Short communication

Galloylated Catechins as Potent Inhibitors of Angiotensin Converting Enzyme

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In the present study, we investigated the inhibitory effects of four tea catechins, including (-)-epicatechin (EC), (-)-epigallocatechin (EGC), (-)-epicatechin gallate (ECg) and (-)-epigallocatechin gallate (EGCg), on angiotensin converting enzyme (ACE) activity *in vitro*. Each catechin treatment significantly reduced the ACE activity with the order of potency being EGCg > ECg > EGC = EC. The addition of 1 mM borate significantly recovered the reduced ACE activities by tea catechins, suggesting that hydroxyl groups at the B-ring or at a galloyl moiety play an important role in the ACE-inhibitory mechanism. The covalent modification of ACE by tea catechins was also observed by a redox-cycling staining experiment. A Lineweaver-Burk plot indicated that EGC and ECg were non-competitive inhibitors. The galloylated catechins might more potently inhibit ACE activity in an allosteric manner through the interaction of the galloyl moiety with the non-catalytic site of ACE.

Keywords: tea catechins, angiotensin converting enzyme, covalent modification, non-competitive inhibition

Introduction

Accumulating epidemiological and intervention studies have indicated that green tea consumption is inversely associated with the risk of hypertension, which might be largely ascribed to the presence of polyphenols (Nakachi *et al.*, 2000; Kuriyama *et al.*, 2008; Arab *et al.*, 2013). Tea catechins, including (-)-epicatechin (EC), (-)-epigallocatechin (EGC), (-)-epicatechin gallate (ECg) and (-)-epigallocatechin gallate (EGCg), are the primary class of polyphenols in green tea. The protective effects of the catechins on cardiovascular diseases might be partially associated with its antioxidant and free radical scavenging properties (Dragsted, 2003). In addition to the antioxidant action, some studies have demonstrated that tea catechins have the ability to inhibit the activity of angiotensin converting enzyme (ACE) (Actis-Goretta *et al.*, 2006; Persson *et al.*, 2006). ACE, a dipeptidyl carboxypeptidase involved in the regulation of blood pressure and electrolyte balance through the renin-angiotensin system, contributes to the conversion of angiotensin I (Ang I) into angiotensin II (Ang II), a strong vasoconstrictor, and to the metabolism of biologically active

peptides such as bradykinin.

Like other well-known artificial ACE inhibitors, tea catechins including EGCg have the capacity to chelate metal ions (Hayakawa *et al.*, 1999; Sun *et al.*, 2008). Alternatively, autoxidation of polyphenols with a catechol structure leads to the formation of polyphenol quinones and ROS such as hydrogen peroxide, both of which can modify protein function through covalent adduct formation and oxidation, respectively (Nakamura and Miyoshi, 2010). We previously demonstrated that catalase significantly abolished the cytotoxicity induced by pyrogallol-type catechins (EGC or EGCg), whereas it did not influence the EC- or ECg-induced effect on human T lymphocytic leukemia Jurkat cells (Tang *et al.*, 2014). These findings suggested that tea catechins are able to induce cytotoxicity in hydrogen peroxide-dependent and -independent manners. It has also been reported that tea catechins could bind and inhibit several cellular enzymes, such as fatty-acid synthase, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and catalase (Wang *et al.*, 2003; Mori *et al.*, 2008; Pal *et al.*, 2014). However, the precise mechanism underlying ACE inhibition by tea

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catechins remains to be clarified.

Tea catechins have a flavan-3-ol structure composed of A-, B-, and C-rings with or without galloyl groups. The number and arrangement of phenolic hydroxyl groups in catechins are regarded to be responsible for their differences in biological activities. Because all tea catechins share several chemical properties, we hypothesized that they would inhibit ACE activity through a common mechanism but to different extents. To test this hypothesis, we compared the inhibitory effect of each catechin on ACE activity *in vitro*. The effect of borate on the inhibition of ACE activity by catechins was also examined, because it is a potent inhibitor of autoxidation of catechins by the formation of a complex with coordinate linkage (Mochizuki *et al.*, 2002). The covalent modification of ACE by tea catechins was determined by redox-cycling staining to confirm its involvement in ACE inhibition. Finally, we employed a Lineweaver-Burk plot to confirm tea catechins as non-competitive inhibitors.

Materials and Methods

(1) *Chemicals* ACE from rabbit lung, hippuryl-histidyl-leucine (Hip-His-Leu) and o-phthalaldehyde (OPA) were purchased from Sigma (St. Louis, MO, USA). EC, EGC, ECg, and EGCg were obtained from Wako Pure Chemical Industries (Osaka, Japan). Nitro blue tetrazolium (NBT) was from Tokyo Chemical Industry (Tokyo, Japan). All other chemicals were obtained from Nacalai Tesque (Kyoto, Japan).

(2) *ACE assay* The enzymatic activity of ACE was determined by a fluorimetric assay as previously reported (Santos *et al.*, 1985) with some modifications. Briefly, 1 mU ACE was incubated with 1 mM Hip-His-Leu in 0.1 M potassium phosphate buffer (pH 8.3) with 0.3 M NaCl in a total volume of 500 μ L at 37°C for 30 min. In an inhibitory assay with tea catechins, ACE was pre-incubated with the chemicals at 4°C for 15 min before the addition of substrate. Enzymatic reaction was terminated by the addition of 1.2 mL of 0.34 N NaOH, followed by a 10-min incubation at room temperature with 100 μ L of OPA (20 mg/mL). The solution was acidified with 200 μ L of 3 N HCl and centrifuged at 3,000 $\times$ g at 4°C for 10 min. The fluorescence intensity of the OPA-His-Leu adduct was measured by a Hitachi F-2500 fluorescence spectrophotometer (Tokyo, Japan) at 360 nm excitation and 490 nm emission wavelengths. As for the effect of borate on the ACE inhibitory activity of tea catechins, tea catechins were pre-incubated with or without borate at 4°C for 15 min, followed by the addition of enzyme and substrate.

(3) *SDS-PAGE and redox-cycling staining* ACE (15 mU) was incubated with 2 mM of tea catechins (EC, EGC, ECg, and EGCg) in 50 mM potassium phosphate buffer (pH 7.4) at 37°C for 60 min. Under this condition, the ACE inhibitory activity of tea catechins was similar to that under a basic condition (data not shown). The reaction between tea catechins and ACE was terminated by the addition of dithiothreitol (50 mM) as previously reported (Ishii *et*

al., 2008). The reaction mixtures were separated by SDS-PAGE using 10% gels. For a redox-cycling staining experiment, the gel bands were transferred onto a PVDF membrane (0.45 μ m, Merck Millipore, Darmstadt, Germany), and then ECG-modified proteins were detected by staining with NBT (0.2 mg/mL in 2 M potassium glycinate, pH 10). The membrane was immersed in the glycinate/NBT solution for approximately 30 min under darkness, resulting in a blue-purple stain of quinoprotein bands and no staining of other proteins. The membrane was also stained with Coomassie Brilliant Blue G-250 (CBB) to confirm the protein loading.

Results and Discussion

The maximum absorption and the elimination metabolism of tea catechins occur very quickly (Scalbert and Williamson, 2000). Therefore, to compare the inhibitory effect of each tea catechin, ACE was incubated with catechins at the indicated concentrations for 15 min and then its enzymatic activity was determined. Each catechin treatment significantly reduced the ACE activity with the order of potency being EGCg > ECg > EGC = EC (Fig. 1); EGCg at the concentration of 50 μ M completely inhibited the ACE activity, whereas ECg and EGC or EC at the same concentration reduced it by 55% and 15%, respectively. This tendency is partly consistent with a previous study using human umbilical vein endothelial cells (Persson *et al.*, 2006), but differs from the tendency for GAPDH inhibition (Ishii *et al.*, 2008) and for hydrogen peroxide production (Tang *et al.*, 2014) by tea catechins. The present result suggested that the presence of the galloyl ester at 3-position might play a more important role in ACE inhibitory activity than the pyrogallol group at the B ring. Galloylated catechins have comparable or higher ACE inhibitory activity than other flavonoids (Guerrero *et al.*, 2012).

To investigate the involvement of *ortho*-dihydroxyl (catechol, pyrogallol or galloyl) groups in tea catechins on their ACE inhibitory activity, we used borate to form a stable complex with these groups under a basic condition (Mochizuki *et al.*, 2002). As shown in Fig. 2, the addition of 1 mM borate partly but significantly recovered the ACE activities reduced by 100 μ M catechins. These results suggested that hydroxyl groups at the B-ring or at a galloyl moiety play an important role in the ACE inhibitory mechanism. Although pyrogallol-type catechins are reported to be more susceptible to autoxidation and subsequent electrophilic reaction than catechol-type catechins (Mori *et al.*, 2010), the borate-treatment had a stronger influence on the ACE inhibition of ECg than that of EGC (Fig. 2). This result also suggested that the galloyl ester might play a significant role in the ACE inhibitory activity independent of autoxidation-related phenomena, such as the formation of polyphenol quinones and hydrogen peroxide. The inhibitory effects of flavonoids on ACE activity have largely been ascribed to the generation of chelate complexes with Zn²⁺ ion at the active center of ACE (Loizzo *et al.*, 2007). Studies of the structure-

Fig. 1. Inhibitory Effect of tea catechins on ACE activity. The enzymatic activity of purified ACE (from rabbit lung) was measured in the presence of tea catechins (EC, EGC, ECg, and EGCg) at the indicated concentrations. The relative values represent means $\pm$ SD of three independent experiments. The maximal SD for the experiments was 5%.

Fig. 2. Counteracting effect of borate on the ACE inhibition by tea catechins. Tea catechins were pre-incubated with or without 1 mM borate at 4°C for 15 min. Then the ACE inhibitory activity of each group was measured. The relative values represent means $\pm$ SD of three independent experiments. *P < 0.05 vs. borate (-) group.

activity relationship and protein-ligand docking also supported this concept (Guerrero *et al.*, 2012). However, we preliminarily observed that the addition of exogenous Zn^{2+} ion did not influence the inhibitory effect of EGCg on ACE activity (unpublished data). This was consistent with the previous study showing that the inhibitory effect of tea catechins on human matrilysin, a metalloproteinase containing a Zn^{2+} ion essential for enzyme activity, was not influenced by the presence of $ZnCl_2$ (Oneda *et al.*, 2003). These results suggested little contribution of the hydroxyl groups to the Zn^{2+} chelation.

It has been widely recognized that tea catechins are able to bind several proteins, which is claimed to be responsible for their biological activities (Ishii *et al.*, 2008; Chen *et al.*, 2011; Pal *et al.*, 2014). At alkaline pH, catechins are readily oxidized into their corresponding quinones, which are able to react with thiol groups

Fig. 3. Covalent modification of ACE by tea catechins. ACE (15 mU) was treated with tea catechins (2 mM) at 37°C for 60 min. The catechin-modified ACE was detected by a redox-cycling staining (above) and CBB staining (below), respectively.

Fig. 4. Lineweaver-Burk plots of ACE inhibition by EGC and ECg. The ACE activity was measured in the absence (closed circle) or presence of 100 μM EGC (closed triangle) or 100 μM ECg (closed square). 1/V and 1/[S] represent the reciprocal of velocity and substrate concentration, respectively.

of cysteine residues in proteins (Bae *et al.*, 2009; Ishii *et al.*, 2009). The autoxidation of catechins is initiated by the one-electron oxidation of the B-ring, which is the crucial step in the autoxidation of catechins (Mochizuki *et al.*, 2002). Next, the covalent modification of ACE by tea catechins was examined by SDS-PAGE/blotting with redox-cycling staining. As shown in Fig. 3, positive bands at about 50 kDa were observed in the tea catechin-treated ACE groups, suggesting that all four tea catechins can covalently bind to ACE (N- or C-domain). The order of potency for redox-cycling staining of ACE was EGCg > EGC > ECg > EC, which is consistent with a previous report showing the modification of GAPDH by tea catechins (Mori *et al.*, 2010). However, there is an inconsistency between the activity inhibition (Fig. 1) and covalent modification of ACE (Fig. 3), supporting the idea that the galloyl ester might contribute to the ACE inhibitory activity in an autoxidation-independent manner. In addition, the stronger activity of EGCg than that of ECg might be attributed to the difference in their covalent modification abilities. This idea was supported by the preliminary observation that the addition of exogenous ascorbic acid partly impaired the inhibitory effect of tea catechins on ACE activity (unpublished data).

To gain further information about the inhibitory mechanism, a Lineweaver-Burk plot was employed for the ACE inhibition by EGC and ECg (Fig. 4). In both cases, there was little effect on K_m

(1.0, 1.1, and 1.1 mM for the absence and presence of EGC or ECg, respectively) and the two straight lines intersected at one point on the $1/[S]$ axis, indicating that both EGC and ECg were non-competitive inhibitors. These results suggested that tea catechins could bind to a non-catalytic but specific site of the ACE molecule and produce a dead-end complex, regardless of whether or not a substrate is bound. Thus, tea catechins might allosterically prevent the formation of the ACE enzyme reaction products.

Some non-competitive inhibitors have been purified from food sources (Ni *et al.*, 2012; Duan *et al.*, 2014). However, the inhibition mechanism and the binding site on ACE of these non-competitive inhibitors have not fully been clarified. Molecular docking has been applied to study the structure-activity relationship between bioactive peptides and ACE (Gentilucci *et al.*, 2012). The binding of inhibitors with ACE is strongly influenced by the three C-terminal amino acids of the peptide, which interact with the subsites S1, S1', and S2' at the ACE active site; hydrophobic amino acids at the C-terminus, such as Leu, Pro, Phe, Trp, and Tyr, would significantly increase ACE-binding affinity to occupy the active site. Additionally, a high content of the amino acids Leu, Tyr, and Val at the N-terminus helps to enhance the ACE inhibitory activity of the peptide, which might contribute to the non-competitive inhibition of ACE. In the present study, we demonstrated that galloylated catechins such as ECg and EGCg exhibited more potent inhibitory activity of ACE than EC and EGC (with no galloyl group). This tendency is the same as that for the binding affinity of tea catechins with human serum albumin (Bae *et al.*, 2009), suggesting that the galloyl moiety is present for the stable interaction with target proteins through hydrogen-bonding forces and electrostatic forces. In addition, a comparative study on the distribution coefficient of tea catechins suggested that the galloyl moiety would increase the hydrophobicity of the catechin molecule (Hashimoto *et al.*, 1999). Taken together, we speculated that the galloylated catechins inhibit ACE activity in a non-competitive manner through the hydrophobic and hydrogen-bonding interaction of the galloyl moiety with the non-catalytic site of ACE.

In conclusion, this study provides basic information concerning the inhibition of ACE enzymatic activity by tea catechins, and enables us to propose the involvement of the galloyl moiety in the stable catechin-protein interaction. Even though several proteins such as serum albumin could interact with galloylated catechins (Bae *et al.*, 2009) and thus act as a competitor of ACE, the present findings encourage further study using not only the plausible structural model based on the docking simulation but also proteomic and reverse genetic approaches to identify amino acid residues of ACE involved in the catechin-protein interaction.

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