

Epigallocatechin-3-gallate (EGCG) inhibits 3-hydroxy-3-methylglutaryl-CoA reductase in the presence of glycerol

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Abstract: The inhibition of 3-hydroxy-3-methylglutaryl CoA reductase (HMGCR) is considered able to decrease serum cholesterol levels and dramatically reduce the risk for cardiovascular and cerebrovascular diseases. The statins, competitive inhibitors of HMGCR, have been employed to control hypercholesterolemia. But their side effects, especially their safety of long-term administration have attracted great attention. Therefore, there is still an urgent requirement for the development of safer inhibitors of HMGCR with less serious side effects. In this study, we cloned and purified the catalytic domain of human HMGCR (Δ HMGCR), and applied the method of Ultra Performance Liquid Chromatography (UPLC) to assay Δ HMGCR activity and screen its inhibitors from natural products. The results indicated that EGCG can inhibit Δ HMGCR in the presence of some glycerol *in vitro* and can decrease cellular total cholesterol in HepG2 cells. As a consequence, it is promising to put EGCG into the development of hypolipidemic health product.

Keywords: HMGCR; EGCG; UPLC; inhibitor; glycerol.

INTRODUCTION

It is widely acknowledged that 3-hydroxy-3-methylglutaryl CoA reductase (HMGCR) has been recognized as the rate-limiting enzyme in synthesis of cholesterol. The degradation of the reductase can reduce serum cholesterol levels and dramatically decrease the risk of cardiovascular and cerebrovascular diseases, essential hypertension, coronary heart diseases, and atherosclerosis (Goldstein and Brown, 1990; Balbisi, 2006). Several HMGCR inhibitors have been developed as cholesterol-lowering therapeutic agents. For example, the statins, competitive inhibitors of HMGCR, have been employed to control hypercholesterolemia for over 25 years (Lytsy *et al.*, 2012; Reiner, 2014), but their side effects are occasionally serious. A well-known complication of statins use is musculoskeletal symptoms. Few patients develop an autoimmune myopathy even after the statins are discontinued (Mammen *et al.*, 2011). In addition, the use of statins may elevate serum transaminases, induce a statin-induced inhibition of proximal tubular reabsorption of protein and have deleterious effects on the peripheral nervous system (Grundy, 2005; Gaist *et al.*, 2002). Recent study has indicated that some people taking statins have an increased risk of amnesia and anxiety (Maggo and Ashton, 2013).

On the basis of data available from above, statins therapy may involve some risks of potential complications. Therefore, the development of safer inhibitors of

HMGCR with less serious side effects has become the main study concern in recent years. Several studies have described that natural products from tomato juice and *Aloe succotrina* can inhibit HMGCR activity, and it is promising to put these compounds into the development of lipid lowering agents (Navarro-González *et al.*, 2014; Dhingra *et al.*, 2014).

In the present study, we cloned and purified the catalytic domain of human HMGCR (Δ HMGCR) and screened its inhibitors from natural products. The results indicated that epigallocatechin-3-gallate (EGCG), the most abundant catechin in tea, is a potent inhibitor against HMGCR in the presence of some glycerol *in vitro*. Further study showed that EGCG can decrease cellular total cholesterol in HepG2 cells without changing HMGCR mRNA and protein levels after 24 hrs incubation compared to control. As a consequence, it is promising to put EGCG into the development of hypolipidemic health product.

MATERIALS AND METHODS

Cloning and expression of Δ HMGCR

Total RNAs were extracted from Human Liver Cells (HepG2) by using 1 mL of RNAiso PLUS (TakaRa), the cDNAs were synthesized from the polyadenylated mRNA by use of a Super RT Kit (Biotek Corporation). The catalytic domain of HMGCR (amino acid residues 447-888) was amplified by a forward primer (5'-CTACAGATACTTGGATCCGCAGAGAAAG-3')

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containing *Bam*H I restriction site and a reverse primer (5'-GTCGCTCGAGTCAGG CTGTCTTC-3') containing *Xho* I site. PCR was performed with high fidelity *Pfu* DNA polymerase (Fermentas) for 35 cycles with 94°C for 30 s, 62°C for 30 s and 72°C for 90 s. The PCR products were sequenced and then cloned into the expression vector pGex-HisC3 (GE Healthcare). Rosseta (DE3) pLysS *E. coli* cells were used as the host for expression of the recombinant GST fusion protein. The cells were cultured overnight in the presence of 0.1 mM isopropyl-1-thio-β-D-galactopyranoside (IPTG) at 28°C in Luria broth (LB) medium supplemented with ampicillin (100mg/L) and chloramphenicol (34mg/L).

Purification and enzymatic activity assays of ΔHMGCR

The *E. coli* cells were broken up by sonication in an ice-cold buffer (pH 7.2) containing 25mM Tris-HCl, 5mM EDTA, 10mM β-ME and a protease inhibitor cocktail (Sigma). The supernatant was collected after centrifugation at 12,000g for 30 min at 4°C. Proteins in the supernatant were loaded onto a Glutathione-Sepharose 4B column equilibrated with PBS (pH 7.2, 50mM Na₂PO₄, 50mM KH₂PO₄), then washed by washing buffer 1 (25mM Tris-HCl, pH 8.0, 2mM β-ME, 150mM NaCl, 2mM EDTA, 0.1% Triton X-100) and washing buffer 2 (25mM Tris, pH 8.0, 2mM β-ME, 150mM NaCl, 2 mM EDTA), eluted with elution buffer (50mM Tris-HCl, pH 8.0, 10mM Glutathione). The active fractions were concentrated by dialysis in buffer C (50 mM Tris-HCl, pH 7.5, 1mM EDTA, 2 mM β-ME and 50% glycerol) and stored in -20°C.

Enzymatic activities were analyzed by monitoring the dynamic changes of NADPH content by use of Ultra Performance Liquid Chromatography (UPLC) equipped with a ACQUITY UPLC BEH Amide column (2.1×150 mm, 1.7 μm) and a UV detector. When HMGCR was mixed with HMG-CoA and NADPH, the NADPH would be oxidized to NADP⁺, causing the decrease in absorbance of NADPH at 340 nm. The chemical reaction is: HMG-CoA + 2NADPH + 2H⁺→mevalonate + CoA + 2NADP⁺. UPLC conditions of analyzing NADPH were as following linear gradient of 90% ACN/water with 10 mM ammonium acetate of pH 9.0 (solvent A) and 5% ACN/water with 10mM ammonium acetate of pH 9.0 (solvent B): 30%-50% B up to 5 min and 50%-30% B for 4 min at flow rate of 0.3mL/min, wavelength detection at 340 nm and the temperature of column at 26°C. One unit activity is defined as 1nmol of NADP⁺ released per minute at 37°C.

The assay was initiated by the addition of 10μL mixture of NADPH and HMG-CoA into 40μL of enzyme solution (100mM PBS pH 7.0, 100mM NaCl, 1mM EDTA, 10 mM DTT, 0.5mM HMG-CoA and 1mM NADPH) and terminated by the addition of 200μL of ACN. Then the reaction liquid was injected into the instrument for UPLC analysis.

Screening the inhibitors of ΔHMGCR

Some natural products, including apigenin, kaempferol, EGCG, myricetin, quercetin, quercetin 3-β-D-glucoside and positive control pravastatin were dissolved in different solvents. 5 μL of inhibitors with various concentrations was mixed to 35 μL of enzyme solution. The assay was initiated by addition of 10 μL mixture of NADPH and HMG-CoA. After incubation for 5 min at 37°C, the reaction was terminated by the addition of 200 μL of ACN. UPLC conditions were the same as enzymatic activities. The changes in the content of NADPH were analyzed, and the inhibitory rate (IR) was calculated according to the following equation:

$$IR = \frac{C_2 - C_1}{C_0 - C_1} \times 100\%$$

C₀ indicates the initial concentration of NADPH. C₁ and C₂ reveal the concentration of NADPH without inhibitors and that with inhibitors after 5-min reaction.

HepG2 Cell Culture

HepG2 cells were grown in monolayer cultures to near confluence in 100 mm×20 mm culture dishes with 10 mL of Dulbecco's modified Eagle's medium (DMEM) containing 10% (v/v) fetal bovine serum (FBS) at 37°C with 5% CO₂. Cells were then incubated for 24 hrs with different concentrations (0-300 μm) of EGCG.

The effect of EGCG on the decrease of cholesterol

Total cholesterol in cells was determined by use of Tissue Free Cholesterol Assay Kit (Applygen Technologies Inc). For details, 7.5×10⁶ cultured cells were lysed in WCEB and cell pellets were collected by centrifugation at 12,000 g for 10 min. 20 μL of samples are incubated with 180 μL of working buffer for 30 min at 37°C. The concentration of cholesterol is determined by measuring the rate of color development at 550 nm.

The effect of EGCG on levels of HMGCR mRNA

HepG2 cells were seeded into 100 mm×20 mm culture plates at a density of 1×10⁶ cells in 10 mL DMEM. The cells were treated with different concentrations of EGCG for 24 hrs. Total RNAs were extracted using Trizol Reagent (Invitrogen). The quantity and purity (260/280 ratio) of RNAs were assessed by UV/VIS spectrophotometer (NanoDrop ND-1000, Thermo Scientific).

The cDNAs were synthesized from 0.27 μg of RNAs by use of Reverse Transcription Kit (Promega). 2 μL of cDNAs was served as a template for PCR. Primers were designed by use of primer premier 5.0, and the sequences were as follows: 5'-CTGTTGGAGTGGCAGGACCCC T-3' and 5'-CCCATGGCATCCCCTGACCTG-3'. The primers 5'-GTCAGTGGTGGACCTG ACCT-3' and 5'-TGAGCTTGACAAAGTGGTTCG -3' were used to amplified GAPDH. PCR was run with Taq DNA polymerase (TakaRa) for 35 cycles with 95°C for 30 s,

62°C for 30 s and 72°C for 45 s. The PCR products were examined by DNA electrophoresis. The grey scales of DNAs indicate HMGCRC mRNA levels and are accurately analyzed by Quantity One software.

The effect of EGCG on the expression of HMGCRC protein

The cells were lysed with the whole cell extraction buffer (WCEB, 25mM glycerolphosphate, pH 7.3, 10mM β -ME, 10mM EDTA, 2mM EGTA, 0.5mM Na_3VO_4 , 100 mM NaCl, 1% Triton X-100 and a protease inhibitor cocktail). The protein samples containing equal amounts of total proteins were separated on 10% SDS-PAGE gel and electroblotted by use of western blotting system (Bio-RAD). Primary Antibody (HMGCRC_C-18, Santa Cruz) was diluted (1 : 500) in TTBS plus 1% BSA and incubated with the membranes overnight in cold room. After 3 times (10 min each) washing with TTBS on a shaker, the membranes were incubated for 2 h at room temperature with 2nd antibody (Rabbit anti-goat IgG, HRP conjugated, BOISYNTHESIS BIOTECHNOLOGY) diluted (1: 2, 500) in TTBS plus 1% BSA and rinsed 3 times (10 min each) with TTBS. Finally, the antibody-bound protein bands were detected with enhanced chemiluminescence (ECL).

RESULTS

Cloning, expression and purification of Δ HMGCRC

A fragment of about 1333 bp was amplified, which

matched human HMGCRC's cDNA sequence 1501-2833, with *Bam*H I and *Xho* I restriction site on both ends. The obtained bands were cloned into pGex-HisC3 vector. The sequence encodes a protein of 441 amino acids with an estimated molecular mass of 47.13 kDa. To purify the truncated enzyme, the supernatant was application to a High-Affinity GST Resin column. The enzyme was eluted with 10 mM glutathione. A phusion protein of about 71 kDa was obtained (fig. 1).

Enzymatic activity assays of Δ HMGCRC

The purified enzyme had a specific activity of 1, 400 U/mg under our assay condition. Enzyme activity demonstrated good linear relationship within 10 min. Commercial enzymes sold in Sigma, for example, contain 50% glycerol as much as the enzyme we used in our experiment, with its specific activity of 2, 000-8, 000 U/mg, higher than that of the enzyme we used in the experiment.

Inhibitory rate of compound candidates to the Δ HMGCRC

The results revealed that except EGCG, no other compound candidates with the concentration of 1 mM have significant inhibitory efficacy to Δ HMGCRC. Pravastatin is regarded as one of HMGCRC inhibitors extensively used in clinical. The comparison demonstrated that EGCG exhibits lower IR than pravastatin on its inhibition of enzyme activity (fig. 2).

Table 1: mRNA levels of HMGCRC in HepG2 cells

EGCG	0 μM	50 μM	100 μM	200 μM	300 μM
HMGCRC (INT \times mm)	119.5 \pm 0.7	123.1 \pm 4.2	122.1 \pm 2.8	111.2 \pm 2.8	111.5 \pm 3.53

Data are the means \pm SD. Non-significant differences are indicated for all values compared with the control ($P > 0.05$).

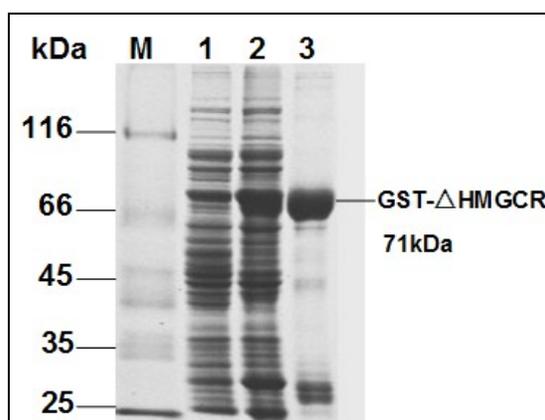


Fig. 1: Expression and purification of Δ HMGCRC as a GST fusion protein in *E. coli* cells. Protein samples from each step were separated by 10% SDS-PAGE and stained with Coomassie blue. **Lane 1**, crude extracts before induction; **Lane 2**, cell extracts after induction with 0.1mM IPTG for 12 h at 28°C; **Lane 3**, Purified proteins from Glutathione-Sepharose column.

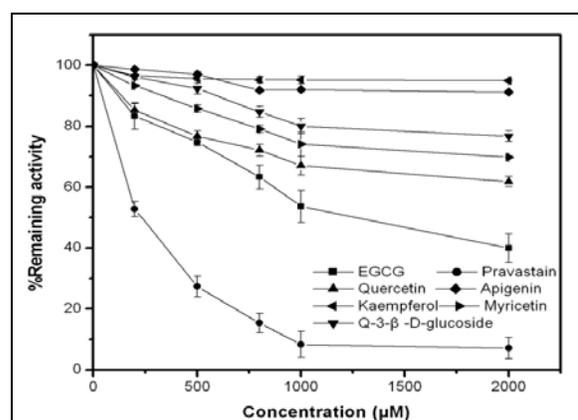


Fig. 2: Comparison of the inhibitory potencies between pravastatin and selected natural products. The activity of Δ HMGCRC was analyzed in the presence of various concentrations of natural products. Data represent relative activity with error bars denoting standard deviation.

EGCG and total Cholesterol

With the increase of EGCG, the total cholesterol decreased gradually (fig. 3). There is no significant change in the reduction of cholesterol at the lower doses of 50µM EGCG. But when the concentration went higher than 200µM, cholesterol decreased to a larger extent.

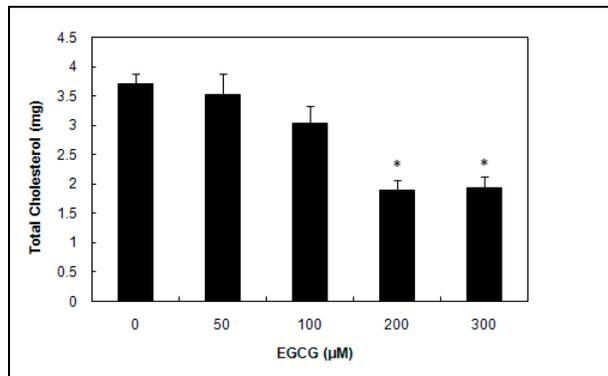


Fig. 3: Dose-dependent effects of EGCG on intracellular total cholesterol. The HepG2 cells were incubated for 24 hrs with various concentrations of EGCG. When the concentration went higher than 200 µM, cholesterol decreased to a larger extent ($P < 0.05$).

Effects of EGCG on the levels of HMGCR mRNAs and proteins

HMGCR mRNAs were of no significant difference from experimental groups treated with EGCG and control group (table 1). The results indicated that EGCG does not change the level of HMGCR mRNAs.

DISCUSSION

EGCG is the most abundant polyphenol compound in green tea, accounting for 58% of the total catechins (Bursill and Roach, 2006). As a natural product with less serious side effects, it has been used to prevent or treat cancer (Landis-Piwowar *et al.*, 2013; Chen, 2011),

obesity (Basu *et al.*, 2010; Wang *et al.*, 2013), diabetes (Hsu *et al.*, 2011; Jiang *et al.*, 2013) and cerebrovascular disease (Yao *et al.*, 2014). EGCG can inhibit some steroid-related enzymes, such as 11β-hydroxysteroid dehydrogenase, 5α-reductase and aromatase (Hintzpeter *et al.*, 2014; Hiipakka *et al.*, 2002; Satoh *et al.*, 2002), but it has been described to be ineffective in the inhibition of HMGCR (Dev *et al.*, 2009). A recent report verified the significant inhibitory effect of EGCG on HMGCR, with the inhibitory mechanism uncovered (Cuccioloni *et al.*, 2011). However, our results showed that EGCG exhibits exciting inhibitory ability against ΔHMGCR. Subsequent study indicated that EGCG is able to inhibit ΔHMGCR with the addition of some glycerol to the reaction system. The inhibitory effects of ΔHMGCR enhanced with the increase in concentration of glycerol. When the concentration reached up to 600mM (5.4%), the best inhibition was observed. Afterwards, the inhibitory effect gradually reduced as the concentration surpassed 600 mM (fig. 5). Initially, equal volume of glycerol was added into enzyme for the convenience of stability during storage. After 1/10 of enzyme stock solution was added into the reaction system, glycerol at the final concentration of about 5% was obtained with the optimum inhibitory effect of EGCG to ΔHMGCR.

In order to explore to role that glycerol played in this process, the analysis of molecular dynamics simulation was conducted on HMGCR-EGCG-glycerol interactions. The 3D structure of Δ HMGCR was adopted from Program Debug Database (PDB, 3cct). The construction and optimization of the structures of both EGCG and glycerol were used PRODRG. The model of HMGCR-EGCG-glycerol complex was obtained by use of the program Auto Dock 4.2 (fig. 6). Through the model of dynamics simulation, we speculate that EGCG and HMGCR form certain complex so that substrates are prevented from penetrating into the active center. The interaction energies of the EGCG with HMGCR are

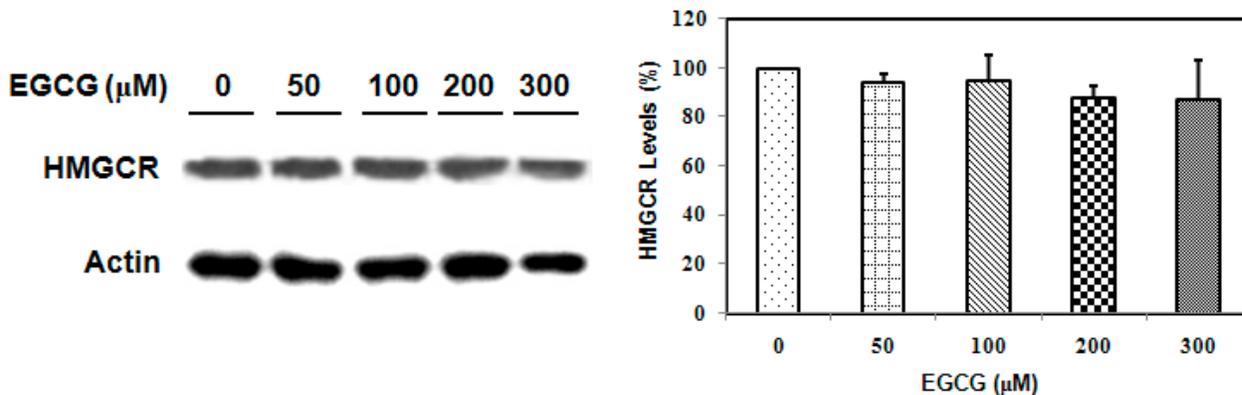


Fig. 4: Effect of EGCG on the expression of HMGCR in HepG2 cells. HepG2 cells were treated with EGCG for 24 hours and the protein expression level of HMGCR was analyzed by western blot. The β-actin was used as the internal control. Data represent relative band intensity with error bars denoting standard deviation ($P > 0.05$).

-175.2 kJ/mol. The interaction energies of the EGCG, glycerol and HMGCR are -211.0kJ/mol. The results revealed that the affinity between EGCG and HMGCR is significantly stronger in the synergy of glycerol.

Moreover, the enzyme we used in this study is GST phusion protein. It might affect the combination of the compounds and enzymes. It might be the main reason why we got higher IC₅₀ of pravastatin than that in other reports (Sarr *et al.*, 2008; Perchellet *et al.*, 2009; McTaggart *et al.*, 2001).

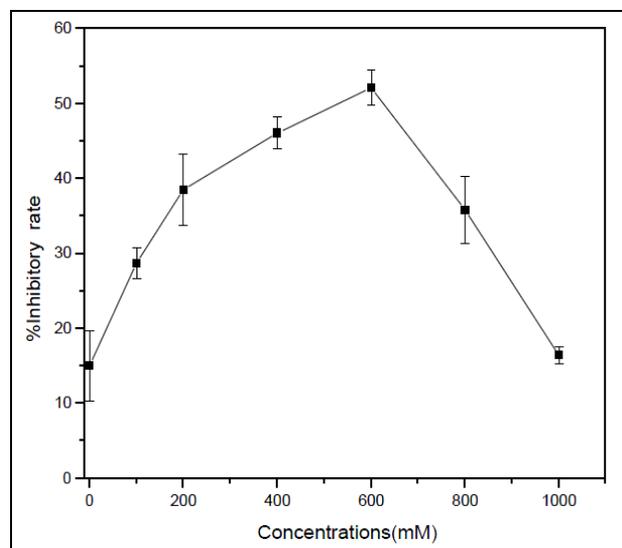


Fig. 5: Inhibition of HMGCR by EGCG in the presence of the glycerol with various concentrations. After adding the glycerol, the assay solutions were incubated at 37°C for 5 min and then analyzed by UPLC.

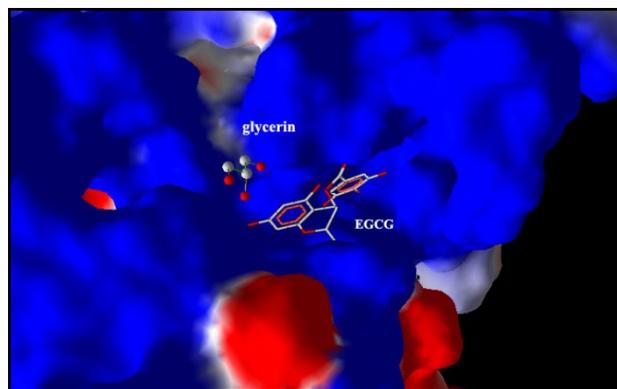


Fig. 6: The interaction schematic diagram of EGCG and glycerol with the HMGCR. The reductase is expressed with the electrostatic potential surface. The negative and positive electrical potential regions are represented by the color of red and blue respectively. Glycerol is represented by ball-and-stick model. EGCG is represented by the pipeline model.

Some studies in animals and humans have verified cholesterol-lowering effects of green tea and catechin

(Wolfram, 2007; Bursill *et al.*, 2007). Our results indicated that EGCG is able to inhibit HMGCR in the presence of glycerol and reduce the total cholesterol with no level change of HMGCR mRNA and protein in HepG2 cells. Glycerol is a precursor for the synthesis of triacylglycerols and of phospholipids in the liver and adipose tissue, which widely exists in human body (Jansson *et al.*, 1992; Dong *et al.*, 2014). Therefore, we supposed that EGCG is extremely possible to inhibit HMGCR with the cooperation of glycerol in human cells so as to reduce the synthesis of cholesterol. It provides new insights into a mechanism for drug synergy *in vivo*.

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