression of gluconeogenic enzymes and might contribute to the change in hepatic gene expression in rats administered the beverage (1). In the present study, we examined whether an EGCG-free fraction from the hot water infusion of green tea leaves affects gene expression related to gluco-

Fig. 4  Effects of GT-W on gene expression of IGFbp1 in H4IIE cells. The cells were incubated with GT-W (0.5 mL/20 mL) for 4 h and the gene expression of IGFbp1 was determined by Q-PCR. The effects of 10 nM insulin and 50 μM EGCG were also examined. The vehicle-treated cells served as a Control. The results were normalized using the level of β-actin, and are expressed as the mean ± SEM relative to that for the Control (100%) from 3 determinations. *Significantly different from the Control at P < 0.05.

Fig. 5  Effects of NAC on the action of GT-W. H4IIE cells stimulated with Dex/dcAMP were treated with GT-W (0.5 mL/20 mL) in the absence or presence of 50 μM NAC for 4 h, and the expression of G6Pase and PEPCK was determined by Q-PCR. The results were normalized using the level of β-actin, and are expressed as the mean ± SEM relative to that for Dex/dcAMP-stimulated cells (100%) from 3 determinations. The effects of NAC alone are also presented. Values not sharing an alphabetic letter are significantly different at P < 0.05. *The levels were much lower for the unstimulated cells (Control) than the cells stimulated with Dex/dcAMP with statistical significance at P < 0.05.

Fig. 6  Effects of the PI3K inhibitor on the action of GT-W. Dex/dcAMP-stimulated H4IIE cells were treated with GT-W (0.5 mL/20 mL) in the absence or presence of 20 μM LY294002 (LY) for 4 h, and the expression of G6Pase and PEPCK was determined by Q-PCR. The results were normalized using the level of β-actin, and are expressed as the mean ± SEM relative to that for Dex/dcAMP-stimulated cells (100%) from 3 determinations. The effects of LY294002 alone are also presented. Values not sharing an alphabetic letter are significantly different at P < 0.05.

Fig. 7  Effects of GT-W on hepatic gene expression in mice. Animals were given either water (Control, n = 5), 0.2% GT-W (n = 5), or 0.5% GT-W (n = 5) for 4 weeks and gene expression was determined by Q-PCR. The results were normalized using the level of β-actin, and are expressed as the mean ± SEM relative to that for the Control (100%) from 3 determinations. *Significantly different from the Control at P < 0.05.
neogenesis.

We separated the dried green tea infusion (GT) with warm ethanol into ethanol-soluble (GT-E) and insoluble (GT-W) fractions. As expected, GT-W was devoid of catechins including EGCG (Fig. 1). When GT-W was included in the culture medium of H4IIE cells, the gene expression of gluconeogenic enzymes, G6Pase and PEPCK, was down-regulated dose-dependently (Figs. 2 and 3). A down-regulation of HNF4α expression was also found, suggesting its involvement in the action mechanism of GT-W. As has been discussed (14), HNF4α is closely associated with the regulation of gluconeogenic enzymes. The suppressive effects of GT-W were also demonstrated at the level of protein expression (Fig. 3B), confirming the results from Q-PCR.

Previous studies have shown that insulin but not EGCG down-regulates the gene expression of IGFBp1 (7, 10). In the present study, GT-W was found to down-regulate IGFBp1 expression like insulin, which was distinguishable from EGCG (Fig. 4). The difference between the actions of GT-W and EGCG was also demonstrated when the effect of an antioxidant NAC was examined. When H4IIE cells were stimulated with Dex/DCAMP according to a method described (12), there were about 2- and 10-fold increases in the gene expression of G6Pase and PEPCK (Fig. 5) as reported previously (14). NAC did not affect GT-W’s activity (Fig. 5). The finding indicates that the action mechanism of GT-W is distinguishable from that of EGCG, since EGCG’s action was attenuated by NAC in our previous study and others (12, 14). These studies also demonstrated that insulin’s action was not affected by NAC (12, 14). As reported previously, the actions of both insulin and EGCG are dependent on PI3K (12). Likewise, the GT-W’s action was dependent on PI3K, because the PI3K inhibitor attenuated the effect of GT-W (Fig. 6).

When GT-W was administered to the mice, no significant differences were observed in food consumptions among the control, 0.2% GT-W, and 0.5% GT-W groups. The GT-W diet caused the down-regulated expression of gluconeogenic enzymes, G6Pase and PEPCK in the mouse liver (Fig. 7). The reduced expression of the HNF4α and PGC1α genes could be correlated to the suppression of the gene expression of G6Pase and PEPCK (15).

Our previous study showed that the catechin-rich green tea beverage contained an EGCG-free fraction with activity to enhance the gene expression of PEPCK but not G6Pase (14). However, a similar fraction from the infusion of the green tea leaves repressed the gene expression of both PEPCK and G6Pase. The reason for this difference is not clear at present, but may be related to the difference between the ways to prepare the catechin-rich green tea beverage and the hot water extract of green tea leaves.

In summary, the present results suggest green tea to be beneficial for the prevention of diabetes by inhibiting the hepatic expression of gluconeogenic enzymes. They also revealed that green tea contains a fraction with insulin-like activity which is distinguishable from that of EGCG. The identification of an active compound may open the way to developing a new drug for diabetes.

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REFERENCES

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