Regeneration of α-Tocopherol in Human Low-Density Lipoprotein by Green Tea Catechin

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Oxidative modification of low-density lipoproteins (LDL) may play an important role in the development of atherosclerosis. α-Tocopherol functions as a major antioxidant in human LDL. The present study was to test whether green tea catechins (GTC) would protect or regenerate α-tocopherol in human LDL. The oxidation of LDL incubated in sodium phosphate buffer (pH 7.4, 10 mM) was initiated by addition of 1.0 mM of 2,2′-azobis(2-amidinopropane) dihydrochloride at 40 °C. It was found that α-tocopherol was completely depleted within 1 h. Under the same experimental conditions, the longjing GTC extracts demonstrated a dose-dependent protective activity to α-tocopherol in LDL at concentrations ranging from 2 to 20 μM. Four pure epicatechin derivatives showed varying protective activity against depletion of α-tocopherol in LDL with (−)-epigallocatechin (EGC) and (−)-epigallocatechin gallate (EGCG) being less effective than (−)-epicatechin (EC) and (−)-epicatechin gallate (ECG). The results showed that addition of longjing GTC extracts, EC, ECG, and EGCG at 5, 10, and 15 min to the incubation mixture demonstrated a gradual regeneration of α-tocopherol in human LDL.

Keywords: *Epicatechin; epicatechin gallate; epigallocatechin; epigallocatechin gallate; α-tocopherol*

INTRODUCTION

The oxidative modification of low-density lipoprotein (LDL) is believed to play a role in the development of atherosclerosis (Brown and Goldstein, 1983; Jiala and Devaraj, 1996; Steinberg et al., 1989; Witztum and Steinberg, 1991). This hypothesis is supported by the observations that oxidatively modified LDL is present in atherosclerosis plaque but absent in normal artery wall (Haberland et al., 1988; Palinski et al., 1989; Shaikh et al., 1988). α-Tocopherol, the major form of vitamin E, is a primary antioxidant protecting LDL from oxidation (Jessup et al., 1990; Knipping et al., 1990). Increased vitamin E intake has been shown to be associated with the reduced oxidation of LDL, platelet adhesivenss, and thrombosis (Hodis et al., 1995; Jialal and Grandy, 1992; Kritchevsky et al., 1995; Princen et al., 1992; Reaven et al., 1993; Stanley et al., 1996). Epidemiological data also suggest that vitamin E supplementation is associated with a lower risk of coronary heart disease in both men and women (Losonczy et al., 1996; Rimm et al., 1993; Stampfer et al., 1993). Growing evidence for a protective effect of vitamin E in humans also includes an inverse association between plasma α-tocopherol and mortality from cardiovascular heart disease (Gey et al., 1992; Riemersma et al., 1991).

Tea is the most widely consumed beverage in the world and is prepared from the leaves of the *Camellia sinensis* plant. There is an increasing interest in green tea catechin (GTC) extracts as dietary antioxidants against oxidation of LDL (Ding et al., 1992; Nanjo et al., 1993; Zhang et al., 1997a). These GTC extracts are mainly epicatechin derivatives including (−)-epicatechin (EC), (−)-epigallocatechin (EGC), (−)-epicatechin gallate (ECG), and (−)-epigallocatechin gallate (EGCG). Increased consumption of green tea has been shown to be inversely associated with serum total cholesterol and triacylglycerols (Kono et al., 1992; Imai and Nakachi, 1995). In addition, ingestion of tea beverage immediately produced a significant increase in human plasma antioxidant capacity in vivo (Serafini et al., 1996). In fact, the Zutphen Elderly Study by Hertog et al. (1993) showed an inverse correlation between tea consumption and coronary heart disease mortality after adjustment for age, total energy intake, total saturated fatty acid intake, physical activity, body-mass index, smoking, serum total cholesterol, and systolic blood pressure.

LDL oxidation will not occur substantially until α-tocopherol is depleted (Knipping et al., 1990; Jessup et al., 1990), implying that maintenance of high α-tocopherol concentration in LDL may be critical in protecting LDL against oxidation. It has been shown that ascorbic acid can recycle or regenerate α-tocopherol from its free radical form, thus sparing this lipophilic antioxidant (Chan et al., 1990; Kagan et al., 1992; Packer et al., 1979). In fact, plasma vitamin C has also been shown to be inversely correlated with coronary heart disease mortality (Gey et al., 1987). In view of the fact that rats fed diet containing polyunsaturated perilla oil diet had a 1-fold increase in plasma α-tocopherol level when GTC was supplemented (Nanjo et al., 1993), we hypothesize that the GTC extracts and the individual epicatechin derivatives are also capable of protecting α-tocopherol in vivo. The present study was therefore to study whether (i) GTC extracts and pure epicatechin isomers isolated from longjing green tea would maintain α-tocopherol levels in human LDL and (ii) GTC extracts and pure epicatechin derivatives would regenerate

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α-tocopherol from its free radical form in human LDL in vitro.

EXPERIMENTAL PROCEDURES

GTC Extraction and HPLC Analysis. Total GTC was extracted from longjing tea (Huangshan Forestry Farm, Xiaoshan, Zhejiang, China), and its individual epicatechin derivatives were analyzed by high-performance liquid chromography (HPLC) as we previously described (Zhang et al., 1997b). In brief, 10 mg of dry longjing tea leaves was extracted three times with 140 mL of hot distilled water (80 °C). The infusion was then cooled to room temperature, filtered, and extracted with an equal volume of chloroform to partially remove caffeine and pigments. The remaining aqueous layer was then extracted twice with an equal volume of ethyl acetate. The ethyl acetate containing GTC was then pooled and evaporated using a vacuum rotary evaporator. The resulting GTC was then dissolved in 10 mL of distilled water and freeze-dried overnight.

The individual epicatechin derivatives in longjing GTC extracts were analyzed using a Shimadzu LC-10AD HPLC (Tokyo, Japan) equipped with a ternary pump delivery system. In brief, 15 μL of longjing GTC solution (0.5 mg/mL) was injected onto the column (Hypersil ODS, 250 × 4.6 mm, 5 μm, Alltech, Deerfield, IL) via a Rheodyne valve (20 μL capacity; Cotati, CA). An eluting mixture of H_2O containing 0.05% H_2SO_4, acetonitrile, and ethyl acetate (86:12:2, v/v/v) was used at a flow rate of 1 mL/min. The individual epicatechin derivatives were separated and quantified using a UV detector at 280 nm (UVIS-205, Alltech) with (+)-catechin (Sigma, St. Louis, MO) as an internal standard. Identification of each derivative was made by comparison of retention time and cochromatography with authentic standards of EC, EGC, ECG, and EGCG (Kurita Industrial Co., Ltd., Tokyo, Japan). The extraction method used in the present study yielded 10.2 g of crude GTC extracts/100 g of longjing tea leaves in which EGCG, ECG, EGC and EC accounted for 68.0, 19.0, 1.4, and 3.2%, respectively, whereas caffeine and other minor components accounted for 8.4%. The average molecular weight (MWt) of longjing GTC extracts was calculated according to the following equation on the basis of the molecular weight of individual epicatechin derivatives and their percentage in GTC extracts: $MWt = MWt_{EGCG} \times 68.0.2\% + MWt_{EGC} \times 19.0\% + MWt_{EC} \times 3.2\% + MWt_{ECG} \times 1.4\%$.

Isolation and Purification of Individual Epicatechin Derivatives. Individual longjing epicatechin derivatives were isolated using a semipreparative column (Spherisorb ODS-1, 250 × 10 mm, 10 μm, Isco, Inc., Lincoln, NE). In brief, 50 mg of longjing GTC extracts in H_2O was loaded onto the column via a Rheodyne valve with a 250 μL sample loop. A 29% methanol solution in H_2O was used at a flow rate of 0.7 mL/min. The eluting peaks were monitored at 280 nm using a UV detector (UVIS-205, Alltech) and collected manually. The fraction containing the individual derivatives was checked immediately using an analytic column as described previously, and the purity of each derivative isolated was estimated to be >99% because only a single HPLC peak or only a single dot using thin-layer chromatography was found. The methanol was then removed in a rotary evaporator. The resulting pure epicatechin derivative were then freeze-dried overnight and stored in the dark at −20 °C until used.

LDL Isolation. Fresh blood was collected from healthy subjects at the Prince of Wales Hospital, The Chinese University of Hong Kong, Shatin, Hong Kong. To prevent lipoprotein modification, EDTA and NaN_3 solutions were added to freshly prepared plasma (final concentrations of EDTA and NaN_3 were 0.1 and 0.05%, respectively). LDL was isolated from plasma according to the method described previously (Havel et al., 1995). To minimize the oxidation of LDL, the centrifuge tube containing plasma was flushed with nitrogen. Briefly, the plasma was first centrifuged at 1500*g* for 15 min to remove cells and cell debris. The density was then increased to 1.019 by addition of a NaCI−KBr solution (dissolve 153 g of NaCI, 354 g of KBr, and 100 mg of EDTA in 1 L of H_2O; density = 1.33 g/mL) and recentrifuged at 160000*g* at 10 °C for 20 h. After the top layer containing chylomicron and very low-density lipoprotein was removed, the density of the remaining plasma fractions was increased to 1.064 and the plasma was recentrifuged at 160000*g* for an additional 24 h. The top LDL fraction was collected and then flushed with nitrogen and stored at −70 °C. The protein content of isolated LDL was determined using Lowry's method (Lowry et al., 1951).

Depletion of α-Tocopherol in LDL. The stock LDL fraction (5 mg of protein/mL) was dialyzed against 100 volumes of the degassed dialysis solution (pH 7.4) containing 0.01 M sodium phosphate, 0.9% NaCI, 10 μM EDTA, and 0.05% NaN_3 in the dark for 24 h. The solution was changed four times during the dialysis. Oxidation of α-tocopherol in LDL was induced by 2,2′-azobis(2-amidinopropane) dihydrochloride (AAPH), a peroxyl radical initiator. In brief, the LDL (150 μg of protein/mL) was incubated in sodium phosphate buffer (pH 7.4, 10 mM) at 40 °C with constant stirring. The various amounts of longjing GTC extracts or individual epicatechin derivatives were added either before, or 5, 10, and 15 min after the addition of 1.0 mM AAPH. An aliquot of the incubation solution (1 mL) was periodically taken and chilled in ice. After immediate addition of 1 mL of ethanol containing 0.5 mg of butylated hydroxyltoluene as an antioxidant and 1.0 μg of α-tocopherol acetate as an internal standard, the mixture was immediately extracted using 2 mL of hexane. The hexane was evaporated under a gentle stream of nitrogen, and the resulting extracts were redissolved into 100 μL of ethanol followed by HPLC analysis for determination of the remaining α-tocopherol in LDL.

HPLC Analysis of α-Tocopherol in LDL. α-Tocopherol in LDL was determined using a Shimadzu LC-10AD HPLC (Tokyo, Japan) equipped with a ternary pump delivery system. In brief, 10 μL of the extracts in ethanol derived from 1 mL of the LDL incubation solution was injected onto a C-18 column (Microsorb MV, 250 × 4.6 mm i.d., 5 μm, Rainin, Woburn, MA) via a Rheodyne valve (20 μL capacity; Cotati, CA). Methanol was used as an eluting solvent at a flow rate of 2 mL/min. α-Tocopherol was monitored using a diode array detector at either 200 or 280 nm and quantified according to the amount of α-tocopherol acetate added. The initial concentration of α-tocopherol in the final LDL incubation solution was found to be 1.44 μM (0.62 μg/mL).

Stability of Individual Epicatechin Derivatives. To explain the varying activity of four epicatechin derivatives against α-tocopherol depletion in LDL, the stability of these epicatechin derivatives (0.15 mg of each/mL) was examined in sodium phosphate buffer (pH 7.4). An aliquot (0.4 mL) of the incubation mixture was periodically sampled, and 0.1 mL of (+)-catechin solution (0.5 mg/mL) was added as an internal standard. The mixture was similarly extracted twice with 1 mL of ethyl acetate and then subjected to HPLC analysis as described above.

RESULTS

The rate of α-tocopherol depletion increased proportionally with increasing concentrations of AAPH from 0.5 to 5 mM (Figure 1). All subsequent LDL oxidation experiments were, therefore, initiated using 1.0 mM AAPH at 40 °C. When 1.0 mM AAPH was added in the LDL incubation mixture, there was a rapid consumption of LDL α-tocopherol, which was almost complete within the first hour (Figure 2). Longjing GTC added to the incubation medium exhibited an inhibitory effect on α-tocopherol consumption (Figure 2). Compared with the control sample, the complete depletion of α-tocopherol in LDL was extended to 3 h instead in the presence of 2 μM longjing GTC. For the same period, only 20% depletion of α-tocopherol in LDL was observed when incubated in the presence of 10 μM. In comparison, α-tocopherol in LDL remained unchanged in the presence of 20 μM for at least 4 h (Figure 2).

All four epicatechin derivatives isolated from longjing tea demonstrated a dose-dependent protecting activity to α-tocopherol in LDL. To simplify the presentation,

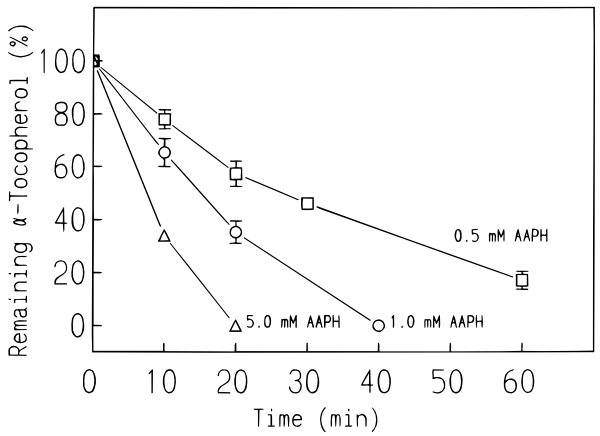

Figure 1. AAPH-dependent depletion of α-tocopherol in human LDL. The LDL (150 μg/mL) was incubated in sodium phosphate buffer (pH 7.4), and the oxidation of α-tocopherol (initial concentration = 1.44 μM) in LDL was induced by various amounts of AAPH at 40 °C as described under Experimental Procedures. Values are means ± SD of $n = 3-5$ samples.

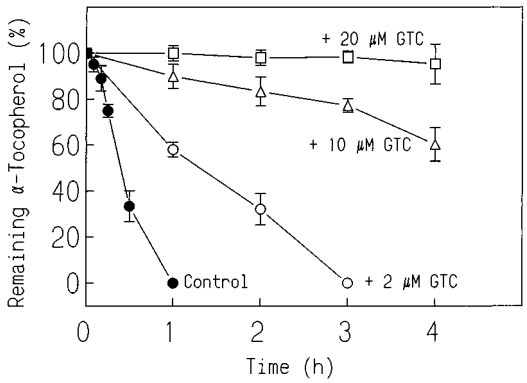

Figure 2. Effect of longjing GTC extracts on depletion of α-tocopherol in human LDL. The LDL (150 μg/mL) was incubated in sodium phosphate buffer (pH 7.4), and the oxidation of α-tocopherol (initial concentration = 1.44 μM) in LDL was induced by 1 mM AAPH at 40 °C as described under Experimental Procedures. Values are means ± SD of $n = 3-5$ samples.

only data for the incubation medium with the presence of 10 μM of these derivatives were shown. Four epicatechin derivatives showed various effectivenesses in protecting α-tocopherol in LDL at the concentration of 10 μM (Figure 3). The results showed that EGC was least effective followed by EGCG. In comparison, ECG was most effective in protecting α-tocopherol from depletion in LDL followed by EC.

To examine whether longjing GTC extracts regenerate α-tocopherol from its free radical form, various amounts were added at 5 or 15 min after the α-tocopherol consumption reaction had been initiated. The initial concentration of α-tocopherol was 1.44 μM in the incubation medium. As shown in Figure 4, α-tocopherol in LDL decreased to 33% (0.48 μM) of its original amount at 10 min followed by a gradual increase to 43% (0.62 μM) at 1 h when 15 μM GTC was added into the incubation medium 5 min after AAPH was added. The similar time course of regenerating α-tocopherol in LDL was also observed when 5 and 30 μM GTC were added 5 min after the reaction had been started (Figure 4). When 5 or 15 μM longjing GTC was added 15 min after the α-tocopherol depletion reaction had been initiated, its role of regenerating α-tocopherol was best illustrated

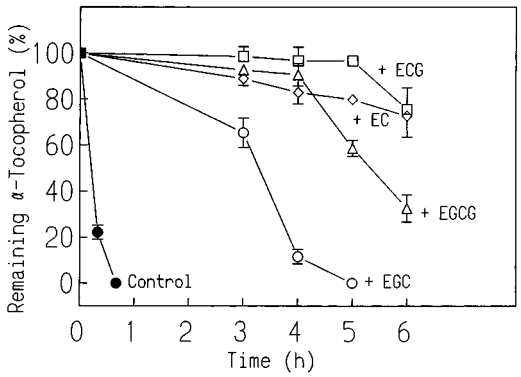

Figure 3. Effect of pure epicatechin derivatives (10 μM) on depletion of α-tocopherol in human LDL. The LDL (150 μg/mL) was incubated in sodium phosphate buffer (pH 7.4), and the oxidation of α-tocopherol (initial concentration = 1.44 μM) in LDL was induced by 1 mM AAPH at 40 °C as described under Experimental Procedures. Values are means ± SD of $n = 3-5$ samples.

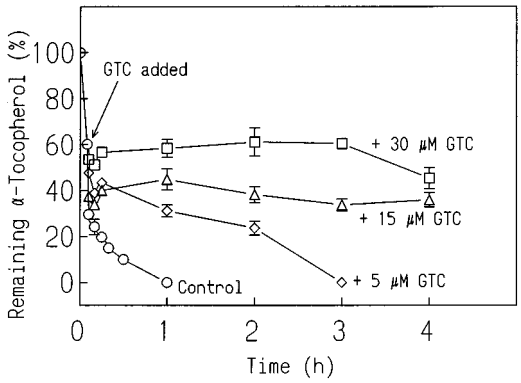

Figure 4. Time course of α-tocopherol regeneration in human LDL by longjing GTC extracts. The LDL (150 μg/mL) was incubated in sodium phosphate buffer (pH 7.4), and the oxidation of α-tocopherol (initial concentration = 1.44 μM) in LDL was induced by 1 mM AAPH at 40 °C as described under Experimental Procedures. Values are means ± SD of $n = 3-5$ samples.

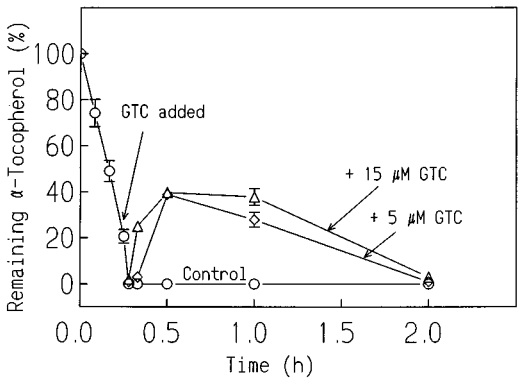

Figure 5. Time course of α-tocopherol regeneration in human LDL by longjing GTC extracts. The LDL (150 μg/mL) was incubated in sodium phosphate buffer (pH 7.4), and the oxidation of α-tocopherol (initial concentration = 1.44 μM) in LDL was induced by 1 mM AAPH at 40 °C as described under Experimental Procedures. Values are means ± SD of $n = 3-5$ samples.

(Figures 5 and 6). α-Tocopherol was almost completely depleted in LDL at 17 min and was regenerated, reaching 39% (0.56 μM) of its original amount at 30 min when 5 or 15 μM GTC was added 15 min during the ongoing α-tocopherol consumption reaction.

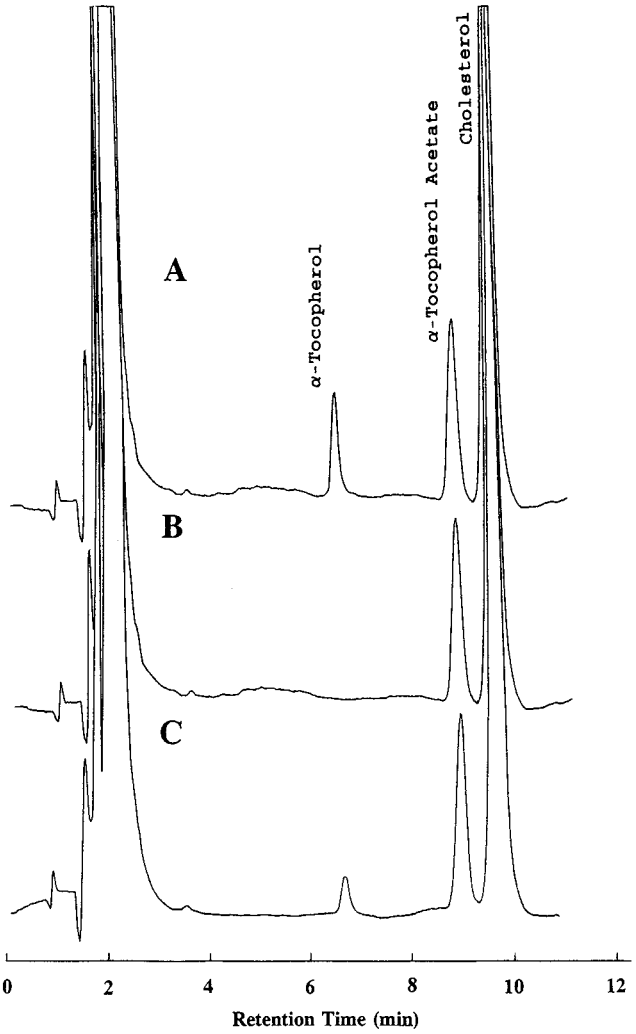

Figure 6. HPLC chromatograms showing the regeneration of α-tocopherol: (A) LDL was incubated in sodium phosphate buffer before addition of 1 mM of AAPH; (B) LDL was incubated in sodium phosphate buffer for 17 min after addition of 1 mM AAPH; (C) LDL was incubated in sodium phosphate buffer for 20 min after addition of 1 mM of AAPH but 15 μM of longjing GTC extracts was added at 15 min.

Under the same conditions, the individual epicatechin derivatives purified from longjing tea also demonstrated similar regenerating activity of α-tocopherol in LDL when 10 μM of these derivatives was added 5 min during the ongoing reaction except for EGC, which only delayed the rate of α-tocopherol depletion, showing no or very little regenerating activity (Figure 7). When the pure epicatechin isomers were added at 10 min, α-tocopherol in LDL dropped to zero and then ∼7−11% (0.10−0.16 μM) was regenerated except for EGC (Figure 8). In comparison to the GTC mixture, ECG, EC, and EGCG differ only in the magnitude of the recycling α-tocopherol in LDL.

Four epicatechin derivatives purified from longjing tea demonstrated various stabilities in sodium phosphate buffer (Figure 9). EGC and EGCG were equally unstable. In contrast, EC was most stable followed by ECG under the same conditions. As shown in Figure 9, EGC and EGCG were almost completely degraded when incubated for 3 h. Under the same conditions, EC remained unchanged while ECG decreased only by 20%.

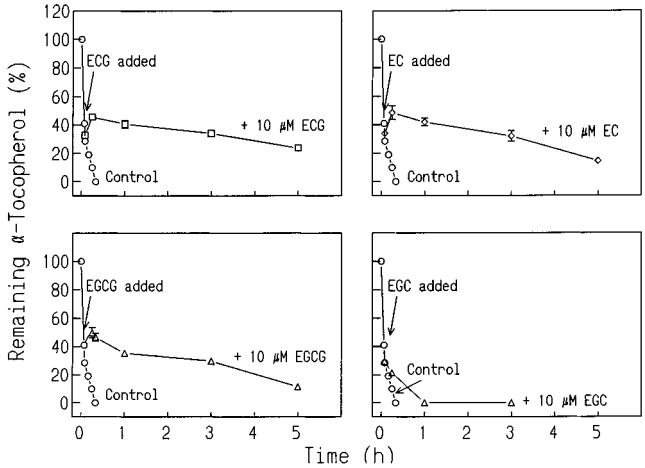

Figure 7. Time course of α-tocopherol regeneration in human LDL by pure epicatechin derivatives. The LDL (150 μg/mL) was incubated in sodium phosphate buffer (pH 7.4), and the oxidation of α-tocopherol (initial concentration = 1.44 μM) in LDL was induced by 1 mM AAPH at 40 °C as described under Experimental Procedures. Values are means ± SD of $n = 3-5$ samples.

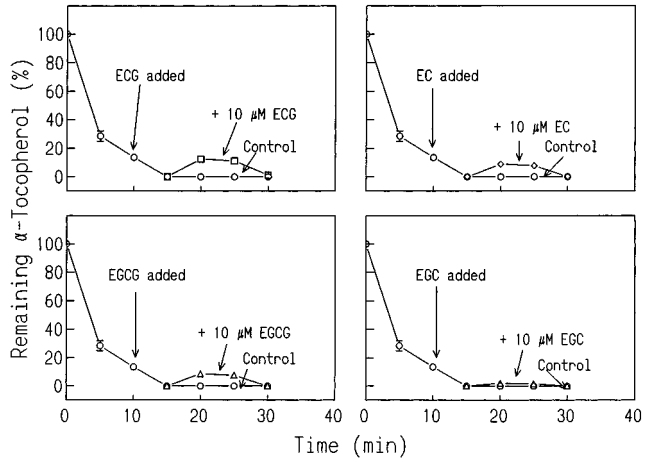

Figure 8. Time course of α-tocopherol regeneration in human LDL by pure epicatechin derivatives. The LDL (150 μg/mL) was incubated in sodium phosphate buffer (pH 7.4), and the oxidation of α-tocopherol (initial concentration = 1.44 μM) in LDL was induced by 1 mM AAPH at 40 °C as described under Experimental Procedures. Values are means ± SD of $n = 3-5$ samples.

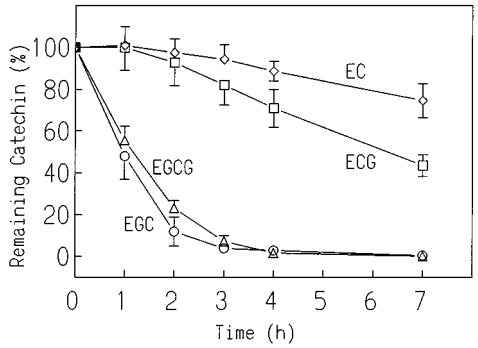

Figure 9. Stability of individual epicatechin derivatives (0.15 mg each/mL) in sodium phosphate buffer (pH 7.4, 60 mM). Values are means ± SD of $n = 5$ samples.

DISCUSSION

The study of the epicatechin derivatives is particularly important in understanding the health effect of

consuming green tea beverage. Together with our previous study (Zhang et al., 1997a), the present results clearly showed that GTC either as a mixture or as individual derivatives not only inhibits LDL oxidation but also prevents α-tocopherol from depletion in LDL in vitro (Figures 2 and 3). Two distinct effects of GTC and its individual derivatives on α-tocopherol were observed. First, longjing GTC and its individual isomers directly protect this lipophilic antioxidant from oxidation in a dose-dependent manner (Figures 2 and 3). This inhibitory effect could be accounted for by a direct interaction between GTC and AAPH free radicals in the aqueous phase. This is because GTC and its individual derivatives are free-radical chain-breaking antioxidants (Salah et al., 1995). Second, longjing GTC and its individual derivatives as reductants can recycle α-tocopherol from its free-radical form (Figures 4−8). This repairing effect on α-tocopherol was best illustrated by the observation that α-tocopherol level in LDL was partially restored when GTC (Figures 4 and 5) and its individual epicatechin derivatives were added during the depletion reaction (Figures 6−8).

Although there is very limited information available as yet on the absorption, metabolism, and excretion, the GTC has been shown to possess antioxidant activity in vivo (Serafini et al., 1996). The concentration of longjng GTC and its individual derivatives used in the present study is physiologically possible. Unno et al. (1996) showed that the concentration of EGCG in serum could reach 0.3 μM in humans after the ingestion of green tea. Our previous study in rats demonstrated that the concentration of total GTC could reach a maximum of 20−30 μM after an oral dose of 300 mg/kg of longjing GTC extracts (Zhang et al., 1997b). The study by Matsumoto et al. (1991) showed that ~20% could be absorbed when rats were orally given 50 mg of EGCG. The present results, although not directly transferable to in vivo conditions, may have implications for individuals who consume green tea beverage daily. In this connection, the ingestion of tea beverage has been shown to be associated with a significant increase in plasma antioxidant capacity in humans (Serafini et al., 1996), indicating uptake of these tea antioxidants into the blood. In addition, plasma α-tocopherol levels could increase by almost 1-fold in rats fed a diet containing polyunsaturated perilla oil when green tea catechin was supplmented (Nanjo et al., 1993). If drinking green tea is associated with a lower risk of coronary heart disease (Hertog et al., 1993), part of the mechanism may involve an increase in plasma antioxidant capacity either by directly protecting LDL from oxidation (Ding et al., 1992; Zhang et al., 1997a) or by maintaining and regenerating α-tocopherol in LDL. It is also well-known that higher plasma cholesterol is associated with an increased mortality of coronary heart disease. In addition to its antioxidant and regenerating α-tocopherol activity in LDL, green tea catechin may be also beneficial in reducing the risk of cardiovascular disease through its hypolipidemic effect. The studies by Imai and Nakachi (1995) and Kono et al. (1992) showed that green tea drinkers have lower plasma total cholesterol and triacylglycerols. The current research in this laboratory has shown that supplementation of GTC as a mixture significantly decreases plasma total cholesterol and triacylglycerols in hamsters (unpublished data).

The regenerating α-tocopherol property is not unique to the GTC and its individual epicatechin derivatives. Ascorbic acid and glutathione (GSH) have been shown to be capable of regenerating α-tocopherol in a number of systems. It is found that protection against the loss of α-tocopherol can be provided either by NADH-cytochrome b_5-dependent enzymatic recycling or by a nonenzymatic regenerating pathway involving ascorbate and dihydrolipoic acid in human erythrocyte membrane (Constantinecu et al., 1993). This repairing effect of ascorbate and dihydrolipoic acid on α-tocopherol was also extended to human LDL (Kagan et al., 1992). The study by Chan et al. (1990) has shown that ascorbate and GSH can also regenerate α-tocopherol in human platelets. To our best knowledge, the present study was the first to demonstrate that green tea epicatechins either as a mixture or as individual derivatives also possess α-tocopherol-repairing activity in human LDL under the present experimental conditions. It is known that a mixture containing both water-soluble and fat-soluble antioxidants will be more effective than a single antioxidant because the former mixture is capable of quenching free radicals in both aqueous and lipid phases (Chen and Tappel, 1996). The anti-free-radical function of α-tocopherol can be therefore augmented when any water-soluble antioxidant, particularly ascorbic acid and possibly green tea epicatechin, is present due to the regenerating activity of α-tocopherol.

Four epicatechin derivatives demonstrated various protective effects on α-tocopherol in LDL. The reason EGC and EGCG were relatively ineffective in comparison to EC and ECG remained unclear at the present time, but it was speculated to be partially related to their stability in the incubation medium. To prove this, we have examined the stability of these derivatives in sodium phosphate buffer. The results demonstrated clearly that EGC and EGCG were less stable than EC and ECG (Figure 9), and the rapid loss of α-tocopherol in LDL incubated with EGC and EGCG at 3−4 h (Figure 3) was coincident with their degradation curve (Figure 9). This indicates that the ineffectiveness of EGC and EGCG as antioxidants against consumption of α-tocopherol in LDL is at least partially due to chemical destruction of these two derivatives. Although EGCG (the major catechin derivative) is a strong antioxidant, its stability has to be considered when the antioxidant activity is assessed.

In conclusion, the study presented here along with other relevant publications suggests that GTC as a mixture and the individual epicatechin derivatives can be absorbed and circulated into the blood, where they may function as antioxidants protecting LDL from oxidation either directly, by protecting LDL from the attack of free radicals, or via the mechanism of maintaining and regenerating α-tocopherol. All of the growing evidence so far suggests the recycling of α-tocopherol by water-soluble antioxidants including ascorbate, thiols, and green tea epicatechin derivatives may be an important mechanism for the enhanced antioxidant protection of LDL and the reduced risk of cardiovascular disease.

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