# Inhibition of Radical Reaction of Apolipoprotein B-100 and α-Tocopherol in Human Plasma by Green Tea Catechins

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(−)-Epicatechin (EC), (−)-epigallocatechin (EGC), (−)-epicatechin gallate (ECg), (−)-epigallocatechin gallate (EGCg), and Trolox inhibited the decreases of apolipoprotein B-100 (apoB) and α-tocopherol in a radical reaction of human plasma initiated by $Cu^{2+}$. The concentrations of EC, EGC, ECg, EGCg, and Trolox for 50% inhibition ($IC_{50}$) of apoB fragmentation were 39.1, 42.2, 14.6, 21.3, and 36.2 μM, respectively. Similar $IC_{50}$ values were observed for α-tocopherol consumption, indicating the close relationship between apoB fragmentation and α-tocopherol consumption. These results demonstrate that tea catechins serve as an effective antioxidant in plasma and that the gallate group has a strong antioxidative activity.

**Keywords:** *Atherosclerosis; catechin; green tea; LDL; protein degradation; a-tocopherol*

## INTRODUCTION

Oxidative modification of low density lipoprotein (LDL) is believed to be involved in atherogenic plaque formation, which is one of the main causes of coronary artery disease (Halliwell and Gutteridge, 1990; Ross, 1993; Palinski et al., 1989). An epidemiological study (Hertog et al., 1993) showed that intakes of dietary antioxidant flavonoids including tea polyphenols (Mukhtar and Ahmad, 2000) were inversely related to mortality due to coronary artery heart disease. Flavonoids are polyphenolic antioxidants naturally present in vegetables, fruits, and beverages such as tea and wine. Among these diets, green tea contains relatively large amounts of polyphenols named catechins. Several in vitro studies (Miura et al., 1995; Vinson et al., 1995; Yamanaka et al., 1997; Yokozawa et al., 1997) have been undertaken to show that tea flavonoids inhibit oxidation of isolated LDL utilizing $Cu^{2+}$ as a radical initiator.

To clarify the role of tea catechins in the prevention of atherosclerosis, it is necessary to evaluate the antioxidative effect in a more physiological condition rather than in the reaction utilizing isolated LDL. Recently, we have reported (Tanaka et al., 1999) that the radical reaction of isolated LDL or human serum caused by $Cu^{2+}$ gives a characteristic pattern of fragmented apolipoprotein B-100 (apoB) based on the immunoblotting assay. We have also demonstrated (Tanaka et al., 1999) that fragmented apoB proteins are present in normal serum and that they tend to increase with age. In addition, apoB shows unusually high reactivity, even comparable to α-tocopherol, in the radical reaction of plasma initiated by $Cu^{2+}$ compared to that of other proteins such as albumin and transferrin (Hashimoto et al., 2000; Yamada et al., 1998). The radical nature of the apoB fragmentation in plasma or serum by $Cu^{2+}$ has been confirmed (Tanaka et al., 1999). On the basis of these observations, the capacity of food factors to inhibit apoB fragmentation caused by radical reaction in plasma can be an effective indicator to evaluate their anti-atherogenic activity. In this study, we evaluated the antioxidative activity of tea catechins by this method. At the same time we estimated the antioxidative effect of catechins based on their interaction with α-tocopherol. As a result, we have found that tea catechins serve as an effective antioxidant in plasma and that the gallate group has a strong antioxidative activity.

## MATERIALS AND METHODS

**Materials.** (−)-Epicatechin (EC), (−)-epigallocatechin (EGC), (−)-epicatechin gallate (ECg), and (−)-epigallocatechin gallate (EGCg) were obtained from Mitsui Norin Co. Ltd. (Shizuoka, Japan). Trolox (6-hydroxy-2,5,7,8-tetramethyl-chroman-2-carboxylic acid) was purchased from Tokyo Kasei Co. Ltd. (Tokyo, Japan). Vectastain ABC-PO (goat IgG) kit was obtained from Vector Lab Inc. (Burlingame, CA). Anti-human apolipoprotein B goat IgG was purchased from Sigma Chemical Co. (St. Louis, MO). Poly(vinylidene difluoride) (PVDF) membrane filters were purchased from Millipore (Tokyo, Japan). Electrophoresis reagents were purchased from Nacalai Tesque Inc. (Kyoto, Japan). All other reagents were of analytical grade and were purchased from Wako Pure Chemical Co. (Osaka, Japan). Blood was taken from healthy volunteers with heparin treatment. Plasma was separated by centrifugation at 600*g* for 10 min.

**Oxidation of Human Plasma.** Human plasma was diluted 4-fold with PBS containing various concentrations of tea catechins or Trolox, and then transferred into a glass vial with a Teflon-covered screw cap. An aliquot (100 μL) was taken to make immunoblot analysis and to measure the α-tocopherol level for the 0 h sample. Oxidation was started at 37 °C by the addition of 40 mM of $CuSO_4$ to a final concentration of 400 μM. Every 2 h, 100 μL of aliquot was taken to make immunoblot analysis of apoB and to measure α-tocopherol as described below. The concentration of catechins and Trolox for 50% inhibition ($IC_{50}$) of apoB fragmentation and α-tocopherol consumption relative to a control was determined graphically as described by Vinson and Hontz (1995) for each plasma

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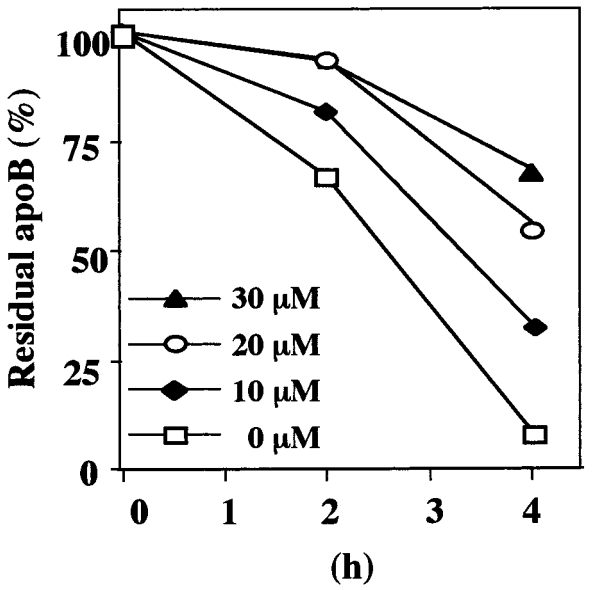


**Figure 1.** Change in apoB by the reaction of human plasma with $Cu^{2+}$ in the absence and presence of EGCg. Human plasma, diluted with PBS containing various concentrations of EGCg by 4-fold, was treated with 400 $\mu$M of $Cu^{2+}$ at 37 °C. From oxidized plasma solutions, samples were withdrawn 0, 2, and 4 h after the addition of $Cu^{2+}$, and the content of apoB was measured as described in the text. Each point is mean ± SE for three plasma samples of three individuals. Where no bars are shown, SE was smaller than the symbol.

sample. Three or four independent runs were made for each plasma. Three plasma samples from three individuals were used.

**Determination of α-Tocopherol and Immunoblot Analysis of apoB.** The level of α-tocopherol was determined as described by Buttriss and Diplock (1984). The conditions of HPLC and a fluorescence detector (Shimadzu RF-535, Kyoto) were reported previously (Kishida et al., 1993; Tokumaru et al., 1997).

A 100-$\mu$L aliquot was taken from the reaction mixture and put into a microtube, and 10 $\mu$L of 4 mM EDTA-2Na (pH 7.4) was added. These samples were treated with 100 $\mu$L of 4% SDS denaturation solution, and SDS gel electrophoresis on 4% polyacrylamide slab gels (1-mm thick) was performed according to the method of Laemmli (1970). Proteins separated on the gel were electrophoretically transferred to PVDF membrane filters as described by Towbin et al. (1979). Immunoblotting analysis apoB was done as described previously (Hashimoto et al., 2000; Yamada et al., 1998). The intensity of the stained band was measured by a densitometer (type CS-930 manufactured by Shimadzu Co. Ltd., Kyoto, Japan).

## RESULTS AND DISCUSSION

Human plasma was subjected to a radical reaction initiated by 400 $\mu$M of $Cu^{2+}$ at 37 °C as described previously (Yamada et al., 1998). ApoB decreased to 5.4% of the initial content after 4 h in the control reaction that was made in the absence of EGCg (Figure 1). When EGCg was added, the decrease of apoB was inhibited in a dose-dependent manner (Figure 1). Other catechins also inhibited the fragmentation of apoB in a dose-dependent fashion. To compare the inhibitory activity among these antioxidants, the concentration of each antioxidant for 50% inhibition ($IC_{50}$) of apoB fragmentation relative to the control was determined graphically at 4 h. The $IC_{50}$ value of EGCg for apoB fragmentation was 21.3 ± 3.8 $\mu$M (mean ± SD for the three plasma samples from three individuals, Table 1). Similar experiments were carried out, and $IC_{50}$ values for EC, EGC, Trolox, and ECg were determined as 39.1 ± 7.1, 42.2 ± 5.9, 36.2 ± 9.4, and 14.6 ± 3.6 $\mu$M, respectively (Table 1). The $IC_{50}$ values of ECg and EGCg were significantly lower than those of EC, EGC, and Trolox. These results indicate that EC and EGC have an activity comparable to that with Trolox as an antioxidant to apoB in the radical reaction of plasma. ECg and EGCg show significantly higher activity than EC, EGC, and Trolox, suggesting that the gallate group functions as a strong antioxidant. The activity sequence of catechins obtained in this study was somewhat different from that of a previous report using isolated LDL and 25 $\mu$M of $Cu^{2+}$ (Vinson et al., 1995). This discrepancy may be due to the difference in the reaction condition.

**Table 1. $IC_{50}$ Values ($\mu$M) of Catechins and Trolox for ApoB and α-tocopherol in the Oxidation of Plasma with $Cu^{2+}$[a]**

| | ApoB | α-tocopherol |
|---|---|---|
| EC | 39.1 ± 4.1[a] | 41.8 ± 13.2[a] |
| EGC | 42.2 ± 3.4[a] | 42.7 ± 3.0[a] |
| Trolox | 36.2 ± 5.4[a] | 60.8 ± 5.4[a] |
| ECg | 14.6 ± 2.1[b] | 11.5 ± 1.1[b] |
| EGCg | 21.3 ± 2.2[b] | 17.3 ± 2.2[b] |

[a] Human plasma, diluted with PBS containing various concentrations of catechins or Trolox by 4-fold, was treated with 400 $\mu$M of $Cu^{2+}$ at 37 °C. From oxidized plasma solutions, samples were withdrawn 0, 2, and 4 h after the addition of $Cu^{2+}$ and the contents of apoB and α-tocopherol were measured as described in the text. $IC_{50}$ was determined graphically at 4 h for apoB and at 2 h for α-tocopherol. Each value is mean ± SE of three plasma samples from three individuals. Different letters indicate significant differences in each column among antioxidants by Bonferroni/Dunn protected least significant difference test ($P < 0.05$).

The antioxidative activity of tea catechins and Trolox to α-tocopherol was evaluated at the same time in the radical reaction of plasma initiated by $Cu^{2+}$ at 37 °C. EGCg dose-dependently inhibited the decrease of α-tocopherol (Figure 2). As shown in Figure 2, the decrease of α-tocopherol preceded that of apoB. Therefore, $IC_{50}$ values for the inhibition of the decrease of α-tocopherol were determined at 2 h for the three plasma samples from three individuals (Table 1). The $IC_{50}$ of each antioxidant for α-tocopherol resembles that for apoB. These $IC_{50}$ values were also divided into two groups, and again gallate groups had a stronger antioxidant activity than other parts of catechins. The present study indicates that catechins have a protective effect on α-tocopherol and apoB in a similar dose-dependent manner. In addition, this observation indicates a sequential relationship between the decrease of α-tocopherol that was caused by lipid peroxidation, and apoB fragmentation. The activity sequence of catechins was similar to that of the reaction of isolated LDL using metmyoglobin (Salah et al., 1995). The protective activity of tea catechins on α-tocopherol in the reaction of LDL with an azo initiator was reported (Zhu et al., 1999).

Yamanaka et al. (1997) reported that EC and EGC had both prooxidant and antioxidant effects on the oxidation of isolated human LDL by 5 $\mu$M of $Cu^{2+}$ based on conjugated diene formation and apoB fragmentation. The prooxidant effect of catechins was not observed in the concentration range of the present study using plasma. Prooxidant effect of polyphenols is very important in some conditions, because polyphenols easily generate hydrogen peroxide by reaction with oxygen, as evidenced by the fact that hydrogen peroxide is indus-

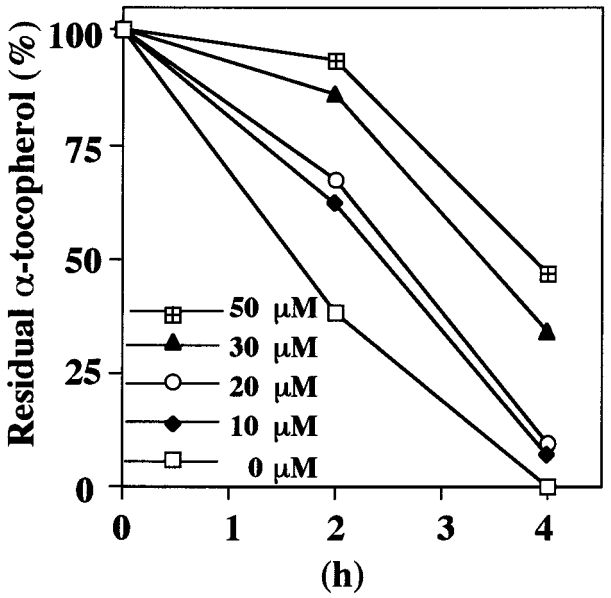


**Figure 2.** Change in α-tocopherol by the reaction of human plasma with $Cu^{2+}$ in the absence or presence of EGCg. Human plasma, diluted with PBS containing various concentrations of EGCg by 4-fold, was treated with 400 μM of $Cu^{2+}$ at 37 °C. From oxidized plasma solutions, samples were withdrawn 0, 2, and 4 h after the addition of $Cu^{2+}$, and the contents of α-tocopherol were measured as described in the text. Each point is mean ± SE for three plasma samples from three individuals. Where no bars are shown, SE was smaller than the symbol.

trially prepared by the reaction of naphthohydroquinone with oxygen. Reaction conditions such as metal and oxygen concentrations, and coexisting substances, may determine whether polyphenols function as a prooxidant or an antioxidant. Therefore, the antioxidant effect of food factors must be evaluated not only in a simple in vitro reaction but also in a more physiological condition.

The reaction condition for isolated LDL is considerably different from that for plasma. First, a similar rate profile of apoB fragmentation to the reaction of isolated LDL is observed when the concentration of $Cu^{2+}$ is higher by 2 orders of magnitude in the oxidation of plasma, probably because of antioxidants and metal-binding proteins contained in the plasma (Hashimoto et al., 2000). This indicates that apoB in plasma is more protected from oxidants than that in isolated LDL, and that a similar fragmentation is initiated once the reaction is initiated by a sufficient amount of $Cu^{2+}$. Second, a comparable concentration of catechins to $Cu^{2+}$ is necessary to observe their antioxidant effect in the reaction of isolated LDL (Yamanaka et al., 1997). However, a much smaller amount of catechins compared to the $Cu^{2+}$ concentration is needed to observe the antioxidant effect of catechins in the reaction of plasma. This result supports the view that the antioxidative effect of catechins does not arise from only a chelating effect to $Cu^{2+}$ (Brown et al., 1998), but that a radical quenching effect by hydrogen donation is also important. This is also supported by the study of Hayakawa et al. (1997) which showed that tea catechins do not inhibit, but rather promote, DNA cleavage in the presence of $Cu^{2+}$.

Finally, there is a physiological meaning of the present study. Piskula and Terao (1998) reported that the rat plasma concentrations of catechins, including their metabolites such as glucuronide and sulfate conjugates, amount to 1−12 μM after oral administration of EC at 172 μmol/kg. Therefore, it is possible that the total concentration of antioxidative catechins including their metabolites in plasma is within the range that displays an antioxidative effect on apoB as well as on α-tocopherol.

**Abbreviations Used.** apoB, apolipoprotein B-100; EC, (−)-epicatechin; EGC, (−)-epigallocatechin; ECg, (−)-epicatechin gallate; EGCg, (−)-epigallocatechin gallate; LDL, low-density lipoprotein; PBS, phosphate-buffered saline.

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