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Prevention of peroxynitrite-induced oxidation and nitration reactions by ellagic acid

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ABSTRACT

Four benzoic acid derivatives and three phenylpropanoids were tested for their ability to suppress oxidation of dichlorodihydrofluorescein (DCDHF) induced by 3-morpholinosydnonimine (SIN-1, a peroxynitrite donor). Ellagic acid was found to potently inhibit this oxidation and to inhibit oxidation of DCDHF induced by peroxynitrite itself. Moreover, this compound prevented peroxynitrite-induced oxidative single strand breaks in pTZ 18U plasmid DNA and nitration of tyrosyl residues in bovine serum albumin. These results show that ellagic acid protects biomolecules against oxidative and nitrative damage induced by peroxynitrite *in vitro*.

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1. Introduction

The most direct interaction between nitric oxide (NO) and superoxide anion is their rapid iso-stoichiometric reaction to form peroxynitrite (Peluffo & Radi, 2007; Ricciardolo, Di Stefano, Sabatini, & Folkerts, 2006). Under physiological conditions, the generation of peroxynitrite is low and potential oxidative damage is prevented by endogenous antioxidant defences. However, in pathological conditions, modest elevations in the simultaneous production of NO and superoxide anion can greatly stimulate the formation of peroxynitrite. Consequently, pathological conditions characterized by oxidative stress can greatly elevate the production of peroxynitrite (Rastaldo et al., 2007). Homolytic cleavage of peroxynitrous acid (protonated peroxynitrite) yields a hydroxyl radical and a nitrogen dioxide radical. Peroxynitrite is considered to be a potent pathophysiologically relevant cytotoxin. The addition of peroxynitrite to biomolecules, cells and tissue at pH 7.4 soon leads to oxidation, nitrosylation and nitration of biomolecules, and eventually to cytotoxicity, cell death and tissue injury. DNA is sensitive to oxidative damage mediated by peroxynitrite. In DNA, peroxynitrite causes extensive base modifications as well as single strand breaks. 8-Nitroguanine is formed from the reaction between guanine and peroxynitrite. Possible mechanisms for the formation of 8-nitroguanine include heterolytic cleavage of peroxynitrite to form a nitronium ion (NO_2^+) and the formation of a high-energy intermediate derived from *trans*-peroxynitrite ($pK_a = 7.9$). The initial reaction leading to DNA strand breaks by peroxynitrite could involve hydrogen abstraction and O_2 attack at either deoxyribose moiety by hydroxyl radical-like intermediate(s) from peroxynitrite or peroxynitrous acid. Next, strand breakage could be induced by bond cleavages (Ohshima, Tatemichi, & Sawa, 2003; Szabó & Ohshima, 1997; Yermilov et al., 1995). In addition to DNA damage caused by peroxynitrite, nitration of free and protein-bound tyrosine to produce nitrotyrosine is a well-established reaction that is used to estimate peroxynitrite-mediated cytotoxicity. Elevated levels of nitrotyrosine have been seen in many inflammatory conditions, neurodegenerative diseases and cancers in humans and in animal models. Peroxynitrite formation and subsequent reactions have been proposed to be involved in the pathogenesis of a series of diseases including carcinogenesis, acute and chronic inflammatory processes, sepsis, and neurodegenerative disorders (Grace, Salgo, & Pryor, 1998; Oldreive & Rice-Evans, 2001; Payne, Bernstein, Bernstein, Gerner, & Garewal, 1999; Radi, Peluffo, Alvarez, Naviliat, & Cayota, 2001).

Ellagic acid (Fig. 1) is a gallic acid dimer found in strawberry (*Fragaria* × *ananassa* Duchesne), both in the free form and esterified to glucose in water-soluble hydrolyzable ellagitannins. Ellagic acid is estimated to comprise 51% of the phenolic compounds in strawberries, and is reported to exhibit antioxidant, antiproliferative, chemopreventive, and antiatherogenic properties in a variety of tissues and cells. It has been also shown to inhibit cancer caused by several types of chemical carcinogens including polycyclic aromatic hydrocarbons, *N*-nitrosamines, aflatoxin B_1, and aromatic amines (Hannum, 2004; Heur, Zeng, Stoner, Nemeth, & Hilton, 1992; Mandal et al., 1987; Papoutsi et al., 2005). Extracts from red raspberry leaves or seeds, pomegranates, or various other sources containing high levels of ellagic acid are commercially available as dietary supplements, and are generally recognized as safe (GRAS) by the Food and Drug Administration (FDA) (Espín, García-Conesa, & Tomás-Barberán, 2007). Four benzoic acid derivatives and three phenylpropanoids were tested for their ability to

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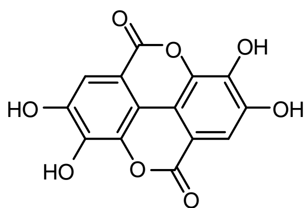

Fig. 1. Structure of ellagic acid.

inhibit the oxidation of dichlorodihydrofluorescein (DCDHF) induced by 3-morpholinosydnonimine (SIN-1, a peroxynitrite donor) to identify those with the ability to decrease oxidative stress induced by peroxynitrite (Crow, 1997; Kirsch & de Groot, 2000). This evaluation revealed that ellagic acid potently suppressed oxidation of DCDHF induced by SIN-1. In this paper, we report the protective effect of ellagic acid on peroxynitrite-induced oxidation and nitration reactions.

2. Materials and methods

2.1. Reagents

All compounds evaluated for antioxidative effect on oxidation reaction induced by SIN-1 were obtained from commercial sources. Bovine serum albumin (BSA), diethylenetriaminepentaacetic acid (DTPA), ellagic acid, pTZ 18U plasmid DNA and SIN-1 were obtained from Sigma–Aldrich Co. (St. Louis, MO, USA). Anti-3-nitrotyrosine mouse antibody and DCDHF were obtained from Cayman Chemical (Ann Arbor, MI, USA). Peroxynitrite was obtained from Dojindo Laboratories (Mashiki, Kumamoto, Japan). The concentration of peroxynitrite was calculated from the absorbance at 302 nm (ε = 1670 M^{-1} cm^{-1}) (Grace et al., 1998) measured by ultraviolet–visible spectrometer (UV-2100, Shimadzu, Kyoto, Kyoto, Japan or DU 800, Beckman Coulter, Fullerton, CA, USA). Peroxynitrite was prepared to the desired concentration by dilution in 0.1 M NaOH from stock solutions. Phosphate-buffered saline (PBS) was prepared to contain 137 mM NaCl, 8.1 mM Na_2HPO_4, 2.68 mM KCl, and 1.47 mM KH_2PO_4 at pH 7.4.

2.2. Oxidation of DCDHF

Oxidation reactions induced by SIN-1 or peroxynitrite were carried out in 96-well plates. SIN-1 (200 μM) was added to DCDHF (100 μM) with test compounds in PBS containing 100 μM DTPA, and then the reaction mixture was incubated for 5 h at 37 °C. The formation of dichlorofluorescein, an oxidative product of DCDHF, was determined by measuring the optical density at 500 nm (reference wavelength 595 nm) using a microplate reader (Model 450, Bio-Rad, Hercules, CA, USA) (Crow, 1997). Three sequential additions of peroxynitrite at 300 μM (final concentration of 45 μM) were made to DCDHF (100 μM) with various concentrations of ellagic acid in PBS containing 100 μM DTPA, and the reaction mixture was incubated for 30 min at 37 °C. The formation of dichlorofluorescein was determined as mentioned above.

2.3. DNA strand breaks

Three sequential additions of peroxynitrite at 5000 μM (final concentration of 750 μM) were made to supercoiled (SC) pTZ 18 U DNA (24 μg/ml) with various concentrations of ellagic acid in PBS containing 100 μM DTPA. The reaction solution was incubated for 30 min at 37 °C and analyzed for single (OC, open circular) strand breaks by agarose gel electrophoresis. The reaction solution (6 μl) was mixed with 1.2 μl of electrophoresis loading buffer and loaded onto a 1.0% agarose gel prepared in TAE buffer (40 mM Tris acetate, 1 mM EDTA, 0.1 μg/ml ethidium bromide). Electrophoresis was carried out at 50 V with a Mupid-21 mini-gel electrophoresis apparatus (Cosmo Bio, Tokyo, Japan). After electrophoresis, the gel was visualized under UV light and photographed. To quantitatively analyze DNA damage, the density of the OC DNA band was measured by Quantity One software (pdi, Huntington Station, NY, USA).

2.4. Tyrosine nitration

Three sequential additions of peroxynitrite at 300 μM (final concentration of 45 μM) were made to BSA (50 μg/ml) with varying concentrations of ellagic acid in PBS containing 100 μM DTPA, then the reaction solution was incubated for 30 min at 37 °C. Tyrosine nitration was measured by Western blotting and densitometric analysis. The reaction solution (5 μl) was treated by 10% sodium dodecyl sulfate (SDS)-polyacrylamide mini-gel electrophoresis at 20 mA/gel with an AE-6400 dual mini slab kit (Atto, Tokyo, Japan), followed by blotting onto a nitrocellulose membrane at 100 V with a mini trans-blot apparatus (Bio-Rad). Western blot analysis was performed using an ECL Western blotting analysis system (Amersham Pharmacia Biotech Inc, Piscataway, NJ, USA) as described by the manufacturer. Anti-3-nitrotyrosine mouse antibody was used at dilutions of 1:500. Signal intensity was quantified with a 420oe optically enhanced laser densitometer (pdi) and Quantity One software.

2.5. Reaction of ellagic acid with peroxynitrite

A solution of 50 mM peroxynitrite (4 ml) was added dropwise to a stirred solution of ellagic acid (100 μmol) in PBS (396 ml), and the reaction solution was incubated for 30 min at 37 °C. The reaction solution was adjusted to pH 3.0 and extracted with ethyl acetate. The ethyl acetate layer was dried *in vacuo*, and analyzed by nuclear magnetic resonance (NMR). The ^{13}C NMR spectrum of the reaction mixture in dimethyl-d_6 sulfoxide (solvent as internal standard, δ 39.5) was recorded on an EX270 spectrometer (JEOL, Akishima, Tokyo, Japan) at 67.5 MHz.

3. Results and discussion

3.1. Protective effect against oxidation of DCDHF by SIN-1

We evaluated the ability of four benzoic acid derivatives and three phenylpropanoids to inhibit the oxidation of DCDHF by SIN-1 (Table 1). The continuous formation of low amounts of peroxynitrite can be simulated in experimental systems with SIN-1 (Kirsch et al., 2000). Peroxynitrite efficiently oxidizes DCDHF to form the sensitive product, dichlorofluorescein ($\varepsilon_{500\,nm}$ = 59,500 M^{-1} cm^{-1}) (Crow, 1997). We used this assay to study the antioxidative effect of test compounds toward peroxynitrite. All tested compounds showed dose-dependent suppression activity on dichlorofluorescein formation. In the benzoic acid derivative class, the order of suppression activity was ellagic acid > 2,3-dihydroxybenzoic acid ≈ gentisic acid > gallic acid. This result exhibits that the number of hydroxy groups in these compounds does not have a correlation with suppression activity. On the other hand, the suppressive activities of three phenylpropanoids decreased in the order caffeic acid > ferulic acid > 3,4-dimethoxycinnamic acid correspondingly to the number of hydroxy groups. Ellagic acid at all concentrations was more effective than caffeic acid, a known antioxidative compound toward peroxynitrite (Pannala, Razaq, Halliwell, Singh, & Rice-Evans, 1998), and other tested compounds.

Table 1
Effect of benzoic acid derivatives and phenylpropanoids on oxidation of DCDHF induced by SIN-1[a]

Compound	Concentration (μM)	Dichlorofluorescein (%)
Benzoic acid derivative		
2,3-Dihydroxybenzoic acid	100	51.6 ± 5.2
	20	83.5 ± 4.6
	4	109.0 ± 3.8
Gentisic acid	100	55.6 ± 6.3
	20	78.4 ± 5.9
	4	100.1 ± 7.7
Gallic acid	100	63.2 ± 4.9
	20	96.2 ± 0.7
Ellagic acid	100	31.4 ± 10.5
	20	36.2 ± 10.9
	4	77.3 ± 9.5
Phenylpropanoid		
Caffeic acid	100	32.7 ± 5.0
	20	58.0 ± 7.7
	4	89.0 ± 5.5
Ferulic acid	100	57.4 ± 5.9
	20	106.6 ± 0.8
3,4-Dimethoxycinnamic acid	100	88.8 ± 4.2
	20	94.4 ± 2.2
	4	96.3 ± 2.7

[a] Oxidation of DCDHF (100 μM) to dichlorofluorescein in the presence of various concentrations of test compounds was started by adding SIN-1 (200 μM), and the reaction mixture was incubated for 5 h at 37 °C. The relative formation of dichlorofluorescein was calculated with reference to the control values in the absence of test compounds. The experiment was performed in triplicate, and the result is expressed as the arithmetic mean ± standard deviation.

Based on this result (Table 1), we studied the ability of ellagic acid to prevent peroxynitrite-induced oxidation and nitration reactions.

3.2. Inhibition of oxidation of DCDHF induced by peroxynitrite

Next, the antioxidative effect of ellagic acid was evaluated on the oxidation of DCDHF induced by peroxynitrite itself. Oxidation reaction of DCDHF (100 μM) to dichlorofluorescein in the presence of various concentrations of ellagic acid was started by adding peroxynitrite (45 μM), and the reaction mixture was incubated for 30 min at 37 °C. Ellagic acid was found to suppress dichlorofluorescein formation in a dose-dependent fashion (Fig. 2). This observation indicates that ellagic acid is an antioxidative compound against peroxynitrite.

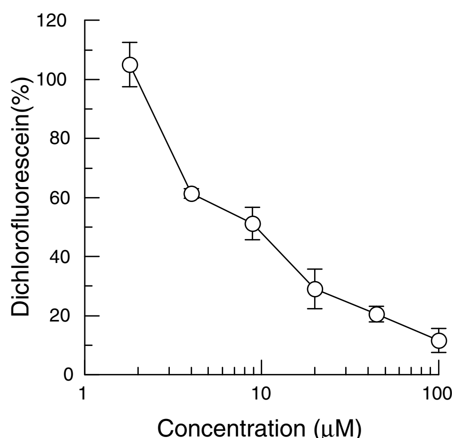

Fig. 2. Effect of ellagic acid on peroxynitrite-induced oxidation of DCDHF. Relative formation of dichlorofluorescein was calculated with reference to the control value in the absence of ellagic acid. The experiment was performed in triplicate, and the result is expressed as the arithmetic mean ± standard deviation.

3.3. Protection against DNA damage

DNA is sensitive to peroxynitrite-mediated oxidative damage. In both SC plasmid DNA and mammalian cellular DNA, peroxynitrite causes extensive base modifications as well as single strand breaks (Grace et al., 1998). Strand breaks of SC pTZ 18U DNA (24 μg/ml) to OC DNA in the presence of various concentrations of ellagic acid was started by adding peroxynitrite (750 μM), and the reaction solution was incubated for 30 min at 37 °C. When pTZ 18U plasmid DNA was exposed to peroxynitrite, the native SC DNA was changed to a relaxed OC DNA with single strand breaks induced by peroxynitrite (lanes 1 vs. 2 in Fig. 3). In the presence of ellagic acid, the conversion of SC DNA into OC DNA decreased in a dose-dependent manner (lanes 3–6 in Fig. 3). This result shows that ellagic acid can protect DNA from oxidative damage caused by peroxynitrite.

3.4. Protection against protein damage

Peroxynitrite is not only a powerful oxidant but also a strong nitrating agent (Radi et al., 2001). Protein tyrosine residues are especially susceptible to peroxynitrite-mediated nitration reactions producing 3-nitrotyrosine (Pannala et al., 1998). To test for the protective effect of ellagic acid against peroxynitrite-induced nitration of tyrosine, we selected BSA as a model target (Fig. 4). Nitration reaction of BSA (50 μg/ml) in the presence of various concentrations of ellagic acid was started by adding peroxynitrite (45 μM), and the reaction solution was incubated for 30 min at 37 °C. Peroxynitrite-induced nitration of tyrosine residues in BSA was examined by Western blotting using anti-3-nitrotyrosine antibody. Exposure of BSA to peroxynitrite resulted in nitrotyrosine immunoreactivity (lanes 1 vs. 2 in Fig. 4). The intensity of the band derived from 3-nitrotyrosine residues in BSA decreased with increasing amounts of ellagic acid (lanes 3–6 in Fig. 4). This result shows the protection of BSA by ellagic acid against peroxynitrite-induced tyrosine nitration.

3.5. Reaction of ellagic acid with peroxynitrite

To elucidate the protective mechanism of ellagic acid on peroxynitrite-induced oxidation and nitration reactions, ellagic acid was treated with peroxynitrite, and the reaction solution was analyzed by reversed-phase high performance liquid chromatography

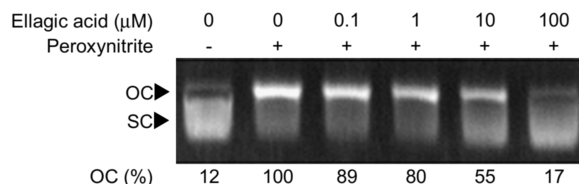

Fig. 3. Protection by ellagic acid against peroxynitrite-mediated strand breaks in DNA. The relative intensity of OC was deduced by dividing the intensity of OC in the peroxynitrite-untreated sample (lane 1) and the ellagic acid-treated samples (lanes 3–6) by that in the sample treated with peroxynitrite only (lane 2).

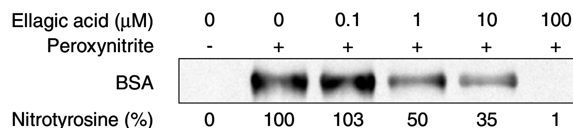

Fig. 4. Protection by ellagic acid against tyrosine nitration of BSA. Tyrosine nitration was measured by Western blotting. The relative formation of nitrotyrosine is expressed as a percentage of control value (lane 2).

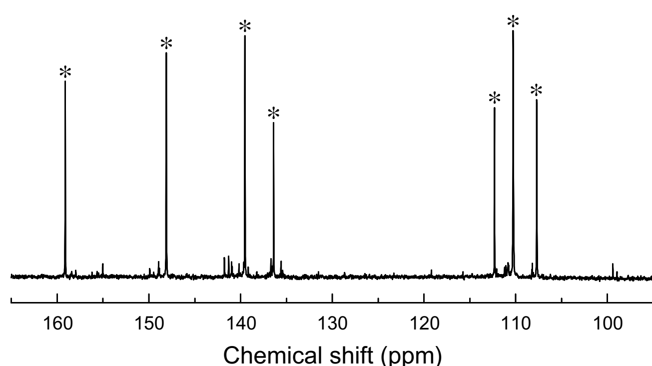

Fig. 5. ^{13}C NMR spectrum of the reaction mixture formed between ellagic acid and peroxynitrite. An asterisk indicates a signal derived from ellagic acid.

(HPLC). However, the HPLC chromatogram did not clearly exhibit a novel peak (data not shown). Next, a large-scale reaction between ellagic acid and peroxynitrite was carried out, and the ^{13}C NMR spectrum of the reaction mixture was measured. The solution of ellagic acid in the NMR tube was yellow, whereas the solution of the reaction mixture was a red-purple color (data not shown). The ^{13}C NMR spectrum of the reaction mixture is shown in Fig. 5. Considering the ^{13}C NMR spectrum of ellagic acid (data not shown), major seven signals denoted by asterisk in Fig. 5 are thought to be derived from ellagic acid unreacted with peroxynitrite. The spectrum exhibited many novel signals with very lower intensity compared with signals of ellagic acid in the region of aromatic or alkenyl sp^2 carbons (100–160 ppm). These minor signals are assumed to be derived from the product(s) formed from the reaction between ellagic acid and peroxynitrite.

In this report, we tested four benzoic acid derivatives and three phenylpropanoids, and found that ellagic acid, a polyphenol found in strawberry, potently suppresses oxidation of DCDHF induced by SIN-1 (a peroxynitrite donor). Furthermore, ellagic acid efficiently prevents peroxynitrite-induced oxidation reaction of DCDHF, oxidative single strand breaks in SC pTZ 18U plasmid DNA, and nitration of protein tyrosyl residues in BSA. These results show that ellagic acid protects against oxidative and nitrative damage induced by peroxynitrite. Pannala et al. (1998) proposed that catecholic hydroxycinnamates (chlorogenic acid and caffeic acid) are changed to the corresponding *o*-quinones through two-electron transfer induced by peroxynitrite. Polyphenol oxidase, an enzyme found in plants, catalyzes the oxidation of chlorogenic acid to the corresponding *o*-quinone, which upon further reaction leads to brown pigments. Dimers or oligomers are reported to be produced by condensation of quinones of phenolic acids. More than a dozen oxidation products were found to be generated from a nonenzymatic oxidative browning reaction of caffeic acid (Oszmianski & Lee, 1990). The presumption that various polymers were generated from *o*-quinone intermediates formed between ellagic acid and peroxynitrite in a similar pathway to that of browning reaction can explain the occurrence of many novel signals in the region of aromatic or alkenyl sp^2 carbons of the ^{13}C NMR spectrum (Fig. 5). Finally, we postulate that ellagic acid scavenges peroxynitrite-derived radicals and consequently inhibits peroxynitrite-induced oxidation and nitration reactions.

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