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Inhibitory Effects of Conjugated Epicatechin Metabolites on Peroxynitrite-mediated Nitrotyrosine Formation

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Summary **Previously, we identified four metabolites of (−)-epicatechin in blood and urine: (−)-epicatechin-3'-*O*-glucuronide (E3'G), 4'-*O*-methyl-(−)-epicatechin-3'-*O*-glucuronide (4'ME3'G), (−)-epicatechin-7-*O*-glucuronide (E7G), and 3'-*O*-methyl-(−)-epicatechin-7-*O*-glucuronide (3'ME7G) (Natsume *et al.* Free Radical Biol. Med. 34, 840-849, 2003). The aim of the current study was to compare the antioxidative activities of these metabolites with that of their parent compound. After oral administration of (−)-epicatechin, E3'G and 4'ME3'G were isolated from human urine, and E7G and 3'ME7G isolated from rat urine. We found that these compounds inhibited peroxynitrite-mediated tyrosine nitration, in the following order of potency: E3'G > (−)-epicatechin > E7G = 3'ME7G. = 4'ME3'G. These results demonstrate that the metabolites of (−)-epicatechin retain antioxidative activity on peroxynitrite-induced oxidative damages to some extent.**

Key Words**: (−)-epicatechin, glucuronide, metabolites, peroxynitrite, nitrotyrosine**

Introduction

Catechins are found in high levels in a variety of foods, including black tea, apples, wine, and chocolate [*1*, *2*]. Flavonoids including (−)-epicatechin have multiple biological functions that are thought to reduce the incidence of atherosclerosis [*3*]. Epidemiological studies have suggested that the intake of catechins correlates with risk reduction for coronary heart disease [*4*, *5*]. (−)-Epicatechin has been shown to protect biological molecules from nitration by peroxynitrite [*6*]. Oxidation of tyrosine residues, which occurs upon the nitration of proteins, is of particular interest in the pathology of inflammation. Upon oral administration, most flavonoids are metabolized in the gastrointestinal tract and absorbed into the blood. There have been a few reports to date on antioxidant activities in these metabolites [*7–9*]. We have shown in humans and rats that, following the ingestion of cocoa powder, (−)-epicatechin is absorbed from the intestinal tract and distributed as a variety of conjugated and methylated forms in the blood before being excreted in the urine [*10*, *11*]. We also isolated and identified (−)-epicatechin glucuronide metabolites from human and rat plasma. In humans, these include (−)-epicatechin-3'-*O*-glucuronide (E3'G) and 4'-*O*-methyl-(−)-epicatechin-3'-*O*-glucuronide (4'ME3'G), and in rat, the metabolites were (−)-epicatechin-7-*O*-glucuronide (E7G) and 3'-*O*-methyl-(−)-epicatechin-7-*O*-glucuronide (3'ME7G) [*12*]. The structures of these compounds are shown in Fig. 1.

The aim of the current study was to examine the antioxidative activities, especially that of nitrotyrosine formation, of the glucuronide metabolites of (−)-epicatechin present in human and rat plasma.

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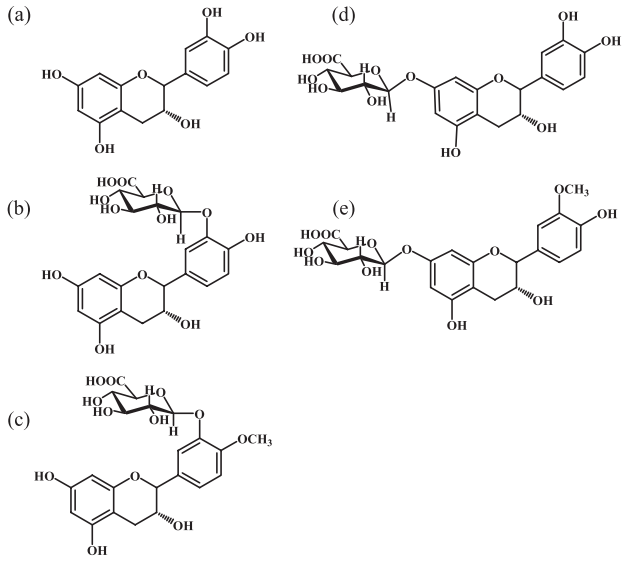

Fig. 1. Structures of (−)-epicatechin and its glucuronide metabolites isolated from human and rat urine. (a) (−)-epicatechin. (b) (−)-epicatechin-3'-*O*-glucuronide (E3'G). (c) 4'-*O*-mehyl-(−)-epicatechin-3'-*O*-glucuronide (4'ME3'G). (d) (−)-epicatechin-7-*O*-glucuornide (E7G), and (e) 3'-*O*-methyl-(−)-epicatechin-7-*O*-glucuonide (3'ME7G).

Materials and Methods

Peroxynitrite was synthesized from sodium nitrite and H_2O_2 using a quenched-flow reactor [*13*]. H_2O_2 was eliminated by passage of the peroxynitrite solution over MnO_2 powder with the final peroxynitrite concentration determined by its absorbance at 302 nm ($\varepsilon = 1620\ M^{-1}cm^{-1}$). Protection against peroxynitrite-mediated tyrosine nitration was performed as described by Arteel *et al.* [*14*]. Briefly, peroxynitrite (0.5 mM) was added as a bolus to a 0.1 mM tyrosine solution in 0.1 M phosphate buffer (pH 7.3) containing 0.1 mM DTPA with constant vortexing. The effects of the tested compounds (0–31.5 μM) were determined by injection of 10 μl of sample, supplemented with 0.1 mM 3-hydroxy-4-nitrobenzoic acid as an internal standard, onto a C-18 reverse-phase column (ODS-UG-Deverosil 4.6 × 150 mm; Nomura Chemical, Aichi, Japan) interfaced with the autosampler from an HP1100 liquid chromatography system (Agilent Technologies, Palo Alto, CA). Separation was performed with a 50 mM potassium phosphate buffer (pH 7.0)/acetonitrile step-gradient delivered by the HP1100 system at a flow rate of 1.0 ml/min. The gradient was initiated at a ratio of 95:5 buffer/acetonitrile, followed in stepwise adjustment to a final ratio of 50:50 buffer/acetonitrile over 5 min, with a return to 95:5 buffer/acetonitrile ratio at 13 min, which was then maintained for an additional 13 min. Formation of 3-nitrotyrosine was monitored with a UV/visible detector set at 430 nm. To determine the concentration of 3-nitrotyrosine in the sample, the peak area ratio of the 3-nitrotyosine to the internal standard was calculated and compared to a standard curve. Each analysis was performed in duplicate.

Results

Figure 2 shows the ability of the test compounds to protect free tyrosine from the nitration by peroxynitrite. The concentration of metabolites required to yield a 50% inhibition of tyrosine nitration increased in the following order: E3'G > (−)-epicatechin > E7G = 3'ME7G. = 4'ME3'G.

Discussion

A variety of foods, particularly green tea, chocolate, cocoa, and red wine have high levels of the flavonoid (−)-epicatechin [*1*]. Flavonoids have multiple biological functions, including the suppression of LDL oxidation [*3*, *15*], a process thought to reduce the incidence of atherosclerosis [*16*, *17*]. In the present study, we compared the peroxynitrite scavenging activities of (−)-epicatechin and its four major metabolites: E3'G and 4'ME3'G, which are found in human plasma, and E7G and 3'ME7G, which are found in rat plasma [*12*]. Peroxynitrite may be generated *in vivo* under pathological conditions, especially in the inflammatory response, due to the reaction of superoxide radicals with nitric oxide. Peroxynitrite causes injury and degeneration of endothelial cells and erythrocytes [*18*]. Moreover, (−)-epicatechin strongly suppresses nitration of tyrosine residues of LDL by Myeloperoxidase (MPO)/nitrite or peroxynitrite [*19*]. Flavonoids protect biological molecules against peroxynitrite-mediated nitration by neutralizing peroxynitrite and its reaction intermediate, the tyrosyl radical [*6*, *20–24*]. In this study, we

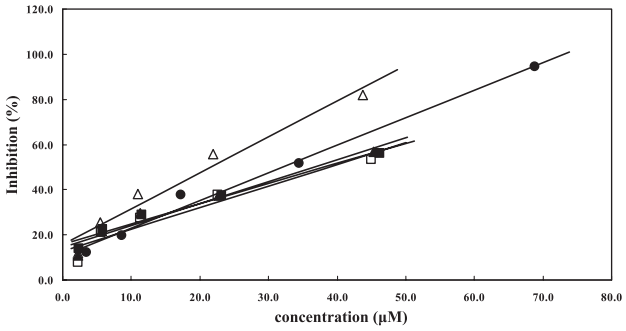

Fig. 2. Protective effect by (−)-epicatechin and its metabolites against nitration of tyrosine residues by peroxynitrite (−)-epicatechin (closed circles), E3'G (open triangles), 4'ME3'G (open squares), E7G (closed triangles) and 3'ME7G (closed squares). Each analysis was performed in duplicate.

found that both (−)-epicatechin and its metabolites inhibited peroxynitrite-mediated nitration. E3'G was more potent than (−)-epicatechin and the other metabolites at the concentrations between 2.1 and 42.8 μM. (−)-Epicatechin glucuronide and methylated forms of epicatechin were similar in their ability to inhibit tyrosine nitration. In the presence of 0.5 mM peroxynitrite, the IC50 values of (−)-epicatechin and E3'G were 32.1 and 21.6 μM, as calculated by linear regression analyses, respectively. Wipple *et al.* [*6*] reported that one equivalent of (−)-epicatechin prevents nitration by 15–30 equivalents of peroxynitrite.

An alternative action mechanism for epicatechin was proposed by Schroeder *et al.* [*21*], who hypothesized that epicatechin acts at the interference with tyrosyl radicals rather than by direct interaction with peroxynitrite. Furthermore, the plasma metabolites of epicatechin may contribute to the antioxidant activity on peroxynitrite attack in blood plasma after administration of catechin rich foods.

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