Short Communication

Epigallocatechin gallate changes mRNA expression level of genes involved in cholesterol metabolism in hepatocytes

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Abstract

Catechins, compounds derived from green tea, have been shown to improve cholesterol metabolism in animal studies, but the molecular mechanisms underlying this function have not been fully understood. We performed DNA microarray analysis in order to clarify the effects of epigallocatechin gallate (EGCG), the dominant catechin in green tea, on cholesterol metabolism in HepG2 hepatocytes. This revealed that the expression levels of several genes related to cholesterol metabolism, including the LDL receptor, were changed by EGCG treatment. Using a real-time PCR technique, we confirmed that EGCG treatment up-regulated mRNA expression level of the LDL receptor. Moreover, EGCG decreased extracellular apoB levels. These findings indicated that EGCG improves cholesterol metabolism through the up-regulation of LDL receptor and also reduces extracellular apoB levels.

Key words: Epigallocatechin gallate: Tea catechins: Cholesterol: LDL receptor: HepG2

Atherosclerosis is a major cause of morbidity and mortality worldwide. Atherogenesis is a complex process, with multiple mechanisms contributing to its initiation and progression; infection, inflammation and autoimmunity have been associated with pathogenesis of the disease[1]. In view of the fact that elevated levels of blood lipids, especially LDL cholesterol, have a central role in the genesis of atherosclerosis, therapeutic and dietary approaches to their treatment and prevention are highly relevant[2,3].

The beneficial health effects of green tea have been attributed mainly to the catechins epigallocatechin-3-gallate (EGCG), epicatechin, epigallocatechin and epicatechin gallate[4,5]. Among these, EGCG has been extensively investigated as it is the dominant catechin, accounting for up to 65 % of the total catechin content in green tea[6]. Catechins have a variety of properties, including antioxidant[7], anti-cancer[6,8], anti-diabetic[9] and anti-atherogenic properties[10], as well as endurance-improving qualities[11]. Moreover, long-term intake of tea catechins reduces diet-induced obesity in mice[12]. Besides these beneficial properties, in epidemiological studies, a significant inverse relationship between tea drinking and plasma cholesterol levels[13,14] has been reported. Animal studies have shown that catechins inhibited

cholesterol absorption and lowered plasma cholesterol[15–21]. Furthermore, several reports have indicated that tea catechins directly influence cholesterol metabolism in hepatocytes[22,23]. Therefore, it appears that the molecular mechanisms underlying the plasma cholesterol-lowering effect of tea catechins are complex and not fully understood.

In the present study, we performed DNA microarray analysis using HepG2 cells treated with EGCG to clarify the effects of EGCG on hepatic cholesterol metabolism, because the studies about the effects of EGCG on cholesterol metabolism in hepatocytes have been very limited and no exhaustive transcriptome analysis has been performed. We demonstrated that EGCG treatment up-regulated mRNA expression of the LDL receptor and decreased extracellular apoB levels.

Experimental methods

Materials

Unless otherwise indicated, all chemicals were purchased from Sigma (St Louis, MO, USA) or Wako (Osaka, Japan) and were guaranteed to be of reagent or tissue culture grade.

Abbreviation: EGCG, epigallocatechin-3-gallate.

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Cell culture

The human hepatoblastoma cell line, HepG2 (from American Type Culture Collection, Manassas, VA, USA), was generated as previously described[24]. Cells were incubated in Dulbecco's modified Eagle's medium (MP Biomedicals Japan, Tokyo, Japan) containing 10% charcoal/dextran-treated fetal bovine serum, 100 units/ml penicillin, 100 μg/ml streptomycin and 10 mM non-essential amino acid solution (Invitrogen, Carlsbad, CA, USA), with and without EGCG. After incubation for 24 h, the cells were harvested for RNA preparation.

RNA preparation and real-time fluorescence monitoring RT-PCR

RNA preparation and real-time PCR were performed as described previously[24]. The level of mRNA expression of the LDL receptor was standardised against 18S ribosomal RNA. Oligonucleotide primer sets and TaqMan® probes for the human LDL receptor and 18S ribosomal RNA were previously described[24].

DNA microarray analysis

RNA samples isolated from HepG2 cells were labelled using the Low RNA Input Linear Amplification Kit (Agilent Technologies, Santa Clara, CA, USA), in accordance with the manufacturer's instructions. Briefly, 500 ng of total RNA were amplified and reverse-transcribed *in vitro* to complementary DNA using T7-polymerase, which was subsequently labelled with cyanine3-labelled cytidine triphosphate dye. After preparation of complementary RNA using RNeasy Mini Kit (QIAGEN, Hilden, Germany), a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA) was used to monitor dye incorporation for the experimental samples (500 ng), which was between 9 and 22 pmol/μg. Targets consisting of amplified and fluorescent-labelled complementary RNA were hybridised using the Agilent Gene Expression Hybridization Kit (Agilent Technologies), following the manufacturer's protocols. In short, 1650 ng of complementary RNA were fragmented for 30 min at 60°C in the dark and hybridised onto Agilent Technologies 44k

(G4112F) whole human genome 60mer oligonucleotide arrays in a rotation oven (65°C, 17 h, 10 rpm in the dark). Following hybridisation, the chambers were disassembled and the slides sequentially washed in Gene Expression Wash Buffers 1 and 2 (Agilent Technologies) and air-dried. The slides were then scanned using an ArrayScan (Agilent Technologies). Spot identification and quantification were performed with Agilent Feature Extraction software (Agilent Technologies). The data were analysed using GeneSpring GX 11.5 Expression Analysis Software (Agilent Technologies).

Measurement of extracellular apoB levels

The level of apoB secreted into the medium was determined using ELISA. Briefly, the media (100 μl) were added onto the ELISA plate (Thermo Fisher Scientific, Rockford, IL, USA) and incubated for 18 h at 4°C. After blocking with bovine serum albumin, mouse anti-human apoB antibodies (Millipore, Bedford, MA, USA) and goat anti-mouse IgG (Sigma), horseradish peroxidase-conjugated antibodies were used for the immunodetection of apoB. The apoB secretion level was normalised to cellular protein content, as determined by the Bradford method (Bio-Rad Laboratories, Hercules, CA, USA).

Statistical analysis

The results were expressed in terms of mean values and standard errors of the mean. The statistical significance of differences was evaluated using Student's *t* test[25] for the data acquired from DNA microarray or ANOVA and using Tukey–Kramer test[26] for the data from quantitative PCR and apoB ELISA. Differences were considered significant when $P < 0.05$.

Results

To comprehensively investigate the effects of EGCG on hepatic cholesterol metabolism, we performed DNA microarray analysis using HepG2 cells treated with 25 μM-EGCG. We selected 24 128 expressed probe sets through filtration based on the expression and Flag analysis. Among these probe sets, we identified 2737 differentially expressed transcripts with a ± 1·5-fold

Table 1. Changes in gene expression of cholesterol metabolic process in HepG2 cells treated with 25 μM-epigallocatechin gallate (EGCG) relative to the vehicle control

Gene	Fold (EGCG/control)	GeneBank accession no.
LDL receptor	2·20	NM_000527
Mevalonate (diphospho) decarboxylase	1·88	NM_002461
7-Dehydrocholesterol reductase	1·85	NM_001360
Sterol-C4-methyl oxidase-like	1·70	NM_006745
ApoL, 2	1·69	NM_145637
Cytochrome P450, family 51, subfamily A, polypeptide 1	1·66	NM_000786
VLDL receptor	1·67	NM_003383
Sterol-C5-desaturase-like	1·62	NM_001024956
Cytochrome b5 reductase 3	1·54	NM_007326
3-Hydroxy-3-methylglutaryl-coenzyme A synthase 1	1·67	NM_002130
PPARδ	1·54	NM_006238
3-Hydroxy-3-methylglutaryl-coenzyme A synthase 2	− 1·58	NM_005518
Aldo-keto reductase family 1, member D1	− 2·12	NM_005989

difference in expression. As shown in Table 1, the expression levels of thirteen genes categorised in sterol metabolic process were changed by EGCG treatment, suggesting that EGCG directly affects cholesterol metabolism in hepatocytes. In particular, the level of expression of the LDL receptor was strongly up-regulated (2·2-fold) by EGCG. The LDL receptor is an integral plasma membrane glycoprotein that is expressed in all cell types, but most abundantly in the liver, and is important for mediating cellular LDL uptake[27]. Thus, in the present study, we focused on investigating the effect of EGCG on LDL receptor expression, and real-time PCR was carried out to confirm the effect of EGCG on mRNA expression levels of the LDL receptor. As expected, the addition of 10 and 25 μM of EGCG increased the level of mRNA expression of the LDL receptor by 1·8- and 1·7-fold, respectively (Fig. 1(a)). Finally, we assessed the effect of EGCG on extracellular apoB protein levels. ApoB is the major apolipoprotein in LDL, present as a single copy per lipoprotein particle[28]. With the addition of EGCG, extracellular apoB protein levels were decreased in a dose-dependent manner (Fig. 1(b)). In the presence of 25 μM-EGCG, extracellular apoB protein levels were decreased by 58 %. These results indicated that, in hepatocytes, EGCG improves cholesterol metabolism through up-regulation of the LDL receptor followed by an increase of cellular uptake of LDL.

Discussion

Several reports have shown that tea catechins directly influence cholesterol metabolism in hepatocytes[22,23]. In fact, DNA microarray analysis revealed that the expression levels of several genes related to cholesterol metabolism were changed through EGCG treatment. Of these genes, the LDL receptor mRNA was strongly up-regulated. The regulation of hepatic LDL receptor expression has been extensively studied using HepG2 cells[29]. LDL receptor levels are suppressed in response to cholesterol and lipoprotein loading[30]. However, LDL receptor expression is also regulated through several signal transduction pathways in HepG2 cells, including the cyclic AMP, diacylglycerol-protein kinase C and mitogen-activated protein kinase pathways[31–33]. In the preliminary LDL receptor promoter assay of the present study, EGCG did not activate the LDL receptor promoter sequence (−4000 to +57 bp). Thus, EGCG may enhance LDL receptor mRNA stability but not activate its transcription. Several recent studies have shown that the 3′ untranslated region of LDL receptor mRNA is important for its stabilisation and this stabilisation is mediated through the activation of the p42/44 extracellular signal-regulated kinase[34] or the c-Jun N-terminal kinase signaling pathway[35]. EGCG was reported to activate both p42/44 extracellular signal-regulated kinase and c-Jun N-terminal kinase pathways in human umbilical vein endothelial cells[36]. Therefore, the up-regulation of LDL receptor mRNA induced by the EGCG treatment may be mediated through the activation of these signalling pathways in HepG2 hepatocytes.

In the present study, extracellular apoB protein levels decreased dose dependently with the addition of EGCG, but LDL receptor mRNA expression levels were not necessarily dose dependent. Molecular mechanisms underlying this difference were unclear; however, it is possible that EGCG treatment may lower extracellular apoB levels via both the LDL receptor-dependent and -independent pathways. Other than LDL receptor, the expression levels of several genes related to cholesterol metabolism, such as VLDL receptor and PPARδ, were up-regulated through 25 μM-EGCG treatment. The VLDL receptor is a member of the LDL receptor family. Unlike the LDL receptor, the VLDL receptor is not regulated by cellular sterols and does not efficiently bind LDL. Rather, it contributes to the delivery of fatty acids that derive from TAG-rich lipoproteins to peripheral tissues. It binds apoE-enriched chylomicrons and VLDL, intermediate-density lipoproteins and lipoprotein lipase[37]. PPARδ belongs to the nuclear hormone receptor superfamily, which comprises a large group of ligand-dependent transcription factors. Interestingly, it has been reported that PPARδ activator lowers serum LDL-cholesterol[38]. Because these genes are closely related to the cellular lipid metabolism, not only LDL receptor but
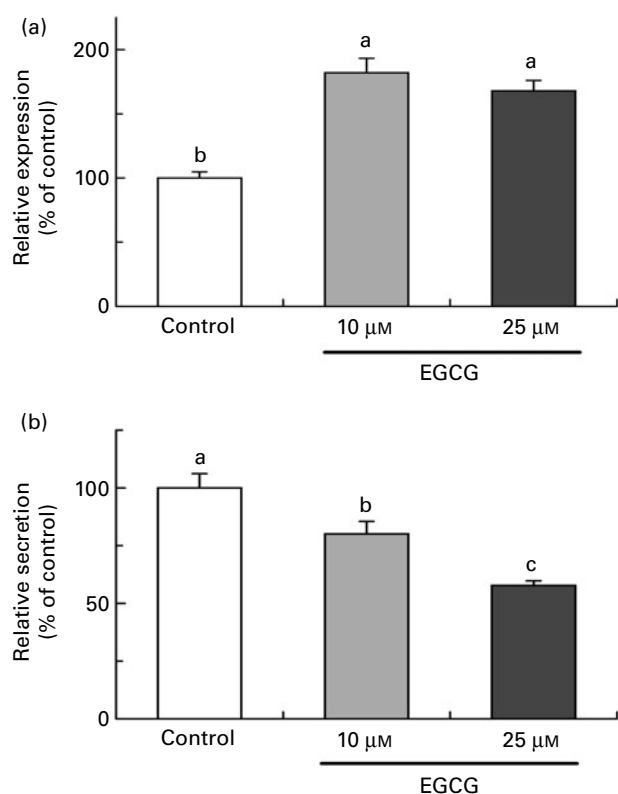
Fig. 1. Effects of the addition of epigallocatechin gallate (EGCG) on the level of LDL receptor mRNA expression and extracellular apoB levels in HepG2 hepatocytes. (a and b) Total RNA and medium were harvested from HepG2 cells treated with vehicle control, 10 or 25 μM-EGCG for 24 h. (a) The level of LDL receptor mRNA expression was measured using real-time monitoring RT-PCR and normalised to the mRNA expression level of the β-actin gene. (b) The extracellular apoB level was measured using ELISA and normalised to cellular protein content. The levels of the vehicle-control-treated group are set at 100 %, and the levels are presented as fold inductions relative to the vehicle-control-treated group. Values are means, with their standard errors (n 6). ^{a,b,c} Mean values with unlike letters were significantly different (P<0·05; one-way ANOVA and Tukey–Kramer test).

also the changes of the expression levels of these genes might be important for the apoB-lowering effect of EGCG.

Several genes encoding enzymes in the cholesterol biosynthetic pathway (3-hydroxy-3-methylglutaryl-coenzyme A synthase 1, mevalonate decarboxylase, cytochrome P450, family 51, subfamily A, polypeptide 1, sterol-C4-methyl oxidase-like, sterol-C5-desaturase-like and 7-dehydrocholesterol reductase) were also up-regulated by EGCG. The expression levels of almost all of these genes were reported to be regulated by a family of transcription factors, sterol regulatory element-binding proteins[39], which are transcription factors under the condition of cholesterol starvation[40]. Thus, EGCG treatment might reduce intracellular cholesterol level followed by the up-regulation of the expression levels of genes related cholesterol uptake and cholesterol biosynthesis.

In the present study, we investigated the effects of EGCG in HepG2 cells at 10 and 25 μM concentrations, because this concentration range of EGCG has been widely used in experiments using cultured hepatocytes[23,41]. These concentrations were considerably high at the thought of the absorbability of EGCG in animal studies[42,43]. For instance, Nakagawa & Miyazawa[43] have shown that EGCG concentration in plasma reaches 12·3 μM at 60 min after a single oral administration of EGCG (500 mg/kg body weight). However, Murase et al.[41] have reported that, whereas AMP-activated protein kinase activation induced by EGCG treatment needs about 50 μM-EGCG in Hepa 1-6 murine hepatocytes, oral administration of EGCG (200 mg/kg body weight) efficiently activates AMP-activated protein kinase in the liver of BALB/c mice. Similar differences in the effective concentration between plasma and cultured hepatocytes are seen in the case of insulin[44]. Because the condition of cultured hepatocytes does not completely reflect that of hepatocytes in vivo, future studies about the effects of EGCG treatment on the gene expression in the liver using animal models are going to be important. Furthermore, the absorbability of epigallocatechin and epicatechin are much higher than that of EGCG[42]. Thus, it might be important for further understanding of the molecular mechanisms of serum cholesterol-lowering effect of tea catechines to clarify molecular specificity of the effect of catechins on the LDL receptor expression in hepatocytes.

In conclusion, the present study indicates that EGCG improves cholesterol metabolism through up-regulation of LDL receptor mRNA and reduction of extracellular apoB levels. The importance of hepatic LDL receptors in systemic cholesterol excretion is exemplified by patients suffering from familial hypercholesterolaemia, an autosomal dominant disorder, whereby one or both LDL receptor alleles do not encode functional receptors[45]. Therefore, the up-regulation of LDL receptor mRNA induced by EGCG may contribute to the cholesterol-lowering effect of tea catechins.

Acknowledgements

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