

Short Communication

Green tea catechin enhances cholesterol 7 α -hydroxylase gene expression in HepG2 cells

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Green tea catechins are known to have hypocholesterolaemic effects in animals and human subjects. In the present study, we investigated the effects of green tea catechins on the mRNA level and promoter activity of hepatic cholesterol 7 α -hydroxylase (CYP7A1), the rate-limiting enzyme in the conversion of cholesterol to bile acids, in human hepatoma cells. Real-time PCR assays showed that different catechins, (–)-epicatechin gallate (ECG), (–)-epigallocatechin-3-gallate (EGCG), (–)-epigallocatechin (EGC) and (–)-epicatechin (EC), up regulated the CYP7A1 mRNA level by 5.5-, 4.2-, 2.9- and 1.9-fold, respectively, compared with the control. The –1312/+358 bp of the CYP7A1 promoter was subcloned into the pGL3 basic vector that includes luciferase as a reporter gene. ECG or EGCG significantly increased CYP7A1 promoter activity by 6.0- or 4.0-fold, respectively, compared with the control. Also, EGCG stimulated CYP7A1 at both mRNA level and promoter activity in a dose-dependent manner. These results suggest that the expression of the CYP7A1 gene may be directly regulated by green tea catechins at the transcriptional level.

Green tea catechins: Cholesterol 7 α -hydroxylase: CYP7A1: mRNA: Promoter activity: HepG2 cells

An increased blood cholesterol level is one of the major risk factors for the development of CVD⁽¹⁾. The level of plasma cholesterol is determined by cholesterol absorption, synthesis, storage and excretion. Cholesterol 7 α -hydroxylase (CYP7A1) is a liver-specific cytochrome P450 isozyme of the CYP7A family that catalyses the rate-limiting step in the classic pathway of bile acid biosynthesis. Conversion of cholesterol to bile acids in the liver is the most important pathway for elimination of cholesterol from the body⁽²⁾. Transcription of CYP7A1 is inhibited in a feedback mechanism by bile acids and is stimulated in a feed-forward mechanism by cholesterol^(3,4).

There is an increasing interest in green tea (*Camellia sinensis*) as a protective agent against CVD⁽⁵⁾. In fact, increased consumption of green tea has been associated with decreased serum total cholesterol and LDL-cholesterol^(6,7). The health-beneficial effects of green tea have been attributed mainly to the catechins, such as (–)-epicatechin (EC), (–)-epigallocatechin (EGC), (–)-epicatechin gallate (ECG) and (–)-epigallocatechin-3-gallate (EGCG). Among them, (–)-epigallocatechin gallate (EGCG) accounts for almost 50%

(w/w) of the catechins in green tea⁽⁸⁾. Several intervention studies using animal models have found that green tea, purified tea catechins, ECG or EGCG have a hypocholesterolaemic effect⁽⁹⁾. Investigations of the hypocholesterolaemic mechanisms of tea catechins have focused on the fact that catechins prevent the absorption of cholesterol and lipid by disrupting micelle formation or promote faecal excretion of total steroids and lipid thus lowering plasma cholesterol levels^(10–12). Additionally, Bursill *et al.*^(13,14) found that green tea EGCG appears to inhibit cholesterol synthesis and increase the LDL receptor, providing an alternative mechanism to explain the hypocholesterolaemic effects of green tea.

Another mechanism of lowering of plasma cholesterol by green tea catechins may involve enhanced conversion of cholesterol to bile acids via up regulation of CYP7A1 gene expression, the rate-limiting enzyme in the conversion of cholesterol to bile acids. Therefore, in the present study, we investigated the effects of green tea catechins on CYP7A1 gene expression at both the mRNA and promoter activity levels using human hepatocarcinoma HepG2 cells.

Abbreviations: CYP7A1, cholesterol 7 α -hydroxylase; EC, (–)-epicatechin; ECG, (–)-epicatechin gallate; EGC, (–)-epigallocatechin; EGCG, (–)-epigallocatechin-3-gallate.

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Materials and methods

Standards and reagents

The standard chemicals, EGC, EC, EGCG and ECG were purchased from Sigma (St Louis, MO, USA).

Cell culture

Human hepatocarcinoma HepG2 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) fetal bovine serum (Invitrogen, Carlsbad, CA, USA) and penicillin–streptomycin (100 units/ml) at 37°C, 5% CO₂. Green tea catechins (EGCG, ECG, EGC, or EC) were dissolved with dimethyl sulfoxide. The dimethyl sulfoxide final concentration in culture medium was 0.01%. The cells were treated with different catechins (5 µmol/l) or different concentrations of EGCG (0, 0.1, 1, 5, 10 or 20 µmol/l) in serum-free media for 24 h. The control cells were treated with 0.01% dimethyl sulfoxide without any catechin supplement. All measurements were performed in triplicate, when testing each treatment (*n* 3).

Quantitative real-time reverse transcription-polymerase chain reaction

Total RNA was extracted from HepG2 cells using the TRIzol Reagent (Promega, Madison, WI, USA). The cDNA were synthesised from 5 µg of RNA using M-MLV RT (Promega). After cDNA synthesis, quantitative real-time PCR was performed in 25 µmol/l of Universal SYBR Green PCR Master Mix (Qiagen, Chatsworth, CA, USA) using a fluorometric thermal cycler (Corbett Research, Mortlake, NSW, Australia). Reaction mixtures were incubated for an initial denaturation at 95°C for 10 min, followed by fifty PCR cycles. Each cycle consisted of 95°C for 10 s, 55°C for 20 s and 72°C for 20 s. Primers were designed using an on-line program (primer3_www.cgi/v0.2)⁽¹⁵⁾. The sequences of the sense and antisense primers were as follows: CYP7A1, 5'-CCTTATGGTATGACAA-GGGA-3' and 5'-TGGATTAATCCATACCTGG-3'; β-actin, 5'-GTTGCCAATAGTGATGACCT-3' and 5'-GGACCTGAC-AGACTACCTCA-3'. Values were expressed as fold change over control and expressed as means with their standard errors.

Construction of human cholesterol 7α-hydroxylase (CYP7A1)/Luc reporter gene

The human CYP7A1 gene promoter from –1312 to +358 bp was generated by PCR using genomic DNA isolated from HepG2 cells. The 5'-primer, bearing a SacI site (GAGCTC), was 5'-nnnnGAGCTCACTGTGGGTGTATGTGTGTG-3' and the 3'-primer, bearing a XhoI site (CTCGAG), was 5'-nnnnCTCGAGTGCCAATACTAAAAAGGGAA-3' (capital letters indicate gene sequence). Amplification of the CYP7A1 promoter consisted of 95°C for 7 min followed by thirty cycles of 95°C for 1 min, 62°C for 2 min and 70°C for 2 min. The CYP7A1 promoter fragment (–1312/+358) was subcloned into pGEM[®] T easy vector (Promega) according to the manufacturer's instructions. The CYP7A1 promoter fragment, corresponding to –1312 to +358 bp, was inserted into the pGL3 basic vector (Promega) that includes luciferase as a reporter gene.

Transfection and luciferase assay

Transfection experiments were carried out with the Superfect reagent (Qiagen, Alencia, CA, USA) according to the manufacturer's instructions. The plasmids used were 2 µg CYP7A1/Luc reporter gene and 1 µg pCMV-β-galactosidase (Clontech, Palo Alto, CA, USA) as an internal standard for the adjustment of transfection efficiency. At 3 h after transfection, HepG2 cells were treated with catechins in serum-free media for 24 h. Cells were harvested with lysis buffer (Promega). CYP7A1 promoter activity in cells was measured with the luciferase reporter assay system (Promega) using a TD 20/20 luminometer (Turner Designs, Sunnyvale, CA, USA). The β-galactosidase activity was assayed enzymically using *o*-nitrophenyl-β-D-galactopyranoside as a substrate⁽¹⁶⁾. Luciferase activity was calculated in relative light units and normalised to β-galactosidase activity.

Statistical analysis

Data are expressed as mean values with their standard errors. The significant differences between groups were determined by one-way ANOVA using the SPSS program (version 11.0; SPSS, Chicago, IL, USA). The results were considered significant if the value of *P* was <0.05, and Tukey's multiple-range test was performed if differences were identified between groups.

Results

Effects of catechin on the cholesterol 7α-hydroxylase (CYP7A1) mRNA expression

Quantitative real-time PCR demonstrated that EGCG and ECG significantly up regulated the CYP7A1 mRNA level by 4.2- and 5.5-fold, respectively, compared with the control (Fig. 1 (A)). EC and EGC treatment resulted in intermediate values that were not different from controls. The order by which green tea catechins enhanced gene expression was shown to be ECG > EGCG > EGC > EC.

Effect of (–)-epigallocatechin-3-gallate on the cholesterol 7α-hydroxylase (CYP7A1) mRNA expression

The effect of EGCG, a principal component of green tea catechins, was investigated at different concentrations. EGCG increased CYP7A1 mRNA concentrations in a biphasic manner (Fig. 1 (B)). The CYP7A1 mRNA level was increased by lower concentrations of EGCG, up to 4.5-fold with 5 µmol/l, compared with the control. However, greater concentrations of EGCG were less effective.

Effects of catechin on cholesterol 7α-hydroxylase (CYP7A1) promoter activity

To determine whether these effects of catechins on mRNA concentrations were mediated by the CYP7A1 promoter, a –1312/+358 CYP7A1 promoter fragment was ligated to a luciferase reporter gene and transfected into HepG2 cells. ECG or EGCG significantly increased CYP7A1 promoter activity by 6.0- or 4.0-fold, respectively, compared with the control (Fig. 1 (C)). Transfected cells were also treated with

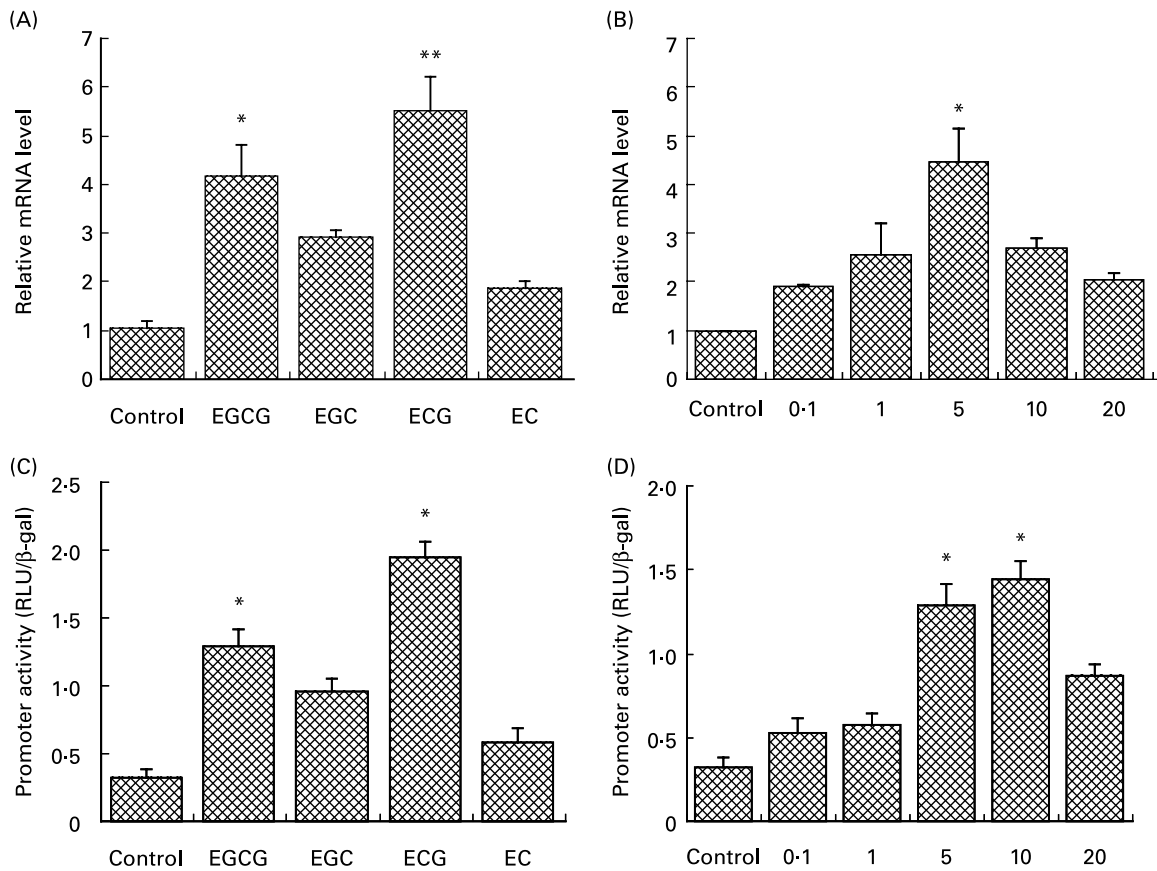


Fig. 1. Effects of green tea catechins on cholesterol 7 α -hydroxylase (CYP7A1) at both mRNA level at different kinds of catechin (A) or different concentrations of (–)-epigallocatechin-3-gallate (EGCG) (B) and promoter activity at different kinds of catechin (C) or different concentrations of EGCG (D) in HepG2 cells. All measurements were performed in triplicate for mRNA and for promoter activity, when testing each treatment (n 3). Data are means, with their standard errors represented by vertical bars. Mean value is significantly different from that of the control treatment: * P <0.05, ** P <0.01. EGC, (–)-epigallocatechin; ECG, (–)-epicatechin gallate; EC, (–)-epicatechin; RLU, relative light units; β -gal, β -galactosidase.

a range of concentrations of EGCG (0–20 μ mol/l). The EGCG stimulated CYP7A1 promoter activity in a dose-dependent manner, with the effect plateauing at 5–10 μ mol/l, and then being reduced at 20 μ mol/l (Fig. 1 (D)).

Discussion

Death rates from CVD have recently been reported to be reduced by drinking about ten cups of green tea per d⁽⁵⁾. HepG2 cells suggested as a model for studies on regulation of CYP7A1 at the molecular level⁽¹⁷⁾, were used in the present study to confirm the effects of catechins in green tea on CYP7A1. However, caution is required because cytotoxic effects have been reported with catechin treatment of HepG2 cells. The maximum non-cytotoxic concentrations of EGCG, ECG, EGC and EC were found to be 15, 5, 80 and 20 μ mol/l, respectively⁽¹⁸⁾. In the experiments reported here, the standard concentration of catechins used was 5 μ mol/l, in the non-toxic range. Study on human subjects has shown the plasma level of EGCG reached 4.4 μ mol/l with the oral administration of 525 mg green tea EGCG (about 50 mg EGCG, an amount that is present in a cup of green tea)^(19,20). The CYP7A1 mRNA concentrations were significantly increased by ECG and EGCG compared with the control in HepG2 cells. The effect of EGCG, the principal catechin found in green tea, was

found to be biphasic, with maximal induction found at 5 μ mol/l and then being reduced at greater concentrations. This may be related to the cytotoxic effect of EGCG⁽¹⁸⁾. This biphasic dose responsiveness is not unusual for bioactive compounds. For example, Yap *et al.*⁽²¹⁾ showed that the *Epimedium brevicornum* extract, used traditionally for bone health in China because of its oestrogenic activity, induced biphasic responses in the mRNA and protein expression of the oestrogen-regulated progesterone receptor gene in breast cancer (MCF-7) cells. Thus, it should be noted that safe use of bioactive compounds for humans must always include consideration of dosage.

Elevation of CYP7A1 mRNA level can be due to an enhancement of transcription and/or an increase in mRNA stability. To distinguish between these possibilities, we examined the effect of different kinds of catechin on CYP7A1 promoter activity. CYP7A1 promoter activity was significantly elevated by ECG and EGCG treatment to an extent similar to that seen with mRNA expression. The dose–response relationship between EGCG and CYP7A1 promoter activity also paralleled the mRNA, with the greatest concentration being less effective than intermediate ones. Thus, it appears reasonable to conclude that the effects of catechin on CYP7A1 gene expression occur at the level of transcription, through response elements yet to be identified in the –1312/+358 bp portion of the promoter.

Historically, the hypocholesterolaemic actions of dietary catechins have been attributed to their ability to inhibit intestinal absorption of bile acids and cholesterol. Yang & Koo⁽¹⁰⁾ reported that supplying rats with 4% Chinese green tea decreased plasma LDL-cholesterol and increased faecal cholesterol and bile acids, thus implying that the hypocholesterolaemic effects of green tea may be due to the enhancement of faecal excretion of bile acids. However, in the present study, EGCG directly increased CYP7A1 transcription in HepG2 cells, untreated with cholesterol or bile acids. These results suggest that in addition to the decreases in absorption of cholesterol and bile acids in the intestine, there may be other mechanisms associated with stimulatory effects of EGCG on CYP7A1 transcription. Bile acids negatively feed back on CYP7A1 transcription through a mechanism mediated in part by the farnesoid X receptor (FXR), a member of the family of ligand-activated orphan receptors⁽²²⁾. When fed diets containing cholesterol, mice lacking oxysterol receptor, liver X receptor (LXR) α , failed to induce transcription of the CYP7A1 gene, demonstrating the existence of a physiologically significant feed-forward regulatory pathway for sterol metabolism⁽²³⁾. Therefore, we propose in addition to the nuclear receptor signalling cascades, there may also be FXR- or LXR-independent mechanisms of stimulation of CYP7A1 expression. Since the hypocholesterolaemic effect of catechins has been associated with increases in bile acids and cholesterol excretion in previous studies^(10–12), this is an important finding that contributes to our knowledge in the hypocholesterolaemic mechanisms of EGCG. We found direct effects of catechin on the hepatocytes themselves and these effects were ultimately localised to the CYP7A1 promoter. Future studies will identify the precise location of the elements within the CYP7A1 promoter that mediate this response and the proteins which bind to these response elements.

Acknowledgements

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