Peroxynitrite-Scavenging Activity of Green Tea Tannin

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Peroxynitrite formed from superoxide and nitric oxide acts as a strong reactive oxidant. However, among green tea components, catechins with a galloyl group inhibited peroxynitrite formation by 3-morpholinosydnonimine and scavenged peroxynitrite itself. Especially when compared with penicillamine as a positive control, the green tea components (−)-epigallocatechin 3-*O*-gallate and (−)-gallocatechin 3-*O*-gallate, which have two galloyl groups, showed the most potent peroxynitrite-scavenging activity, indicating that the galloyl group may contribute to this activity. A structure of flavan-3-ol linked to gallic acid may be essential for the peroxynitrite-scavenging activity.

Keywords: *(−)-Epigallocatechin 3-O-gallate; (−)-gallocatechin 3-O-gallate; green tea; peroxynitrite*

INTRODUCTION

Although nitric oxide (NO) is a free radical that has an unpaired electron, its reactivity is considerably low, showing no typical radical reactions such as hydrogen abstraction or radical addition. However, in vascular endothelial cells and macrophages in which NO and superoxide (O_2^-) are produced simultaneously, NO reacts promptly with O_2^- to produce peroxynitrite ($ONOO^-$) (Blough and Zafiriou, 1985; Malinski et al., 1993).

$ONOO^-$ is a potent oxidant for the SH group, and peroxynitrous acid (ONOOH) ($pK_a = 6.8$), that is, proton-linked $ONOO^-$, induces lipid peroxidation as it passes through an activated state with reactivity equivalent to the hydroxyl radical ($^{\bullet}OH$) in the process of epimerization to nitric acid (Radi et al., 1991a,b). In addition, in the presence of transition metal ions contained in superoxide dismutase (SOD), $ONOO^-$ produces the nitronium ion (NO_2^+), which has potent nitrating activity and attacks aromatic amino acid residues such as tyrosine (Beckman et al., 1994).

Available mechanisms for preventing oxidation injuries by such active oxygen series include prophylactic antioxidants, which prevent the formation of radicals, and chain-terminating antioxidants, which promptly scavenge radicals. Miura et al. (1994), Serafini et al. (1996), Zhang et al. (1997a,b), Chan et al. (1997), Pannala et al. (1997), and Haenen et al. (1997) have recently published data showing that tea, a time-honored drink which has been widely favored, and its component are useful antioxidants. We have also demonstrated using in vivo and in vitro experimental systems that green tea exerts radical-scavenger activity and have attributed this activity to the active component flavan-3-ol and its gallate compounds (Yokozawa et al., 1993, 1996a,b, 1997, 1998). In the present study, we investigated whether flavan-3-ol and its gallate compounds also scavenge $ONOO^-$.

MATERIALS AND METHODS

Green Tea Tannin. Fifty grams of dry green tea leaves, which had been produced in the Haibara district (Shizuoka, Japan), were added to 1 L of hot distilled water (70 °C) and shaken for 5 min. The resulting supernatant was freeze-dried to obtain a green tea extract. (−)-Epigallocatechin 3-*O*-gallate, (−)-gallocatechin 3-*O*-gallate, (−)-epicatechin 3-*O*-gallate, (−)-epigallocatechin, (−)-epicatechin, and (+)-catechin were prepared from a hot-water extract of green tea. For purification of these components, recycling high-performance liquid chromatography (HPLC) was done on a JAI-LC-908 high-performance liquid chromatograph (Japan Analytical Industry Co., Tokyo, Japan) equipped with JAI RI and JAI UV detectors, operating at 280 nm, as described previously (Sakanaka et al., 1989). A prepacked PVA HP-GPC column (JAIGEL GS-320, 50 × 2 cm i.d.) was used. Methanol was employed as the eluting solvent at a flow rate of 3 mL/min. The component isolated was identified by analysis by fast atom bombardment mass spectroscopy (FAB-MS) and HPLC. FAB-MS was recorded on a mass spectrometer (JMS-DX 303, JEOL, Tokyo, Japan) using glycerol as the matrix. The chemical structures of these constituents are illustrated in Figure 1.

Reagent. Peroxynitrite was synthesized in a quenched flow reactor, as previously described (Radi et al., 1991).

Measurement of Peroxynitrite. According to the method of Crow (1997), all oxidation reactions were carried out in a stirred 3-mL glass cuvette at 37 °C. Solutions contained 100 μM diethylenetriaminepentaacetic acid in a total volume of 1 mL of 100 mM phosphate buffer, pH 7.4, and then the reaction mixture was incubated for 1 min. Each of the green tea components, 100 μM dichlorodihydrofluorescein and 200 μM 3-morpholinosydnonimine (SIN-1), or 5 μM peroxynitrite was added, and then the reaction mixture was analyzed for 5 min. A spectrophotometer that exposed samples to the full wavelength range of light was used for absorbance measurement at 500 nm.

Statistics. Results are presented as means ± SE of five determinations. The data were analyzed for statistical significance using Dunnett's method. Differences at $p < 0.05$ were considered statistically significant.

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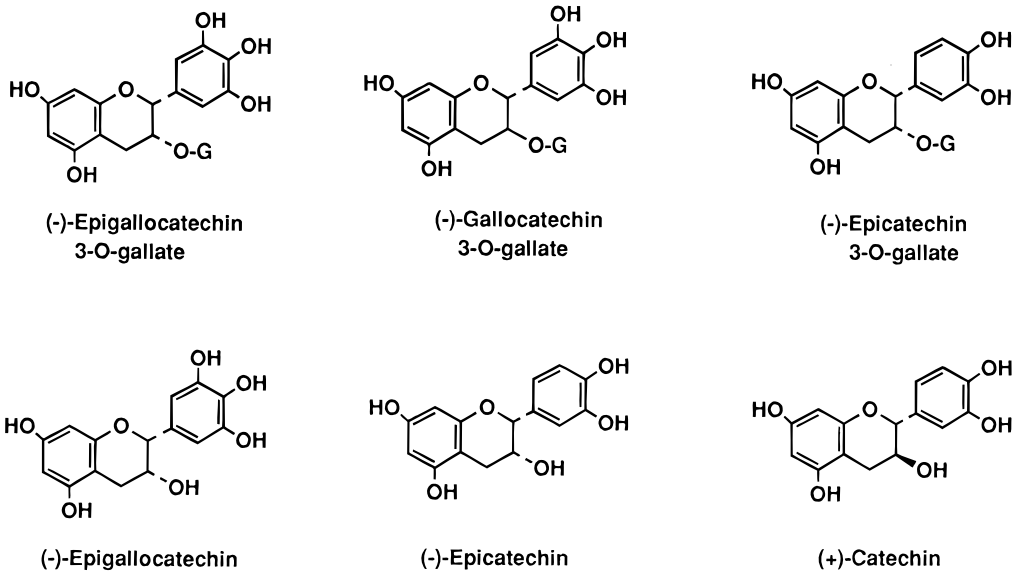

Figure 1. Chemical structures of green tea tannin.

Table 1. Effect of Green Tea Components on Peroxynitrite Formation from SIN-1

compound	absorbance at 500 nm[a]
none	0.242 ± 0.005
(−)-epigallocatechin 3-*O*-gallate	0.074 ± 0.003[b]
(−)-gallocatechin 3-*O*-gallate	0.089 ± 0.004[b]
(−)-epicatechin 3-*O*-gallate	0.128 ± 0.004[b]
(−)-epigallocatechin	0.144 ± 0.002[b]
(−)-epicatechin	0.205 ± 0.003[a]
(+)-catechin	0.221 ± 0.008

[a] Statistical significance: [a]$p < 0.01$, [b]$p < 0.001$ vs nonadditive value.

Table 2. Effect of Green Tea Components on Peroxynitrite-Scavenging Activity

compound	absorbance at 500 nm[a]
none	0.214 ± 0.004
(−)-epigallocatechin 3-*O*-gallate	0.101 ± 0.006[b]
(−)-gallocatechin 3-*O*-gallate	0.105 ± 0.003[b]
(−)-epicatechin 3-*O*-gallate	0.138 ± 0.002[b]
(−)-epigallocatechin	0.159 ± 0.005[b]
(−)-epicatechin	0.185 ± 0.004[a]
(+)-catechin	0.206 ± 0.002

[a] Statistical significance: [a]$p < 0.01$, [b]$p < 0.001$ vs nonadditive value.

RESULTS

The addition of each green tea component at 20 μM caused a significant decrease in peroxynitrite formation from SIN-1, as shown in Table 1. The most effective component was (−)-epigallocatechin 3-*O*-gallate, followed in order of potency by (−)-gallocatechin 3-*O*-gallate > (−)-epicatechin 3-*O*-gallate > (−)-epigallocatechin > (−)-epicatechin > (+)-catechin.

To clarify whether or not green tea components directly scavenge peroxynitrite itself, the peroxynitrite-scavenging activities of green tea components were examined at the same concentration. As shown in Table 2, (−)-epigallocatechin 3-*O*-gallate and (−)-gallocatechin 3-*O*-gallate scavenged peroxynitrite markedly, even though (−)-epicatechin 3-*O*-gallate, (−)-epigallocatechin, (−)-epicatechin, and (+)-catechin showed a weaker scavenging effect than (−)-epigallocatechin 3-*O*-gallate and (−)-gallocatechin 3-*O*-gallate, indicating that green tea components also had the ability to directly scavenge peroxynitrite itself.

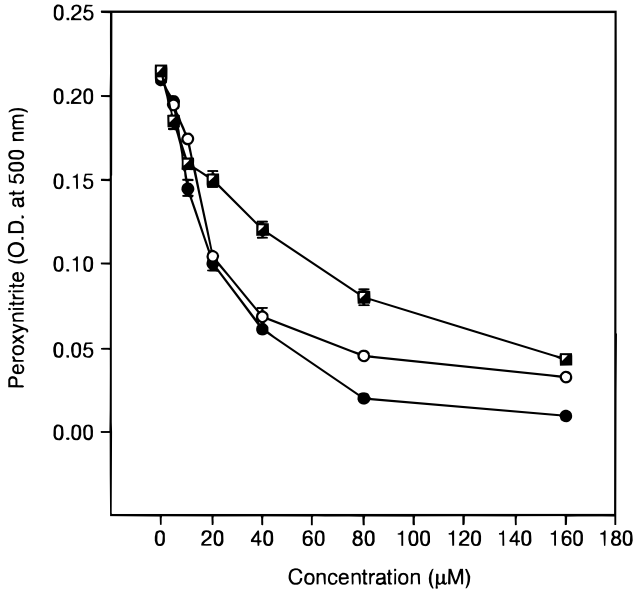

Figure 2. Dose−response curve of (−)-epigallocatechin 3-*O*-gallate (●), (−)-gallocatechin 3-*O*-gallate (○), and penicillamine (◪) on peroxynitrite-scavenging activity.

(−)-Epigallocatechin 3-*O*-gallate and (−)-gallocatechin 3-*O*-gallate, showing the stronger scavenging effects, were further investigated by comparison with penicillamine as an effective scavenger of peroxynitrite in vitro (Althaus et al., 1994), and the results are shown in Figure 2. Both of these compounds efficiently scavenged peroxynitrite, the concentration necessary for ∼50% inhibition being 20 μM, compared to 60 μM for penicillamine. Thus, (−)-epigallocatechin 3-*O*-gallate and (−)-gallocatechin 3-*O*-gallate were ∼3 times more effective in scavenging peroxynitrite.

DISCUSSION

It is known that active oxygen species attack proteins, lipids, nucleic acids, and enzymes in the body, independently or in cooperation, and show various toxic effects. The effect of antioxidants on damage caused by NO acting directly on the target molecule or resulting from cooperation of NO with other free radicals is now

attracting attention (Blough and Zafiriou, 1985; Halliwell, 1987). Using lipopolysaccharide and interferon-γ-activated mouse peritoneal cells, Chan et al. (1997) demonstrated that (−)-epigallocatechin 3-*O*-gallate suppresses the production of NO and gene expression of inducible nitric oxide synthase (iNOS), and Haenen et al. (1997), in their recent study, observed the $ONOO^-$-eliminating activity of flavonoid and pointed out the important role of the catechol group (ring B) and hydroxyl group at position 3 in the manifestation of this scavenger activity. On the basis of our previous finding that green tea tannin components that have flavonoid-like structures showed O_2^--eliminating activity (Yokozawa et al., 1997, 1998), we examined their effects on $ONOO^-$. We found that (−)-epigallocatechin 3-*O*-gallate was the most potent scavenger; (−)-gallocatechin 3-*O*-gallate and (−)-epicatechin 3-*O*-gallate also had high scavenger activity, showing that compounds with a catechol group and a hydroxyl group were effective scavengers, consistent with the results reported by Haenen et al. (1997). However, gallate-free tannin proved to have low activity, suggesting that, in tannin, gallate might play a more important role in the manifestation of scavenger activity. This scavenger activity was found to be dose-dependent and involved not only inhibition of $ONOO^-$ production but also the ability to directly scavenge $ONOO^-$.

As a highly reactive radical, $ONOO^-$ is reported to have carcinogenicity and cytotoxicity due to release of vasoconstrictive lipids (e.g., thromboxane A_2, platelet activating factor) and elimination of NO, in addition to causing activation of protease and reduction of proteoglycan (Moncada et al., 1991). Therefore, much attention is now focused on the biological activity of $ONOO^-$ as a NO-related radical. The $ONOO^-$-eliminating activity of the major component of green tea tannin, (−)-epigallocatechin 3-*O*-gallate, as demonstrated in the present study, seems to alleviate peroxidant damage. (−)-Epigallocatechin 3-*O*-gallate was also proved to have the ability to scavenge O_2^-, hydrogen peroxide (H_2O_2), hydroperoxyl radical ($^{\bullet}O_2H$) and $^{\bullet}OH$ (Yokozawa et al., 1993, 1996a,b, 1997, 1998) as well as $ONOO^-$, indicating its potential as a promising natural antioxidant having a wide spectrum of activity.

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