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Synergistic effect of green tea polyphenols with trolox on free radical-induced oxidative DNA damage

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Abstract

The antioxidant effect of the principal polyphenolic components extracted from green tea leaves, namely (−)-epicatechin (EC), (−)-epigallocatechin (EGC), (−)-epicatechin gallate (ECG) and (−)-epigallocatechin gallate (EGCG), and their synergistic antioxidant effects with trolox against oxidative DNA damage were studied. The oxidative DNA damage was initiated by a water-soluble azo initiator, 2,2′-azobis (2-amidinopropane hydrochloride) (AAPH) and the ability of green tea polyphenols and/or trolox (a water-soluble analogue of α-tocopherol) to inhibit the oxidative damage of DNA was assessed, in vitro, by measuring the conversion of supercoiled pBR322 plasmid DNA to the open circular and linear forms. It was found that these green tea polyphenols could significantly inhibit the oxidative damage of DNA synergistically with trolox, with an activity sequence of EC = ECG > EGCG > EGC.

Keywords: Green tea polyphenols; Trolox; DNA damage; Antioxidant activity; Synergism

1. Introduction

Epidemiological, biological and clinical studies have provided various lines of evidence in the past decade that free radical-induced oxidative damage of cell membranes, DNA and proteins might play a causative role in aging and several degenerative diseases, such as cancer, atherosclerosis and cataract, and that antioxidants, such as α-tocopherol (vitamin E), L-ascorbic acid (vitamin C) and β-carotene, might have beneficial effects in protecting against these diseases (Barnham, Masters, & Bush, 2004; Cooke, Evans, Dizdaroglu, & Lunec, 2003; Finkel & Holbrook, 2000; Perwez Hussain, Hofseth, & Harris, 2003). Therefore, inhibition of free radical-induced oxidative damage by supplementation of antioxidants has become an attractive therapeutic strategy for reducing the risk of these diseases (Brash & Harve, 2002; Rice-Evans & Diplock, 1993; Surh, 2003).

Green tea has been the most popular beverage in China for thousands of years. Drinking tea, especially green tea, is believed to be associated with a lower incidence of human cancer (Cao & Cao, 1999; Jankun, Selman, Swiercz, & Skrzypczak-Jankun, 1997; Nakachi et al., 1998; Yang & Wang, 1993). For example, in western countries, 1 in 10 women will develop breast cancer, while, in Japan, where drinking green tea is an integral art of lifestyle, only 1 in 40 women will develop breast cancer (Nakachi et al., 1998). We recently found that green tea polyphenols (GOHs) could reverse malignant phenotypic characteristics and induce redifferentiation of human hepatoma cells (SMMC-7721) (Zhou et al., 2004a). The mechanisms of cancer inhibition by green tea polyphenols remain an active area of research. (−)-Epigallocatechin gallate (EGCG), the most abundant constituent of green tea polyphenols, was reported to

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be active in preventing cancer by inhibiting angiogenesis (Cao & Cao, 1999), by inhibiting urokinase activity (Jankun et al., 1997), and by accelerating the apoptosis of cancer cells, whilst healthy cells are left unharmed (Chen, Schell, Ho, & Chen, 1998). A recent review summarized effects of tea polyphenols on signal transduction pathways related to cancer chemoprevention (Hou, Lambert, Chin, & Yang, 2004). The desirable cancer protective or putative therapeutic properties of green tea polyphenols have also been considered to depend on their antioxidant properties (Mitscher et al., 1997).

We have recently found that green tea polyphenols are good antioxidants against free radical initiated lipid peroxidation in solution (Jia, Zhou, Yang, Wu, & Liu, 1998a), in micelles (Zhou et al., 2000a; Zhou et al., 2000b; Zhou, Yang, & Liu, 2004b; Zhou, Wu, Yang, & Liu, 2005), in human red blood cells (Ma, Liu, Zhou, Yang, & Liu, 2000), in human low density lipoprotein (Liu, Ma, Zhou, Yang, & Liu, 2000), and in rat liver microsomes (Cai et al., 2002), and that the antioxidant activities of these polyphenols depend significantly on the structure of the molecules, the initiation conditions and the microenvironment of the reaction medium (Cai et al., 2002; Jia et al., 1998a; Liu et al., 2000; Ma et al., 2000; Zhou et al., 2000a, 2000b, 2004b, 2005). It was also found that these green tea polyphenols could interact with α-tocopherol (vitamin E), synergistically, to enhance their antioxidant activity (Jia et al., 1998a; Liu et al., 2000; Zhou et al., 2000a, 2000b, 2004b, 2005).Therefore, it is of interest to extend these researches to other oxidative substrates such as DNA, since DNA is also an important target for free radical attack. We herein examined the effects of polyphenols extracted from green tea, namely (−)-epicatechin (EC), (−)-epigallocatechin (EGC), (−)-epicatechin gallate (ECG) and (−)-epigallocatechin gallate (EGCG), and the synergistic effects of polyphenols with trolox (a water-soluble analogue of α-tocopherol), on the inhibition of AAPH-induced oxidative damage of supercoiled pBR322 plasmid DNA.

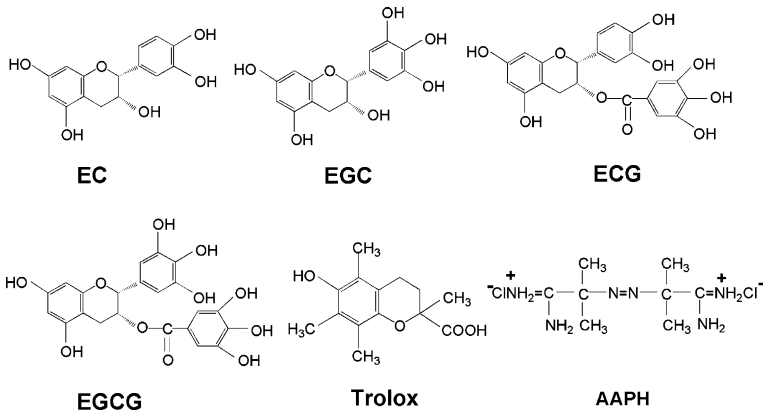
2. Materials and methods

2.1. Materials

(−)-Epicatechin (EC), (−)-epigallocatechin (EGC), (−)-epicatechin gallate (ECG) and (−)-epigallocatechin gallate (EGCG), were isolated from green tea leaves by extraction with methanol, water and ethyl acetate, consecutively, and chromatographic separation on a Sephadex LH-20 column, with reference to procedures previously reported (Nonaka, Kawakami, & Nishioka, 1983). Their structures and purity were confirmed by ^{1}H and ^{13}C NMR spectra and HPLC, respectively, as reported previously (Jia, Zhou, Yang, Wu, & Liu, 1998b). pBR322 was obtained from MBI. 2,2′-Azobis(2-amidinopropane hydrochloride) (AAPH, from Aldrich) and trolox (from Merck) were purchased with the highest purity available and used as received.

2.2. Assay for oxidative DNA strand breaks

Induction of DNA strand breaks by AAPH was measured by the conversion of supercoiled pBR322 plasmid DNA to open circular and linear forms, according to the procedure described previously (Li & Trush, 1993). The relative mobilities of the three DNA forms depend on agarose concentration, ionic strength of buffer, strength of the applied current, and relative densities of the three DNA forms (Jhnson & Grossman, 1977). Briefly, 100 ng of pBR322 DNA was incubated with the indicated concentration of AAPH in phosphate-buffered saline (PBS) (consisting of 137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4 and 1.5 mM KH_2PO_4) for 1.5 h at 37 °C at a final volume of 25 μl (i.e., DNA 5 μl, AAPH 10 μl, antioxidants 10 μl, if without antioxidants, 10 μl PBS for complement) in 1.5 ml microcentrifuge tubes. In the inhibition experiments, various GOHs and/or trolox were preincubated before addition of AAPH. Following incubation, the samples were mixed with 5 μl of gel loading buffer (0.13% bromophenol blue and 40% (w/v) sucrose) and immediately loaded into a 1% agarose gel



containing 40 mM Tris, 20 mM sodium acetate and 2 mM EDTA, and electrophoresed in a horizontal slab gel apparatus in Tris/acetate/EDTA gel buffer for 1 h (45 V/20 mA). After electrophoresis, the gels were stained with 0.6 μg/ml of a solution of ethidium bromide for 30 min, followed by another 30 min destaining in water. The gels were then photographed under UV light. DNA strand breaks were evaluated using the untreated DNA under the same incubation condition as a control.

In this study, we used Gel_Pro Analyzer (version 3.0 from Media Cybernetics USA) to quantify the density of supercoiled DNA form.

3. Results and discussion

3.1. DNA strand breaks initiated by AAPH

Conversion of the supercoiled form of plasmid or bacteriophage DNA to the open-circular and further linear forms has been used as an index of DNA damage (Ehrenfeld et al., 1987; Lewis, Stewart, & Adams, 1988). The formation of circular form of DNA is indicative of single-strand breaks and the formation of a linear form of DNA is indicative of double-strand breaks (Inouye, 1984; Zhang & Omaye, 2001). Since AAPH is water-soluble and the rate of free radical generation from AAPH can be easily controlled and measured, it has been extensively used as a free radical initiator for biological studies. It has been demonstrated previously that the presence of AAPH is able to cause strand breaks in pBR322 DNA (Zhang & Omaye, 2001). Thermal decomposition of AAPH at physiological temperature generates alkyl radicals which can react with oxygen and give alkylperoxyl radicals (ROO·). Alkylperoxyl radicals (ROO·) then attack plasmid DNA and lead to DNA oxidative damage (Eqs. (1)–(3)).

$$\mathrm{AAPH} \rightarrow 2\mathrm{R}^{\cdot} + \mathrm{N}_2 \quad (1)$$

$$\mathrm{R}^{\cdot} + \mathrm{O}_2 \rightarrow \mathrm{ROO}^{\cdot} \quad (2)$$

$$\mathrm{ROO}^{\cdot} + \mathrm{DNA} \rightarrow \mathrm{DNA\ damage\ product} + \mathrm{ROOH} \quad (3)$$

Fig. 1 shows AAPH-induced pBR 322 DNA strand breakings. In each lane, the lower band is due to the supercoiled DNA, the upper band to the open-circular DNA and in between is a band which sometimes appears due to the linear DNA. It is generally assumed that it takes only the single stand scission event to convert supercoiled DNA to open-circular DNA (Inouye, 1984) and the latter seems always to be present (in variable amounts, depending on the batch) in supercoiled DNA. A second strand scission event converts open-circular DNA into linear DNA provided this event occurs on the other (i.e., the uncut) strand and probably within about 5 bp of the break in the first strand. As

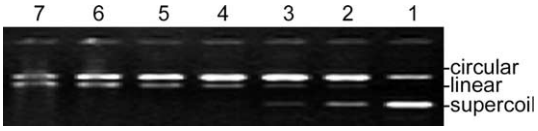


Fig. 1. Agarose gel electrophoretic pattern of pBR322 DNA (4 ng/μl) after treatment with indicated concentration of AAPH in PBS at 37 °C and pH 7.4 for 90 min. Lane 1: control; Lane 2: 2.5 mM AAPH; Lane 3: 5 mM AAPH; Lane 4: 10 mM AAPH; Lane 5: 20 mM AAPH; Lane 6: 40 mM AAPH; Lane 7: 80 mM AAPH.

shown in Fig. 1, the supercoiled DNA was gradually converted to open-circular DNA with the increase of concentration of AAPH (from 2.5 to 5 mM), and that open-circular DNA was gradually converted to linear DNA with the increase of concentration of AAPH (from 10 to 80 mM). It can be concluded that the damage of pBR322 DNA is concentration-dependent on AAPH.

3.2. The inhibition of AAPH-initiated DNA strand breaks by green tea polyphenols or trolox

Green tea polyphenols have been well studied as excellent antioxidants and good free radical-scavengers (Cai et al., 2002; Jia et al., 1998a; Liu et al., 2000; Ma et al., 2000; Zhou et al., 2000a, 2000b, 2004b, 2005). It was recently reported that GOHs could direct interact with DNA radicals to repair DNA by a mechanism of electron transfer (or H-atom transfer) (Anderson et al., 2001). As shown in Figs. 2 and 3, incubation of DNA with 10 mM AAPH for 90 min resulted in the formation of open circular and linear forms of DNA, indicating both single-strand and double-strand DNA breaks. Addition of trolox at 2.5–40 μM (Fig. 2) and EGCG at 0.625–40 μM (Fig. 3) to DNA resulted in a partial or complete inhibition of the conversion of supercoiled DNA to open circular and linear forms, indicating that trolox and EGCG are able to protect plasmid DNA against AAPH-initiated oxidative damage. With the concentrations of trolox or EGCG increasing, the formation of the open linear and circular forms DNA decreased (lanes 3–7 in Fig. 2 and lanes 3–9 in Fig. 3). The inhibition of AAPH-initiated DNA strand breaks by EGCG and trolox exhibited a concentration-dependent relationship. The inhibition effects produced

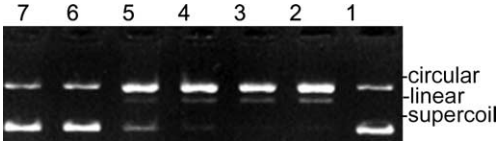


Fig. 2. Effects of trolox on pBR322 DNA (4 ng/μl) strand breaks induced by 10 mM AAPH in PBS at 37 °C and pH 7.4 for 90 min. Lane 1: control; Lane 2: AAPH; Lane 3: AAPH + 2.5 μM trolox; Lane 4: AAPH + 5.0 μM trolox; Lane 5: AAPH + 10.0 μM trolox; Lane 6: AAPH + 20.0 μM trolox; Lane 7: AAPH + 40.0 μM trolox.

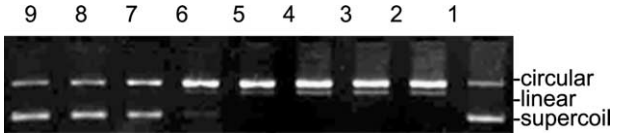


Fig. 3. Effects of EGCG on pBR322 DNA (4 ng/μl) strand breaks induced by 10 mM AAPH in PBS at 37 °C and pH 7.4 for 90 min. Lane 1: control; Lane 2: AAPH; Lane 3: AAPH + 0.625 μM EGCG; Lane 4: AAPH + 1.25 μM EGCG; Lane 5: AAPH + 2.5 μM EGCG; Lane 6: AAPH + 5.0 μM EGCG; Lane 7: AAPH + 10.0 μM EGCG; Lane 8: AAPH + 20.0 μM EGCG; Lane 9: AAPH + 40.0 μM EGCG.

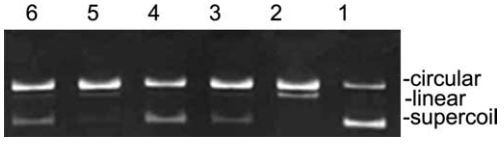


Fig. 4. Effects of green tea polyphenols (10 μM) on pBR322 DNA (4 ng/μl) strand breaks induced by 10 mM AAPH in PBS at 37 °C and pH 7.4 for 90 min. Lane 1: control; Lane 2: AAPH; Lane 3: AAPH + EGCG; Lane 4: AAPH + ECG; Lane 5: AAPH + EGC; Lane 6: AAPH + EC.

by green tea polyphenols (10 μM) are shown in Fig. 4 with the activity sequence of EC = ECG > EGCG > EGC.

3.3. Synergistic inhibition of AAPH-initiated DNA damage by green tea polyphenols with trolox

We found recently that GOHs could act synergistically with α-tocopherol to inhibit the peroxidation of lipid in homogeneous solution (Jia et al., 1998a), in micelles (Zhou et al., 2000a, 2000b, 2004b, 2005) and in human low density lipoprotein (Liu et al., 2000). The antioxidant synergism has been rationalized to be due to the reduction of the α-tocopheroxyl radical ($TO^{\cdot}$) by the coexistent GOH to regenerate α-tocopherol (Eq. (4)) (Zhou et al., 2000a, 2000b, 2005). The kinetic and mechanistic details of the tocopherol regeneration reaction by GOHs has recently been confirmed by electron paramagnetic resonance (EPR) spectroscopy in this laboratory (Zhou et al., 2000b, 2005). Therefore, it is desirable to investigate whether GOH and trolox (a water-soluble analogue of α-tocopherol) show similar synergistic antioxidant effects on DNA damage. As shown in Fig. 5, when 10 μM trolox (lane 3) and 2.5 μM EGCG (lane 4) were used alone, the weak supercoiled DNA appeared. But when 10 μM trolox and 2.5 μM EGCG were added together, the supercoiled DNA became clear and intense (lane 5), indicating that EGCG and trolox have a distinctly synergistic antioxidant efficiency. Furthermore, we used Gel_Pro Analyzer (version 3.0 from Media Cybernetics USA) to quantify the density of supercoiled DNA form. Values are shown in Fig. 6. This antioxidant synergism can be quantified by the percentage increment of the density of the supercoiled DNA form when the two antioxidants are used in combination with reference to the sum of the densities of the supercoiled DNA form when the two antioxidants are used individually, termed as synergistic efficiency (SE%) (Eq. (5)). For example, addition of trolox (10 μM) and ECG (2.5 μM) together gave the density of the supercoiled DNA form as 52.1% of control (lane 7) which was 172% more intense than the sum of the density of the supercoiled DNA form of trolox (10 μM, 9.6% of control, lane 3) and ECG (2.5 μM, 9.5% of control, lane 6) when they were used separately. Therefore, the SE% for ECG and trolox was 172%. The inhibition effects of GOHs and their synergistic efficiencies of GOHs with trolox on DNA damage are listed in Table 1. It can be seen from Table 1 that GOHs could inhibit the oxidative damage of DNA, used either alone or in combination with trolox, with an activity sequence of EC = ECG > EGCG > EGC. The sequence is similar to the activity sequence of GOHs against free radical-initiated lipid peroxidation in human low density

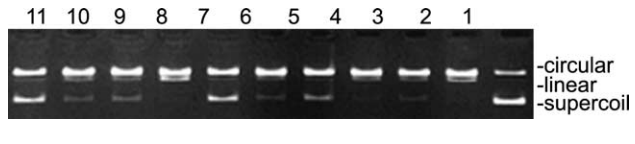


Fig. 5. Synergistic effects of green tea polyphenols (2.5 μM) with trolox (10 μM) on pBR322 DNA (4 ng/μl) strand breaks induced by 10 mM AAPH in PBS at 37 °C and pH 7.4 for 90 min. Lane 1: control; Lane 2: AAPH; Lane 3: AAPH + trolox; Lane 4: AAPH + EGCG; Lane 5: AAPH + EGCG + trolox; Lane 6: AAPH + ECG; Lane 7: AAPH + ECG + trolox; Lane 8: AAPH + EGC; Lane 9: AAPH + EGC + trolox; Lane 10: AAPH + EC; Lane 11: AAPH + EC + trolox.

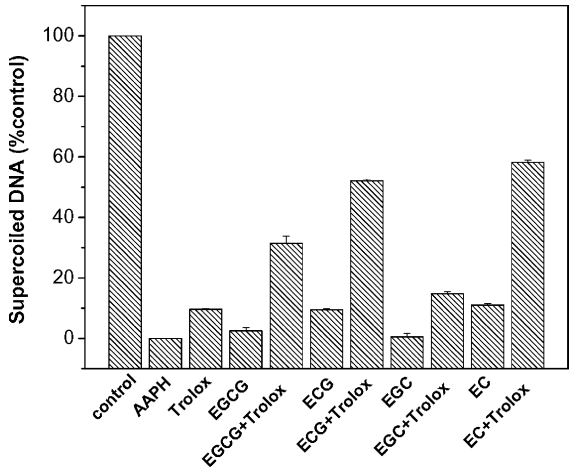


Fig. 6. Synergistic effects of green tea polyphenols (2.5 μM) with trolox (10 μM) on pBR322 DNA (4 ng/μl) strand breaks induced by 10 mM AAPH in PBS at 37 °C and pH 7.4 for 90 min. The density of the supercoiled DNA form was quantified by Gel_Pro Analyzer (version 3.0 from Media Cybernetics USA). Data are the average of three determinations.

Table 1
The synergistic efficiencies of GOHs (2.5 μM) with trolox (10 μM) on DNA damage

Compounds	Supercoiled DNA (%control)	SE%
Control	100	
Trolox	9.6 ± 1.3	
EGCG	2.5 ± 1.1	
EGCG + trolox	31.5 ± 2.4	159
ECG	9.5 ± 1.4	
ECG + trolox	52.1 ± 2.5	172
EGC	0.6 ± 1.1	
EGC + trolox	14.8 ± 2.7	45
EC	11.1 ± 3.6	
EC + trolox	58.1 ± 0.8	181

lipoprotein (Liu et al., 2000) and in rat liver microsomes (Cai et al., 2002).

$$TO^{\cdot} + GOH \rightarrow GO^{\cdot} + TOH \quad (4)$$

$$SE\% = \{\text{density(trolox} + GOH) - [\text{density(trolox)} + \text{density(GOH)}]\}/[\text{density(trolox)} + \text{density(GOH)} \times 100 \quad (5)$$

In conclusion, the principal polyphenolic components of green tea and trolox are effective antioxidants against free radical-induced oxidative DNA damage. The observation that GOHs and trolox can synergistically protect plasmid DNA from free radical-induced oxidative damage gives us useful information for antioxidant drug design.

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