Green Tea Polyphenols React with 1,1-Diphenyl-2-picrylhydrazyl Free Radicals in the Bilayer of Liposomes: Direct Evidence from Electron Spin Resonance Studies

Chang Chen,† Hui-Ru Tang,‡ Leslie H. Sutcliffe,‡ and Peter S. Belton*,‡

Institute of Biophysics, Academia Sinica, Beijing 100101, People's Republic of China, and Institute of Food Research, Norwich Research Park, Colney Lane, Norwich NR4 7UA, United Kingdom

Free radical scavenging reactions of green tea polyphenols (GTP) were investigated with electron spin resonance (ESR) spectroscopy in the phospholipid bilayer of liposomes, using 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical as a model. The results showed that (1) GTP reacts with DPPH radicals in the bilayer of liposomes of both 1-hexadecanoyl-2-[(*cis*,*cis*,*cis*,*cis*,*cis*,*cis*)-4,7,10,13,16,19-docosahexaenoyl]-sn-glycero-3-phosphocholine (DHAPC) and 1,2-di[*cis*-9-hexadecenoyl]-sn-glycero-3-phosphocholine) (DPPC); and (2) GTP protects DHAPC liposomes effectively from the oxidation initiated by DPPH radicals. These results provide direct evidence that GTP reacts with free radicals in the model membrane and support the hypothesis that GTP protects unsaturated phospholipids from oxidation by reacting directly with the radicals.

Keywords: *Green tea polyphenols (GTP); 1,1-diphenyl-2-picrylhydrazyl (DPPH); electron spin resonance (ESR); liposomes; free radicals*

INTRODUCTION

Recent epidemiological studies have shown an inverse association between the intake of green tea polyphenols (GTPs) and the risk of diseases such as cancer (Hertog et al., 1994; Yang et al., 1993; Chung et al., 1999; Kim et al., 1994), coronary heart disease, and stroke (Hertog et al., 1993, 1997; Hollman et al., 1996; Keli et al., 1996). It is believed that this protective action results from the bioactivity of GTPs acting as antimutagenesis (Wang et al., 1989; Teel et al., 1992; Hour et al., 1999; Kuroda et al., 1999; Mukhtar et al., 1992), antiinflammatory, and anticarcinogenesis agents (Kuroda et al., 1999; Mukhtar et al., 1992; Hertog et al., 1993; Li et al., 1999). Because tea is a popular beverage, the potential beneficial aspects of consuming tea and other polyphenol-containing foods have led to an explosion of interests in their bioactivity throughout the world. The term GTP describes a water-extractable mixture of green tea leaves which often accounts for up to 30% of the dry weight (Wang et al., 1994; Rice-Evans et al., 1996). Although the understanding of the chemical composition of GTP is far from complete, it is now generally accepted that it is mostly composed of monomeric species of flavan-3-ols such as catechin, its epimers, and esters of gallic acid (Wang et al., 1994; Rice-Evans et al., 1996; Nonaka et al., 1984; Hashimoto et al., 1987). In addition, some oligomeric components of catechin or its derivatives (Nonaka et al., 1983, 1984; Hashimoto et al., 1989; Lakenbrink et al., 1999) (also known as proanthocyanidins) and hydrolyzable tannins (Nonaka et al., 1984; Yoshida et al., 1989a) have been identified. To determine the relative effectiveness and possible synergistic effects, numerous research publications have focused on the structure−activity relationships of the individual polyphenolic components (Salah et al., 1995; Rice-Evans et al., 1996; Nanjo et al., 1996, 1999; Guo et al., 1996, 1999; Hashimoto et al., 1999).

Although beneficial aspects of tea have been demonstrated epidemiologically, no acceptable mechanistic conclusions have been established as to its biological activity. It is not clear whether tea polyphenols are acting in their original form or in the form of metabolites. It also remains to be understood whether direct chemical antioxidant effects or the elicitation of gene expression (Uda et al., 1997) are more important. Although it has been shown that GTP can react with a lipophilic radical in ethanol, no direct studies have been carried out on the scavenging potential of GTP in model membrane systems. If GTP does work by radical scavenging, it must be able to protect membranes in real systems and thus tests of its activity in membrane materials are important. Docosahexaenoic acid (DHA, $22{:}6\Delta^{4,7,10,13,16,19}$) is one of the polyunsaturated fatty acids (PUFA), which have recently received considerable attention because they are associated with health benefits for neurologic development, cardiovascular disease, cancer, and arthritis (Burns et al., 1990; Martinez et al., 1993). In the membranes of tissues including brain, sperm, and retinal rod outer segments, DHA can account for up to 50 mol % of the total fatty acids (Hendriks et al., 1976; Salem et al., 1986). DHA-containing phospholipids have a powerful influence on membrane structure and properties (Stillwell et al., 1993). Phospatidylcholine is quantitatively the most important phospholipid in mammary tissue (Hawke et al., 1995). Therefore, saturated (DPPC) and unsaturated 1-hexadecanoyl-2-[(*cis*,*cis*,*cis*,*cis*,*cis*,*cis*)-4,7,10,13,16,19-docosahexaenoyl]-sn-glycero-3-phosphacholine (DHAPC) were chosen separately as the model membrane materials. In this paper we report an ESR study of the reactions between GTP and DPPH radical located in the

† Academia Sinica.
‡ Institute of Food Research.
* Corresponding author (telephone 44-1603-255226; fax 44-1603-458939; e-mail Peter.Belton@bbsrc.ac.uk).

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bilayer of liposomes of DPPC and DHAPC in order to determine if the protective effect of GTPs, as present in tea, can protect phospholipids from oxidation by free radicals.

MATERIALS AND METHODS

Materials. DHAPC, DPPC, DPPH, and 2-thiobarbituric acid (TBA) were purchased from Sigma Co. (+)-Catechin was purchased from Sigma as monohydrate with minimum purity of 98%. Extruder 610000 and 0.1 μm membranes were bought from Avanti Inc. Other chemicals used were of analytical grade made in the UK.

GTP's were obtained from Zhejiang Agricultural University, P. R. China. GTP was extracted by the following method: the raw material was soaked in hot water with a solid-to-liquid ratio of 1:10. The mixture was then filtered to remove the residue. Addition of saturated aqueous calcium hydroxide to the solution precipitated polyphenols, leaving the caffeine in solution. The precipitate was separated from the solution and dissolved in hydrochloric acid solution at pH 1−3. The solution was extracted with ethyl acetate and the organic phase was concentrated at low pressure and finally dried in a vacuum to obtain crystalline GTP. We refer to this mixture as GTP rather than "tea catechins" to avoid confusion with catechin which is a pure compound ([2*R*,3*S*]-2-[3,4-dihydroxyphenyl]-3,4-dihydro-1[2H]-benzopyran-3,5,7-triol). GTP we used in this study had as major components: 58% epigallocatechin gallate (EGCG), 18% epicatechin gallate (ECG), 12% epigallocatechin (EGC), and 1% epicatechin (EC). Because this paper is focused on the behavior of whole GTP, no attempt has been made to separate and test each of the components.

Sample Preparation. *Liposomes*. Appropriate amounts of DHAPC or DPPC and DPPH (1 mol %) were dissolved in chloroform/methanol (2/1, v/v). After the solvents were evaporated off under a nitrogen stream, multilamellar vesicles were prepared by addition of water. After the multilamellar vesicles were frozen and thawed five times, they were then filtered through the membrane (0.1 μm) by extrusion to obtain small unilamellar vesicles. Normally, this process took approximately 30 min. The final concentration of the liposomes was 150 μM. Liposomes were always tested using ESR to ensure that DPPH free radical was dissolved in the bilayer of the liposomes, as indicated by the characteristic five peaks.

DPPH Water Suspension. A chloroform/methanol solution of DPPH was evaporated to dryness in a nitrogen stream, followed by the addition of water and vortex mixing. This suspension gives a relatively narrow single line in the ESR spectrum which indicates that the DPPH is in the microcrystalline state.

All measurements were carried out immediately after the liposomes were prepared.

HPLC-Based TBA Assay. The degree of the lipid peroxidation of DHAPC liposomes initiated by DPPH was analyzed by an HPLC-based TBA method (Hansen et al., 1998) with some modification as described below. Liposomes were treated with TBA to develop a colorimetric reaction as described previously (Guo et al., 1996). Then the malondialdehyde (MDA)−TBA adduct was quantified with a Gilson 715 HPLC system equipped with a 5 μm supelcosil LC18 column (25 cm × 4.6 mm). The mobile phase employed was 25 mM potassium phosphate buffer (pH 7.0)/methanol (65/35, v/v) with a flow rate of 1.0 mL min^{-1}). A 20 μL sample was injected. The elution was monitored with an UV detector at the wavelength of 532 nm. The MDA−TBA adduct was observed with a retention time of 5.4 min.

ESR Measurements. All measurements were carried out immediately after the reaction mixture was prepared; generally it took 2 min to make the samples. All ESR spectra were recorded at the ambient temperature (298 K) on a Varian E-4 X-band spectrometer. A personal computer fitted with a Real Time Devices ADA2000 digitizer card and EPRWare software was used to record the spectra: the hardware and software were supplied by Scientific Software Services (Bloomington, IL). ESR instrumental settings were as follows: microwave power 10 mW; modulation amplitude 0.125 mT; modulation frequency 93 kHz; time constant 0.3 s; scan time 4 min; scan range 10 mT; center field 0.3227 T.

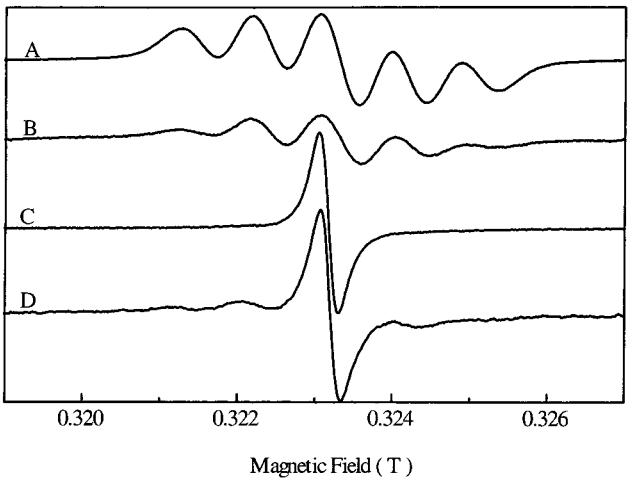

Figure 1. ESR spectra of DPPH free radical. (A) 100 μM DPPH chloroform/methanol (2/1, v/v) solution; (B) DPPH dissolved in the lipid bilayer of DPPC liposomes (DPPH 1 mol %.); (C) DPPH aqueous suspension (0.5 mg DPPH in 1 cm^3 H_2O); (D) DPPH distributed in both aqueous phase and the lipid bilayer of DPPC liposomes (DPPH 15 mol %).

RESULTS AND DISCUSSION

ESR Spectra of DPPH and Its Reaction with GTP. Figure 1 shows the ESR spectra of DPPH in both aqueous and lipid phase. DPPH is a stable lipophilic free radical which has been used in antioxidant activity analysis (Hatano et al., 1989; Yoshida et al., 1989b; Sawai et al., 1998; Yamaguchi et al., 1998; Nanjo et al., 1996). When it is solubilized in chloroform/methanol solvents, five clearly resolved peaks can be observed in its ESR spectrum (Nanjo et al., 1999) due to the interactions with two ^{14}N magnetic nuclei (Figure 1A). The characteristics of the ESR spectrum of DPPH distributed evenly in the lipid bilayer of DPPC liposomes (Figure 1B) are similar to those in organic solvents. This indicates that DPPH is solubilized in the bilayer of the liposomes. However, DPPH is not water soluble and the ESR spectrum of its water suspension shows one intense peak which is the typical signal of solid DPPH (Figure 1C). If DPPH is suspended in the aqueous phase of the liposomes, a composite spectrum is obtained, as shown in Figure 1D: the strong peak in the center of the spectrum is due to the contribution of solid DPPH suspended in water and the multiplet structure is due to DPPH in liposomes. Therefore, observation of the multiplet structure in a reaction mixture may be taken as characteristic of DPPH in liposomes.

Before undertaking a study of the behavior of DPPH and GTP in liposomes, it is useful to ascertain that good spectra could be obtained from organic media. Thus, a preliminary study of the system in chloroform/methanol was carried out.

Addition of an aqueous solution of GTP to a chloroform/methanol mixture results in a clear solution, indicating dissolution of all the GTP components. Figure 2 shows the ESR spectra of the DPPH chloroform/methanol solutions with added GTP aqueous solution. The intensity of ESR signal of DPPH decreases with the increase of the GTP concentration. The 50% scavenging dose for DPPH (100 μM) is about 5 μg cm^{-3} for GTP. This result is consistent with previously reported observations in

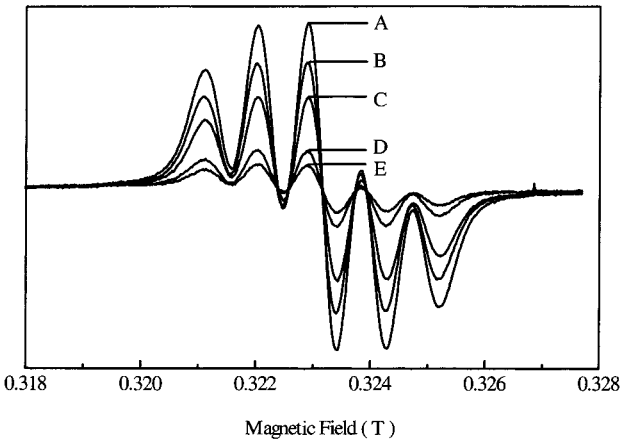

Figure 2. ESR spectra of DPPH with different concentrations of GTP in chloroform/methanol (2/1, v/v) solution. (A) 0 μg cm^{-3}; (B) 2 μg cm^{-3}; (C) 5 μg cm^{-3}; (D) 10 μg cm^{-3}; (E) 12.5 μg cm^{-3}.

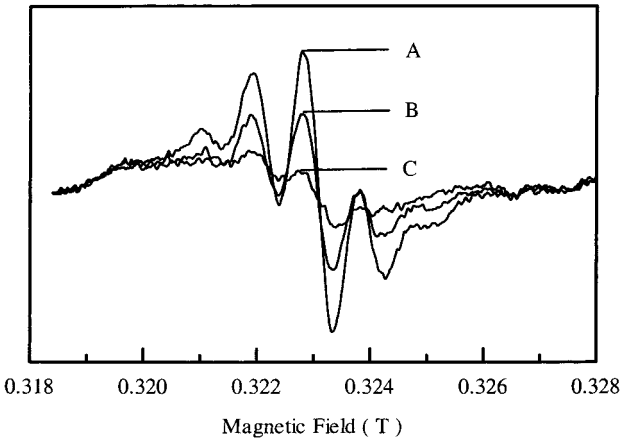

Figure 3. ESR spectra of DPPH in the lipid bilayer of DPPC liposomes with different concentrations of GTP. (A) 0 μg cm^{-3}; (B) 2 μg cm^{-3}; (C) 4 μg cm^{-3}.

ethanol (Nanjo et al., 1996, 1999), and indicates that GTP can scavenge free radicals in a number of organic solvents.

The Reaction of GTP with DPPH in the Bilayer of DPPC Liposomes. Figure 3 shows the ESR spectra of DPPH in the bilayer of DPPC liposomes with and without aqueous GTP solution. The multiplet structures of the spectra clearly indicate that the DPPH is dissolved in the bilayer and not suspended in the aqueous phase. When GTP solution was added, the intensity of the ESR spectrum of DPPH in the bilayer of the liposomes clearly decreased. This implies that the DPPH radical in the lipid bilayer of the liposomes was scavenged by GTP because there was no observable signal decrease for DPPH in DPPC liposomes in the absence of GTP (data not shown). The scavenging effect of GTP on the DPPH radical in the bilayer is dose-dependent (Figure 5A). The 50% scavenging dose is about 5 μg cm^{-3}, and is comparable to that for the chloroform/methanol mixture.

That green tea polyphenols are not unique among phenolic compounds in their ability to scavenge free radicals in the bilayer is shown by the behavior of (+)-catechin. Figure 4 shows that catechin also scavenges the DPPH in the lipid bilayer of DPPC liposomes, although the dose-dependent studies (Figure 5B) show that it is not as efficient as GTP. This may be because of the presence in GTP of other polyphenols such as

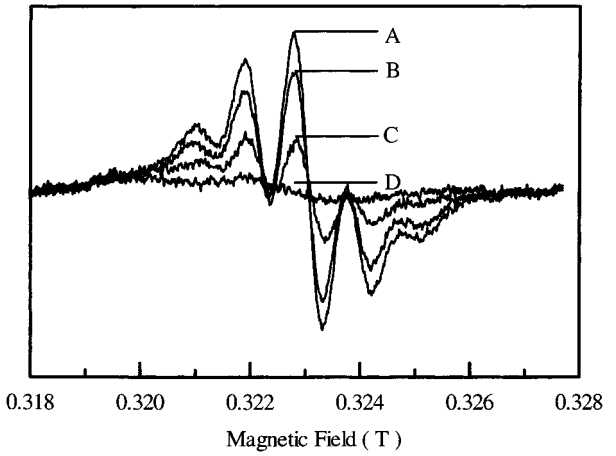

Figure 4. ESR spectra of DPPH in the lipid bilayer of DPPC liposomes with different concentrations of catechin. (A) 0 μg cm^{-3}; (B) 4 μg cm^{-3}; (C) 8 μg cm^{-3}; (D) 15 μg cm^{-3}.

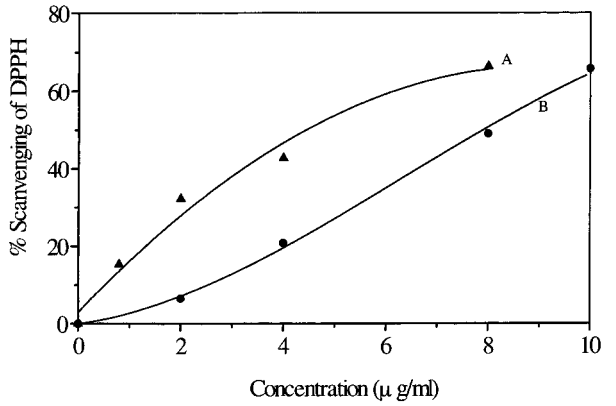

Figure 5. Dose-dependent scavenging effects of GTP and catechin on DPPH in the bilayer of DPPC liposomes (solid lines are a guide for the eye only). (A) GTP; (B) catechin.

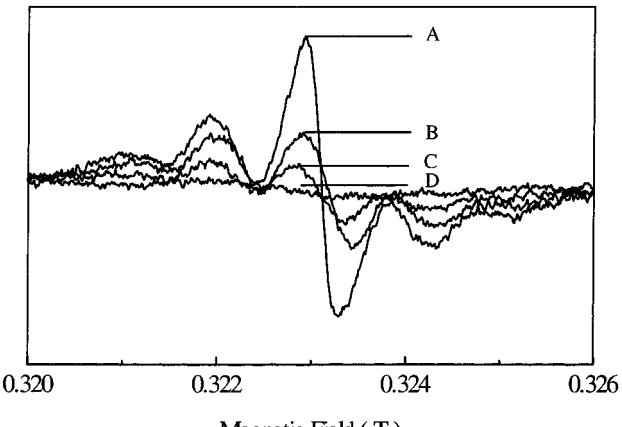

Figure 6. ESR spectra of DPPH in DHAPC liposomes. The reaction time: (A) 2 min; (B) 15 min; (C) 45 min; (D) 2 min with addition of 50 μg cm^{-3} GTP aqueous solution.

EGCG, ECG, and EGC. However, a synergistic effect (Li et al., 1999; Qi et al., 1999) of combined GTP components cannot be completely excluded.

DPPH in DHAPC Liposomes with and without GTP. In contrast with DPPC liposomes, the liposomes of the unsaturated DHAPC are liable to react with DPPH. Figure 6 shows the ESR spectra of DPPH changing with time in the DHAPC liposomes. The DPPH signal decreased drastically within 15 min and DPPH was effectively consumed in about 1 h. This

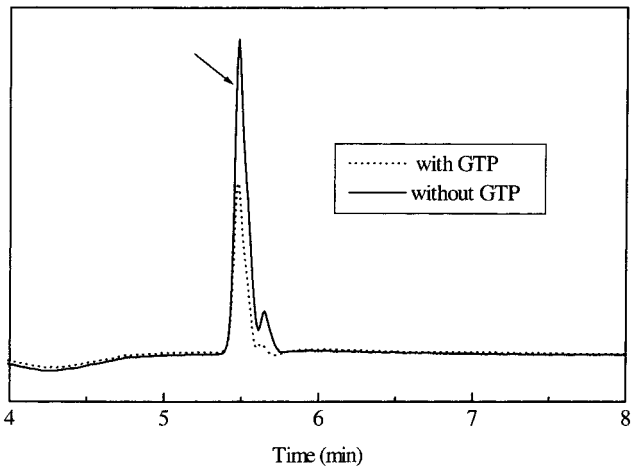

Figure 7. HPLC analysis of MDA−TBA production in DPPH-containing DHAPC liposomes with and without GTP. GTP concentration is 50 μg cm^{-3} (the arrow indicates the eluent with retention time of 5.4 min).

indicates the reaction of DPPH with unsaturated phospholipids in the liposome system. When GTP was added, DPPH in the bilayer of the liposomes was quickly scavenged within 2 min (Figure 6D). The presence of peroxides in DHAPC can be monitored by the reaction with TBA and MDA. Because the DPPH−DHAPC reaction presumably starts when DPPH is mixed with with DHAPC during the liposome preparation, some reaction will have taken place before GTP is added. This was shown by the HPLC result (Figure 7) which showed some MDA−TBA adduct in the system with GTP. However, when GTP was added, the amount of MDA−TBA adduct was reduced to about 48% of that without the protection of GTP (Figure 7). These results demonstrate that the peroxidation of the DHAPC liposomes was inhibited with the addition of GTP by direct scavenging of the DPPH in the bilayer of the liposomes.

In conclusion, this study provides direct evidence that GTP reacts with DPPH free radicals in the bilayer of liposomes of both DPPC and DHAPC. We infer therefore that GTP is active in the lipid bilayer and not merely the aqueous phase. The results clearly demonstrated that polyphenols can act as "chain breakers" (Salah et al., 1995) in the process of free radical attack on cells by scavenging lipid free radicals in the bilayer.

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