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Green tea polyphenol epigallocatechin-3-gallate (EGCG) induced intermolecular cross-linking of membrane proteins

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ABSTRACT

Increasing evidence has demonstrated that EGCG possesses prooxidant potential in biological systems, including modifying proteins, breaking DNA strands and inducing the generation of reactive oxygen species. In the present study, the prooxidant effect of EGCG on erythrocyte membranes was investigated. SDS–PAGE and NBT-staining assay were utilized to detect the catechol-protein adducts that generated upon treating the membranes with EGCG. The results indicated that EGCG was able to bind covalently to sulfhydryl groups of membrane proteins, leading to the formation of protein aggregates with intermolecular cross-linking. We suggested that the catechol-quinone originated from the oxidation of EGCG acted as a cross-linker on which peptide chains were combined through thiol-S-alkylation at the C2- and C6-sites of the gallyl ring. EGC showed similar effects as EGCG on the ghost membranes, whereas ECG and EC did not, suggesting that a structure with a gallyl moiety is a prerequisite for a catechin to induce the aggregation of membrane proteins and to deplete membrane sulfhydryls. EDTA and ascorbic acid inhibited the EGCG-induced aggregation of membrane proteins by blocking the formation of catechol-quinone. The information of the present study may provide a fresh insight into the prooxidant effect and cytotoxicity of tea catechins.

Introduction

Natural polyphenols, the most common and widely distributed phytochemicals in fruits and vegetables, are secondary plant metabolites that are involved in a wide range of biochemical processes [1]. Increasing evidence has demonstrated that natural polyphenols possess beneficial effects to human health. The antioxidant properties and free radical-scavenging activity of these compounds have been recognized to be responsible for their protective effects against neurological disorders, heart dysfunction, cancer, inflammation, and many other diseases [2–5]. However, a wealth of research data suggested that some natural polyphenols acted as prooxidants *in vitro* and *in vivo* [6–8]. The prooxidant effects of these compounds involve the generation of reactive phenoxyl intermediates and oxygen radicals which attack proteins, DNA strands and other cellular components, triggering a cascade of events leading to cytotoxicity and apoptosis. Actually, some health-beneficial effects of polyphenols, such as in cancer chemoprevention, are not related to their antioxidant properties but rather to their prooxidant action and direct interaction with target molecules [9–11].

Green tea is one of the most popular beverages which are rich in natural polyphenols. Its major biological active components are catechins (Scheme 1) including (−)-epicatechin (EC),[1] (−)-epicatechin-3-gallate (ECG), (−)-epigallocatechin (EGC) and (−)-epigallocatechin-3-gallate (EGCG). Among them EGCG is the most abundant catechin in green tea, and many of the biological properties of the beverage have been attributed to this compound [12]. In addition to its antioxidant activity and free radical-scavenging capacity, EGCG can also play a prooxidant role in biological systems [9–11,13]. Several studies have demonstrated that EGCG exhibited carcinogenic potential and genotoxicity [14–16]. Moreover, EGCG is reported to induce oxidative damage to macromolecules such as DNA and proteins through the generation of reactive oxygen species and quinonoid intermediates, resulted in inhibiting activity of numerous protein kinases [12,17], blocking transcription factors [9,12,18] and enhancing DNA cleavages [8,16]. Recent investigations indicated that EGCG was converted to a catechol-quinone upon auto-oxida-

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[1] *Abbreviations used:* EGCG, (−)-epigallocatechin-3-gallate; EGC, (−)-epigallocatechin; ECG, (−)-epicatechin-3-gallate; EC, (−)-epicatechin; DTNB, 5,5-dithiobis(2-nitrobenzoic acid); GSH, reduced glutathione; HMWAs, high molecular weight aggregates; EDTA, ethylenediaminetetraacetic acid; BHT, butylated hydroxytoluene; DTT, dithiothreitol; NEM, N-ethylmaleimide; NBT, nitroblue tetrazolium; ROS, reactive oxygen species; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis.

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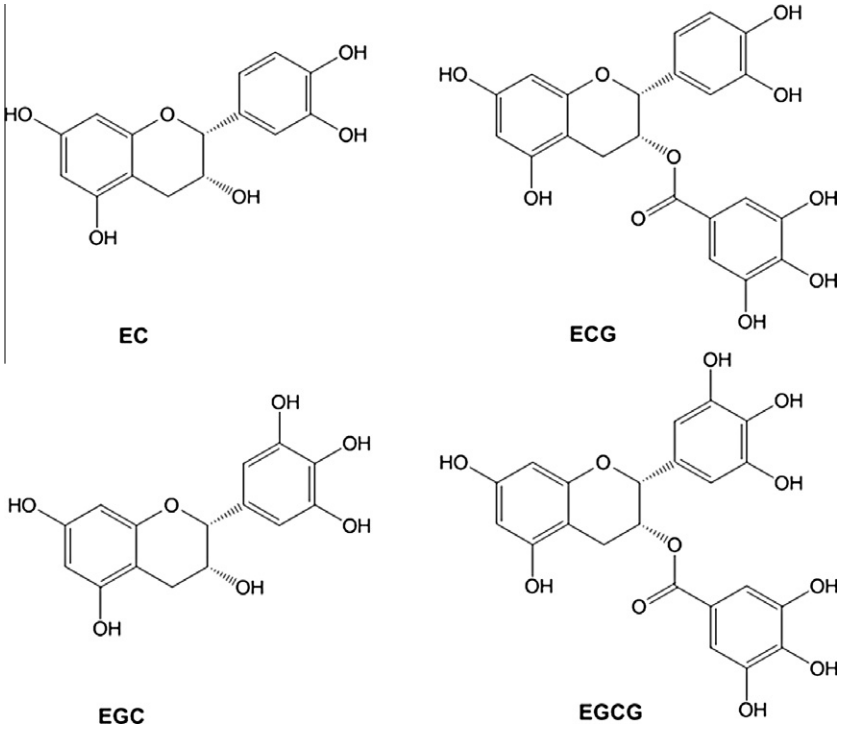

Scheme 1. Structures of the tea catechins used in the present study. EC, (−)-epicatechin; ECG, (−)-epicatechin-3-gallate; EGC, (−)-epigallocatechin; EGCG, (−)-epigallocatechin-3-gallate.

tion, and the resultant quinone moiety rapidly reacted with sulfhydryl group of a protein to form cysteinyl-flavonoid adducts [19].

In the present study, by using human erythrocyte as an *in vitro* model, the prooxidant effect of EGCG on the cell membranes has been explored. SDS–PAGE and the NBT-staining assay were utilized to detect the quinoprotein adducts that generated upon treating the membranes with EGCG. The roles of other catechin analogs and benzoquinone in the cell membranes have also been investigated for a comparison with the effects of EGCG.

Materials and methods

Chemicals

Nitroblue tetrazolium (NBT), dithiothreitol (DTT), N-ethylmaleimide (NEM), 5,5-dithiobis(2-nitrobenzoic acid) (DTNB), reduced glutathione (GSH), phenylmethanesulfonyl fluoride, cysteine, ascorbic acid and all the catechin compounds were purchased from Sigma & Aldrich (St. Louis, MO, USA); 1,4-benzoquinone and butylated hydroxytoluene (BHT) were from Fluka (Buchs, Switzerland). Electrophoresis related reagents were from Bio-Rad (Hercules, CA). Other reagents were of analytical grade.

Isolation of human erythrocytes

Fresh blood was drawn from the antecubital vein of healthy volunteers using sodium citrate as an anticoagulant. Serum and buffy coats were removed by centrifugation at 1000*g* for 10 min, and the packed erythrocytes were washed three times in cold isotonic phosphate-buffered saline (135 mM NaCl and 5 mM KCl in 10 mM phosphate buffer, pH 7.4). The suspensions of erythrocytes used in this study were freshly prepared daily.

Preparation of erythrocyte ghost membranes

Erythrocyte ghost membranes were prepared by hypotonic hemolysis. Packed erythrocytes were lysed by diluting the cells in hypotonic phosphate buffer in the presence of phenylmethanesulfonyl fluoride. The lysates were centrifuged at 30,000*g* for 20 min at 4 °C, and the pellets were resuspended and washed with phosphate buffer. Centrifugation and washing were repeated three times, and the final white pelleted ghost membranes were resuspended in the same buffer and stored at −40 °C.

Quantification of free sulfhydryl groups

Quantification of free sulfhydryl groups of ghost membranes was performed according to the Ellman's method [20]. Briefly, ghost membranes were treated with EGCG or other reagents for 30 min at 37 °C. The samples were then supplemented with DTNB (0.47 mM) and SDS (3%) and the absorbance was measured at 412 nm after further incubation for 15 min at room temperature. The concentrations of free sulfhydryl were calculated from a standard curve of absorbance versus concentration of cysteine.

Gel electrophoresis of ghost membranes and NBT staining for quinoproteins

SDS–PAGE was performed by using a 5% stacking gel and a 10% separating gel (2.6% C). Aliquots of ghost membranes were incubated at 37 °C for 30 min in the presence or absence of a catechin and other chemicals. Some samples were treated in the presence of 1 mM DTT for disulfide reduction or 1 mM NEM for sulfhydryl blocking. Bands were visualized by Coomassie brilliant blue R-250 staining. For blotting assay, the gel bands were transferred onto a polyvinylidene fluoride membrane (0.45 μm, Millipores) with a mini transfer cell (GE Healthcare). Quinoproteins were detected by staining with NBT (0.24 mM in 2 M potassium glycinate,