Tea catechins as potent and selective inhibitors of cholesterol biosynthesis.

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SUMMARY

The green tea gallocatechins; (-)-epigallocatechin-3-*O*-gallate (EGCG) (IC_{50} = 0.69 μM), (-)-gallocatechin-3-*O*-gallate (GCG) (IC_{50} = 0.67 μM), (-)-epicatechin-3-*O*-gallate (ECG) (IC_{50} = 1.3 μM), and theasinensin A (IC_{50} = 0.13 μM), were found to be potent and selective inhibitors of rat squalene epoxidase (SE), a rate-limiting enzyme of cholesterol biogenesis. On the other hand, flavan-3-ols without galloyl group at C-3 did not show significant enzyme inhibition. It was for the first time demonstrated that the cholesterol lowering effect of green tea may be attributed to their potent SE inhibition activities. Inhibition kinetics revealed that EGCG inhibited SE in non-competitive (K_I = 0.74 μM), and non-time dependent manner. The potent enzyme inhibition may be caused by specific binding to the enzyme, and by scavenging reactive oxygen species required for the oxygenase reaction.

KEYWORDS

Cholesterol, Squalene epoxidase, Enzyme inhibition, (-)-Epigallocatechin-3-*O*-gallate, Galloyl esters.

INTRODUCTION

Squalene epoxidase (SE) is a non-metallic, flavoprotein monooxygenase that catalyzes the conversion of squalene to (3*S*)2,3-oxidosqualene. In addition to oxygen, vertebrate SE requires FAD, NADPH, NADPH-cytochrome P-450 reductase, and a supernatant protein factor. SE is the only known non-cytochrome P-450 enzyme that epoxidizes an unactivated alkene. Vertebrate SE, a membrane-bound protein with molecular mass of 55 kDa, has been purified, cloned from several sources including rat and human, and recombinant proteins are now available for further characterization of the enzyme. SE has been considered to be a rate-limiting enzyme of the cholesterol biogenesis. Since the HMG CoA reductase inhibitors, that are currently clinically used as cholesterol-lowering drugs, could in principle suppress all post-mevalonate biosynthetic pathways, selective inhibition of cholesterol biosynthesis is a desirable pharmaceutical goal. Thus, the enzyme inhibitors for SE have been potential target for the design of such therapeutic agents. To-date, several potent and specific SE enzyme inhibitors including chemically synthesized squalene analogs and allylamine derivatives have been developed, however, there are as yet no reports of human clinical trials.

Here we describe that green tea polyphenols (GTP) are potent and selective inhibitors of SE. GTP are well known chemopreventive agents with a variety of biological effects including cholesterol lowering activity. This is the first report of the potent SE inhibition by naturally occurring compounds, and it was for the first time demonstrated that the cholesterol lowering effect of green tea may be attributed to the potent inhibition activities of SE, a rate-limiting enzyme of cholesterol biogenesis.

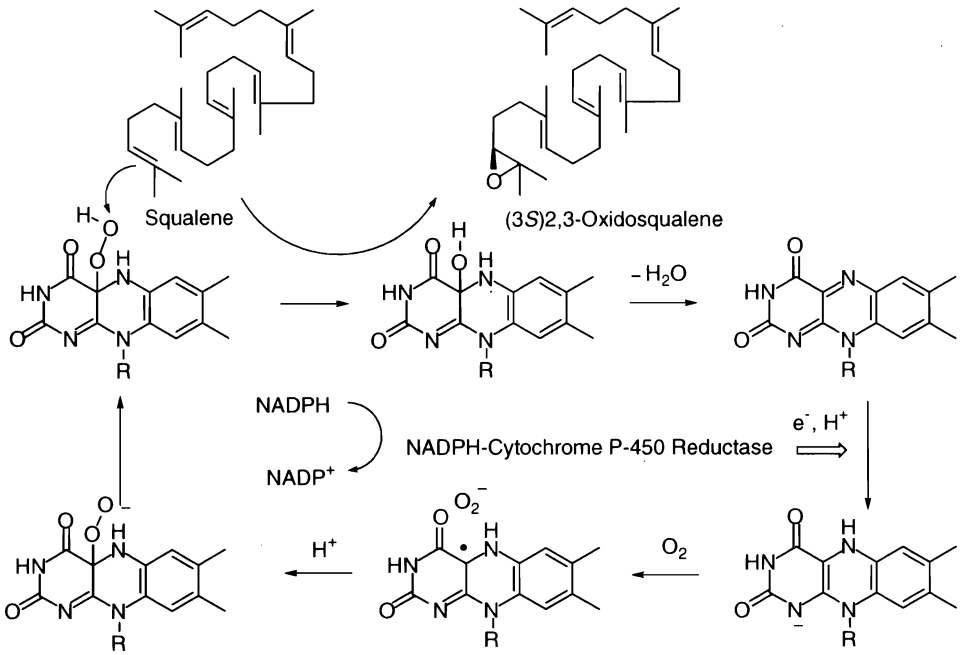

Fig. 1 Proposed mechanism of epoxidation of squalene to (3S)-2,3-oxidosqualene by SE.

MATERIALS AND METHODS

Chemicals. [1,25-^{14}C]Squalene (57.1 mCi/mmol) was synthesized according to the published method. Tea catechins were isolated from green tea (*Camellia sinensis*) leaves. Plant and herbal medicine extracts from ca. 300 plant species were prepared by refluxing with 10 volumes of methanol at 80 °C for 1 hour, and the solvent was removed by evaporation.

Enzyme. A truncated recombinant rat SE (Glu^{100}-His^{573}) without the N-terminal putative membrane domain and with an additional hexahistidine tag at the C-terminal was expressed in *E. coli*, and purified by Ni-NTA agarose and Blue Sepharose CL-6B columns.

Enzyme assay. Inhibitors were preincubated for 10 min at 37 °C in the assay mixture containing in a total volume of 200 μL of 20 mM Tris-HCl, pH 7.4, the recombinant rat SE (1.5 mg/mL), NADPH-cytochrome P-450 reductase (0.05 U), 1 mM NADPH, 0.1 mM FAD, 0.1% Triton X-100, and [1,25-^{14}C]squalene (5 μM, 2 x 10^4 dpm). After incubation at 37 °C for 60 min, the enzyme reaction was quenched by addition of 200 μL of 10% KOH in methanol, and 10 μL of 0.1% cold carrier solution of squalene and oxidosqualene in ethanol. The lipids were then extracted with 400 mL of CH_2Cl_2, and separated by TLC which was developed with 5% ethyl acetate in hexane. The Rf values were 0.84 for squalene and 0.54 for oxidosqualene. Radioactivities were analyzed by radio-TLC scanning.

RESULTS AND DISCUSSION

Out of ca. 300 plant and herbal medicine extracts tested for SE enzyme inhibition activities, tea plant (*Camellia sinensis*) extract showed significant enzyme inhibition (IC_{50} = 5 μg/mL) toward recombinant rat SE. The outstanding characteristics of the chemical composition of the green tea is its

very high concentration of polyphenolic metabolites, and further examination revealed that most of the activities were indeed recovered in the polyphenol fractions. A crude extract of green tea polyphenols (GTP) showed SE inhibition at almost the same concentration level (IC_{50} = 5 μg/mL). On the other hand, caffeine, another major component of green tea, and L-theanine, a unique amino acid found almost solely in tea plant, did not show significant SE inhibition activity.

The GTP was composed of (-)-epigallocatechin-3-*O*-gallate (EGCG) (**1**) (18.0%), (-)-gallocatechin-3-*O*-gallate (GCG) (**5**) (11.6%), (-)-epicatechin-3-*O*-gallate (ECG) (**3**) (4.6%), (-)-epigallocatechin (EGC) (**7**) (15.0%), (+)-gallocatechin (GC) (**9**) (14.8%), (-)-epicatechin (EC) (**8**) (7.0%), and (+)-catechin (C) (**10**) (3.5%). Among these catechins, flavan-3-ols with galloyl group at C-3; EGCG (IC_{50} = 0.69 μM), GCG (IC_{50} = 0.67 μM), and ECG (IC_{50} = 1.3 μM), showed potent enzyme inhibition of SE. This accounted for the total inhibition activities of the crude GTP extract. In contrast, flavan-3-ols without galloyl group, and with a catechol B-ring; EC (IC_{50} > 1000 μM) and C (IC_{50} >1000 μM), did not show significant enzyme inhibition, while flavan-3-ols with a pyrogallol B-ring; EGC (IC_{50} = 3.2 μM) and GC (IC_{50} = 44 μM), showed moderate inhibition. Furthermore, theasinensin A (**11**), a dimeric flavan-3-ol gallate, showed the most potent inhibition (IC_{50} = 0.13 μM).

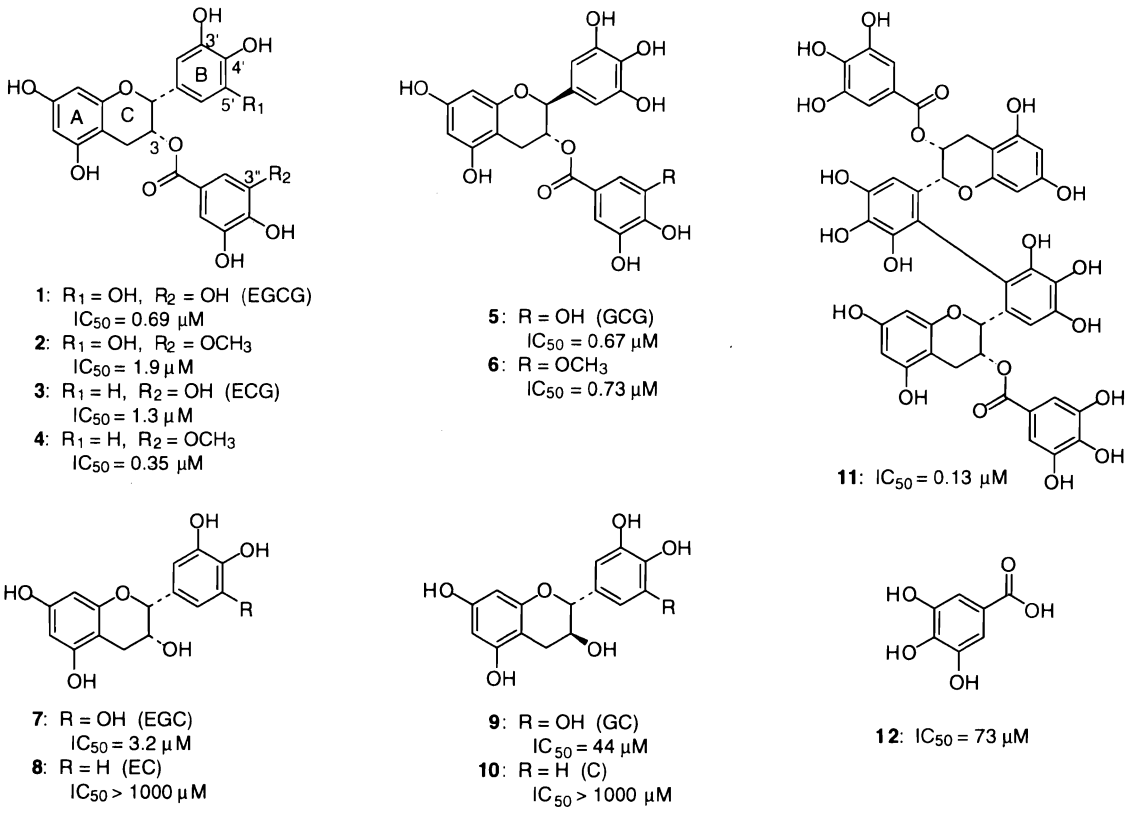

Fig.2 Structures and SE inhibition activities of green tea polyphenols.

The tea gallocatechins are the first examples of the potent SE inhibitors from natural sources. The inhibition activities were far more potent than those of known vertebrate SE inhibitors such as

chemically synthesized squalene analogs; trisnorsqualene alcohol (IC_{50} = 4 μM for pig SE), and trisnorsqualene cyclopropylamine (IC_{50} = 2 μM for pig SE).

EGCG (**1**), the major component of GTP, has been reported to be also a good inhibitor of several enzymes including acetyl-CoA carboxylase (IC_{50} = 310 μM), angiotensin I converting enzyme (IC_{50} = 90 μM), steroid 5α-reductase (IC_{50} = 15 μM), NADPH-cytochrome P450 reductase (K_I = 9.7 μM) and telomerase (K_I = 0.1 μM). The submicromolar level SE enzyme inhibition was therefore more potent than that of above described enzymes except telomerase. Further, in our assay system, EGCG did not show effective enzyme inhibition toward two other key enzymes in cholesterol biosynthesis; oxidosqualene:lanosterol cyclase and lanosterol 14α-demethylase (CYP51) (IC_{50} > 100 μM for both enzymes from rat). Thus, the potent SE enzyme inhibition by EGCG seemed to be highly selective. It has been demonstrated that both crude GTP and EGCG decreased plasma total cholesterol and LDL levels, and increased HDL concentration in rats when supplemented to the diets. We propose that the cholesterol lowering effect of green tea may be attributed to the potent inhibition activities of SE, a rate-limiting enzyme of cholesterol biogenesis.

Inhibition kinetics revealed that EGCG inhibited SE in non-competitive (K_I = 0.74 μM), and non-time dependent manner (k_{inact} < 0.01 min^{-1}). The non-metallic flavoprotein-mediated epoxidation has been proposed to proceed *via* formation of flavin C(4a)-hydroperoxide intermediate. The potent enzyme inhibition would be caused by specific binding to the enzyme, possibly in close proximity of the FAD binding domain, and by scavenging reactive oxygen species required for the enzyme reaction. Interestingly, antioxidative vitamins; α-tocophenol and L-ascorbic acid, did not inhibit SE at 1000 μM concentration, as in the case of the catechol-type polyphenols, suggesting that, in these cases, there is no specific binding to the enzyme involved. Finally, it is likely that the antioxidative GTP also inhibit other oxygenase reactions in the cholesterol biogenesis such as oxidative removal of methyl groups catalyzed by cytochrome P-450 systems. However, as described, rat lanosterol 14α-demethylase (CYP51) did not suffer significant enzyme inhibition (IC_{50} > 100 μM). Further analysis of the SE inhibition mechanism by tea gallocatechins are now in progress in our laboratories.

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