Theaflavins in Black Tea and Catechins in Green Tea Are Equally Effective Antioxidants[1]

(Manuscript received 2 March 2001. Initial review completed 26 April 2001. Revision accepted 31 May 2001.)

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ABSTRACT Green tea catechins, including (-)-epicatechin (EC), (-)-epicatechin gallate (ECG), (-)-epigallocatechin (EGC) and (-)-epigallocatechin gallate (EGCG), are oxidized and dimerized during the manufacture of black tea and oolong tea to form orange-red pigments, theaflavins (TF), a mixture of theaflavin (TF_1), theaflavin-3-gallate (TF_2A), theaflavin-3′-gallate (TF_2B) and theaflavin-3,3′-digallate (TF_3). The present study was designed to compare the antioxidant activities of individual TF with that of each catechin using human LDL oxidation as a model. All catechins and TF tested inhibited Cu^{+2}-mediated LDL oxidation. Analysis of the thiobarbituric acid–reactive substances (TBARS) and conjugated dienes produced during LDL oxidation revealed that the antioxidant activity was in the order: TF_3 > ECG > EGCG ≥ TF_2B ≥ TF_2A > TF_1 ≥ EC > EGC. Four TF derivatives also demonstrated a dose-dependent antioxidant activity in Cu^{+2}-mediated LDL oxidation at concentrations of 5–40 μmol/L. These results demonstrate that the TF present in black tea possess at least the same antioxidant potency as catechins present in green tea, and that the conversion of catechins to TF during fermentation in making black tea does not alter significantly their free radical–scavenging activity. J. Nutr. 131: 2248–2251, 2001.

KEY WORDS: • black tea • catechins • green tea • low density lipoprotein • oxidation • theaflavins.

Catechins are a group of natural polyphenols found in green tea. There are four main catechin derivatives, including (-)-epicatechin (EC),[3] (-)-epigallocatechin (EGC), (-)-epicatechin gallate (ECG) and (-)- epigallocatechin gallate (EGCG) (**Fig. 1**). Catechins account for 6–16% of the dry green tea leaves (1). Theaflavins (TF) are another group of polyphenol pigments found in both black and oolong teas. TF are formed from polymerization of catechins at the fermentation or semi-fermentation stage during the manufacture of black or oolong tea (2). TF contribute to the characteristic bright orange-red color of black tea, accounting for ~2 g/100 g of the dried water extract of black tea. The major TF in black and oolong tea are theaflavin (TF_1), theaflavin-3-gallate (TF_2A), theaflavin-3′-gallate (TF_2B) and theaflavin-3,3′-digallate (TF_3) (**Fig. 1**). Both catechins and TF have recently received much attention as protective agents against cardiovascular disease and cancer (3–5). They are also believed to have a wide range of other pharmaceutical benefits, including antihypertensive (6,7), antioxidative (8,9) and hypolipidemic (10,11) activities.

The oxidation of human LDL may contribute to the development of atherosclerosis (12,13). This idea is supported by the following observations: *1*) oxidatively modified LDL (ox-LDL) are present in atherosclerotic plaque but absent in the normal artery wall (14,15); *2*) ox-LDL are taken up by macrophage scavenger receptors, promoting cholesterol ester accumulation and foam cell formation (16); and *3*) ox-LDL are toxic to endothelial cells and stimulate monocyte adhesion to the endothelium (13). Catechins are effective inhibitors of LDL oxidation induced by copper and peroxyl free radicals (17,18). However, information is very limited concerning the relative anti-LDL oxidation activity of individual TF derived from black tea compared with that of individual catechins found in green tea. The objective of the present study was to isolate theaflavins from Chinese black tea and examine the relative antioxidant potencies of TF_1, TF_2A, TF_2B and TF_3 in comparison with those of EC, ECG, EGC and EGCG, using human LDL oxidation as a model.

MATERIALS AND METHODS

Isolation and purification of individual catechins and TF. Total catechins from Chinese longjing green tea (Huangshan Forestry Farm, Xiaoshan, Zhejiang, China) were extracted and isolated as previously described (17). To purify TF, qimen black tea (2.25 kg) was purchased locally and was first extracted three times using 13.5 L of 70% ethanol. After the removal of ethanol in a rotary evaporator, the remaining water solution was extracted subsequently using chloroform (3 L), ethyl acetate (2 L) and butanol (2 L). The ethyl acetate extract was then applied onto a silica gel column (80 × 6.5 cm i.d.; silica gel 60M, 230–240 mesh). The total TF fraction was obtained when the column was eluted with a mixture of chloroform and ethyl acetate (1:1, v/v) followed by increasing the ratio of chloroform to

[1] Supported by the Hong Kong Research Grant Council (Project No. CUHK 4237/00M).
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[3] Abbreviations used: EC, (-)-epicatechin; ECG, (-)-epicatechin gallate; EGC, (-)-epigallocatechin; EGCG, (-)-epigallocatechin gallate; MDA, malondialdehyde; ox-LDL, oxidatively modified LDL; TBARS, thiobarbituric acid–reactive substances; TF_1, theaflavin; TF_2A, theaflavin-3-gallate; TF_2B, theaflavin-3′-gallate; TF_3, theaflavin-3,3′-digallate.

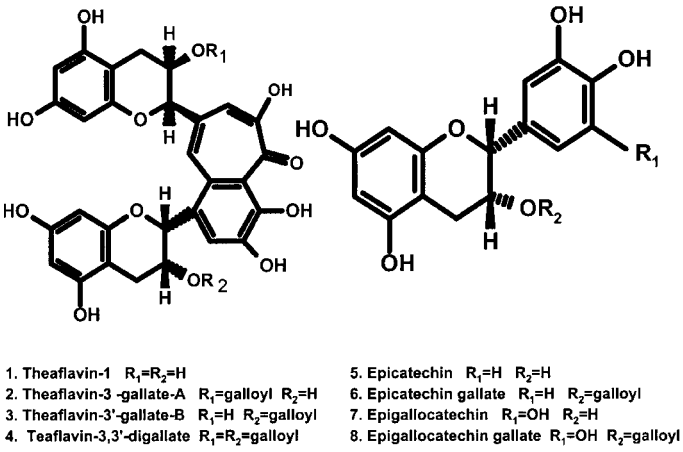

FIGURE 1 Chemical structures of theaflavin (TF_1), theaflavin-3-gallate (TF_2A), theaflavin-3′-gallate (TF_2B), and theaflavin-3,3′-digallate (TF_3), (-)-epicatechin (EC), (-)-epigallocatechin (EGC), (-)-epicatechin gallate (ECG) and (-)-epigallocatechin gallate (EGCG).

ethyl acetate to 4:1 (v/v). The total TF fraction was then subjected to a Sephadex LH-20 column (50 ×6.0 cm i.d.) and eluted with 15 L of 70% ethanol to obtain crude TF_1, TF_2A, TF_2B and TF_3 fractions. TF_1 was purified on a Sephadex LH-20 column B (50 × 2.5 cm i.d.) and eluted using 4 L of 30% acetone in water containing 2% acetic acid. TF_2A, TF_2B and TF3 were similarly isolated and purified. The chemical structures of the four purified TF were verified using the melting point test, TLC, UV spectrometry, liquid chromatography-mass spectrometry and ^1H nuclear magnetic resonance spectrometry. The results were in agreement with those previously reported (19).

LDL isolation. Fresh blood was collected and pooled from healthy subjects (n = 20) at the Prince of Wales Hospital, The Chinese University of Hong Kong, Shatin, Hong Kong. To prevent the lipoprotein from oxidative modification, EDTA (2.7 mmol/L) and NaN_3 (7.7 mmol/L) solutions were immediately added before LDL were isolated from serum according to the method previously described (17). The protein content of isolated LDL was determined using the method of Lowry et al. (20). The protocol was approved by the Committee of Human Ethics, The Chinese University of Hong Kong.

LDL oxidation. Oxidation of LDL was conducted as previously described by Puhl et al. (21). In brief, the stock LDL fraction (5 g protein/L) was dialyzed against 100 volumes of the degassed dialysis solution (pH = 7.4) containing 0.01 mol/L sodium phosphate, 9 g/L NaCl, 10 μmol/L EDTA and 7.7 mmol/L NaN_3 in the dark for 24 h. The dialysis solution was changed at least four times. Then, the dialyzed LDL were diluted to 250 mg protein/L with 0.01 mol/L sodium phosphate buffer (pH = 7.4). For the control incubation tubes, 0.4 mL LDL (250 mg/L) was mixed with 50 μL of 50 μmol/L $CuSO_4$ solution and 50 μL of 0.01 mol/L sodium phosphate buffer (pH = 7.4), and incubated at 37°C for up to 24 h. For the experimental tubes, 0.4 mL LDL (250 mg protein/L) was preincubated with 50 μL of varying concentrations of individual catechins and TF for 5 min. Then, 50 μL of 50 μmol/L $CuSO_4$ solution was added to initiate the oxidation, followed by incubation at 37°C for up to 24 h. The oxidation was then stopped by the addition of 25 μL of 27 mmol/L EDTA and cooled to 4°C. The degree of LDL oxidation was monitored by measuring the production of thiobarbituric acid–reactive substances (TBARS) as previously described (17). The LDL-incubated tubes were immediately combined with 2 mL of 0.67% thiobarbituric acid and 15% trichloroacetic acid in 0.1 mol/L HCl solution. The incubation mixture was then heated at 95°C for 1 h, cooled on ice and centrifuged at 1000 × g for 20 min. TBARS were then determined by measuring the absorbance at 532 nm. The calibration was done using a malondialdehyde (MDA) standard solution prepared from tetramethoxylpropane. The value of TBARS was expressed as nmol MDA/mg LDL protein.

Conjugated dienes. Oxidation of LDL was also monitored by measuring the production of conjugated dienes. In brief, the same amount of human LDL was incubated at 37°C and the oxidation was initiated by the same concentration of Cu^{+2} as described above. The absorbance of conjugated dienes at 234 nm was recorded at 0, 4, 8, 12, 16, 20 and 24 h. The change in the absorbance (Δ234 nm) was used as an index of LDL oxidation.

Statistics. Data were expressed as means ± SD, n = 6–7. For the dose effect of individual TF on Cu^{+2}-mediated LDL oxidation, the linear regression was conducted between the induction time and the dose of each TF. The trends were considered significant when $P < 0.01$. For the TBARS assay, differences were analyzed by a three-factor ANOVA with concentrations, types of catechins and TF, and the time of incubation as factors. Sigmastat (Jandel Scientific Software, San Rafael, CA) was used for all analyses. The differences were considered significant when $P < 0.01$.

RESULTS

Longjing is one of the most popular green teas consumed by Chinese. Total catechins, including EGCG, ECG, EC and EGC, accounted for 10.1 g/100 g dry longjing tea leaves (17). In contrast, qimen black tea is one of the most popular Chinese black teas exported. In this study, it was found by HPLC that the total theaflavins in qimen black tea were 1.58 g/100 g dry leaves. TF_3 was the most abundant (1.05%) followed by TF_2A (0.34%), TF_2B (0.11%) and TF_1 (0.08%).

TBARS were used as an index of LDL oxidation. When all catechins and TF were compared at 5 μmol/L, the antioxidant activity was in the order: TF_3 > ECG > EGCG ≥ TF_2B ≥ TF_2A > TF_1 ≥ EC > EGC (**Fig. 2**). A similar order was seen when oxidation was measured by conjugated diene production (data not shown) Under the same experimental conditions, vitamin C (ascorbic acid) had little or no protective activity for human LDL.

Four TF isolated from qimen black tea demonstrated dose-dependent antioxidant activities in Cu^{+2}-mediated LDL oxidation (**Fig. 3**, P for trend < 0.01). When 5 μmol/L concentrations of TF_1, TF_2A, TF_2B and TF_3 were incubated with

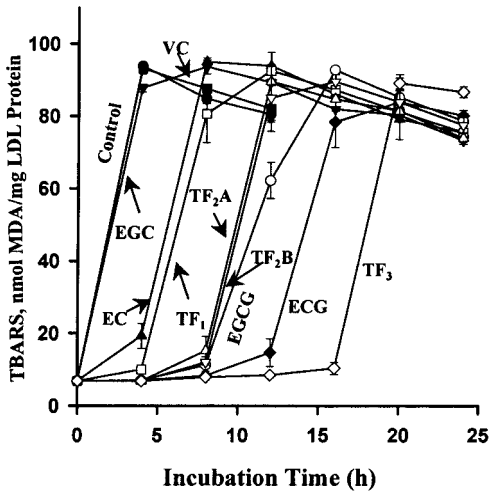

FIGURE 2 Comparison of the inhibitory effects of 5 μmol/L theaflavin (TF_1), theaflavin-3-gallate (TF_2A), theaflavin-3′-gallate (TF_2B), and theaflavin-3,3′-digallate (TF_3), (-)-epicatechin (EC), (-)-epigallocatechin (EGC), (-)-epicatechin gallate (ECG), (-)-epigallocatechin gallate (EGCG) and ascorbic acid (VC) on production of thiobarbituric acid–reactive substances (TBARS) in Cu^{+2}-mediated oxidation of human LDL. The LDL (100 mg protein/L) were incubated in sodium phosphate buffer (pH 7.4) containing 5 μmol/L $CuSO_4$. The oxidation was conducted at 37°C. Data are expressed as means ± SD, n = 6–7.

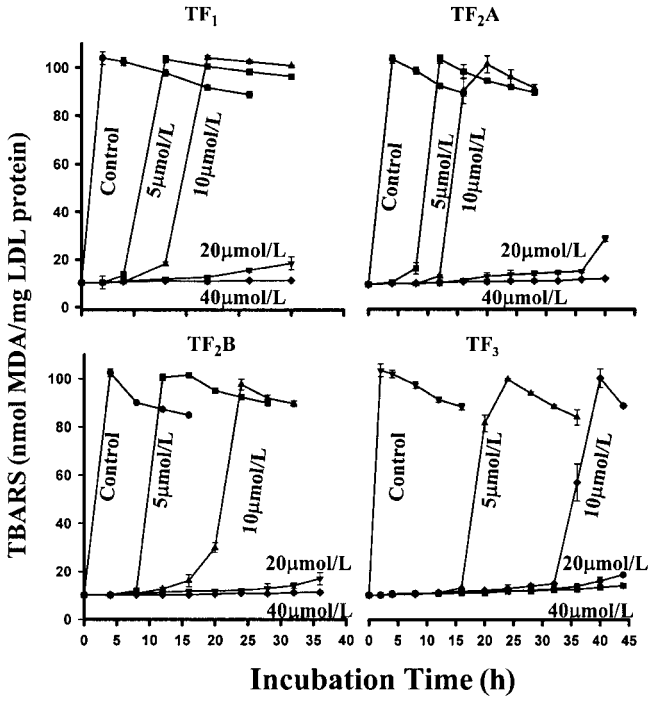

FIGURE 3 Dose-dependent inhibitory effects of theaflavin (TF_1), theaflavin-3-gallate (TF_2A), theaflavin-3′-gallate (TF_2B), and theaflavin-3,3′-digallate (TF_3) on production of thiobarbituric acid–reactive substances (TBARS) in Cu^{+2}-mediated oxidation of human LDL. The LDL (100 mg protein/L) were incubated in sodium phosphate buffer (pH7.4) containing 5 μmol/L $CuSO_4$. The oxidation was conducted at 37°C. Data are expressed as means ± SD, n = 6–7.

LDL, the lag times were 4, 7, 7.5 and 16 h, respectively. When 10 μmol/L concentrations of the four TF derivatives were incubated, the lag times were extended to 8, 12, 16 and 32 h, respectively. As the concentrations of the four TF were increased to 20 and 40 μmol/L, LDL showed little or no oxidation during the test period.

DISCUSSION

Tea is one of the most widely consumed beverages in the world; it can be grouped into three types, i.e., green, black and oolong tea. Green tea is not fermented and is a major beverage consumed in Asian countries. Black tea generally refers to the fermented tea that is more popular in North America and Europe. Oolong tea is a partially fermented product whose production and consumption are confined to China. Catechins are the major components of green tea leaves. In black tea, they are oxidized and dimerized during fermentation to the yellow-orange "pigments," TF, or polymerized to the red "pigments" called thearubigins. In contrast, oolong tea contains a mixture of catechins, TF and thearubigins. Many studies have demonstrated that both catechins and TF have strong free radical–scavenging activity both in vitro and in vivo. We found that little information exists concerning the relative antioxidant activity among individual catechins and TF derivatives. The present results demonstrated that the antioxidant activity was $TF_3 > ECG \geq EGCG \geq TF_2B \geq TF_2A > TF_1 \geq EC > EGC$ in decreasing order using human LDL as the oxidation model. Together with the study by Yoshino et al. (22), who showed that green tea and black tea infusions had similar antioxidant activities in rat liver homogenates, the present study clearly suggests that the TF present in black tea possess at least the same antioxidant potency as the catechins present in green tea.

TF are the products formed when catechins are oxidized and dimerized. TF_1 was formed by the oxidation and dimerization of EC and EGC (23). As shown in Figure 2, the antioxidant activity of TF_1 was similar to that of EC (lag time = 4 h) but it was stronger than that of EGC ($P < 0.01$). Similarly, TF_2A had an antioxidant activity similar to that of EGCG but it was more effective than EC; the former was produced when the latter two underwent oxidation and dimerization (23). Compared with the two precursors, TF_2B was weaker than ECG but stronger than EGC in protecting LDL from oxidation (Fig. 2). Interestingly, TF_3 was more effective than its two precursors, ECG and EGCG, in preventing LDL from oxidation (Fig. 2). The present comparisons indicate that TF are at least as effective as their precursors in protecting human LDL from oxidation. The conversion of catechins to theaflavins during the manufacture of black tea does not affect their free radical–scavenging potency on the same molar basis.

It is interesting that TF_3 containing two gallate groups inhibited LDL oxidation more than TF_2A and TF_2B, which have only one gallate group. Similarly, TF_2A and TF_2B had stronger antioxidant activities than TF_1 because the former two have one gallate group, whereas the latter contain no gallate group. This observation is consistent with that of Shiraki et al. (24), who showed that the galloyl moiety of TF was essential for their potent antioxidant activities. Similarly, EC and EGC were less active than their corresponding gallate derivatives, ECG and EGCG, as antioxidants against LDL oxidation (17). Perhaps an additional group increases the total number of phenyl hydroxyl groups and makes the gallate-containing catechins and TF more able to donate a proton due to the resonance delocalization.

The present study in vitro, although not directly applicable to humans, may have some implications for individuals who often consume black or green tea. It was previously demonstrated that the ingestion of green and black teas significantly increased human plasma antioxidant capacity in vivo (25). This was also in agreement with the observation that total plasma lipid peroxides and oxidation of LDL were significantly reduced in hamsters fed a high cholesterol diet and given green and black tea (26). All data presented here suggest that drinking black tea has benefits equal to those of drinking green tea in terms of their antioxidant capacity.

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