Tea catechins as potent non-competitive inhibitors of angiotensin converting enzyme

September, 2016

Zhe Liu

Graduate School of Environmental and Life Science

(Doctor's Course)

OKAYAMA UNIVERSITY, JAPAN

CONTENTS

LIST OF FIGURES

ABBREVIATIONS

CVD, cardiovascular disease

EC, (-)-epicatechin

EGC, (-)-epigallocatechin

ECg, (-)-epicatechin gallate

EGCg, (-)-epigallocatechin gallate

LDL, low-density lipoprotein

ACE, Angiotensin converting enzyme

sACE, somatic ACE

tACE, testis ACE

RAS, renin-angiotensin system

Ang I, angiotensin I

Ang II, angiotensin II

HHL, hippuryl-histidyl-leucine

OPA, *o*-phthaldialdehyde

NBT, nitro blue tetrazolium

CBB, coomassie Brilliant Blue G-250

GAPDH, glyceraldehyde 3-phosphate dehydrogenase

rh-ACE, recombinant human ACE

EDTA, ethylenediaminetetraacetic acid

ROS, reactive oxygen species

FBS, fetal bovine serum

FOX, ferrous ion oxidation-xylenol orange

DOPAC, 3,4-Dihydoxyphenylaceetic acid

Q4'G, quercetin 4'-glucoside

TBTMA, tris[(1-benzyl-1H-1,2,3-triazol-4-yl)methyl]amine

BNPP, bis(4-nitrophenyl) phosphate

DPE, 3,4-dihydroxyphenylacetic acid propargyl ester

CHAPTER 1

General Introduction

1.1 Cardiovascular disease (CVD)

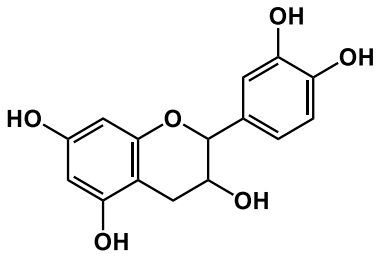
Cardiovascular disease (CVD) is a group of disorders that involve the heart and blood vessels. Cardiovascular disease includes hypertension, coronary heart disease, cerebrovascular disease, peripheral arterial disease, rheumatic heart disease, as well as congenital heart disease. CVDs are the leading cause of death worldwide. According to the World Health Organization, an estimated 17.5 million people died from CVDs in 2005, which accounted for 30% of all global deaths. It has been estimated that, by 2030, almost 23.6 million people will die from CVDs, mainly from heart attack and stroke. However, most of the premature heart attacks and strokes are preventable by checking and controlling the risk factors, such as high blood pressure, high cholesterol, and diabetes.

1.2 Tea and tea catechins

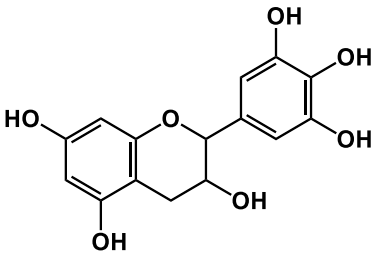
Tea is considered to be one of the most popular beverages in the world. The consumption of tea has a long history in Asia, which was literally recorded nearly 5,000 of years ago. Nowadays, tea is becoming very popular in North America and Europe. It is cultivated in more than 30 countries around the world. Although there are many types and grades of tea available in the market, based on the manufacturing process, three main types of tea can be classified: green tea which does not undergo fermentation, oolong tea which is subject to partial fermentation, and black tea which is made by full fermentation of plant.

Tea leaves are produced from the Camillia Sinensis plant. Fresh tea leaves contain a considerable amount of polyphenols (about 30% of the dry leaf weight), which include flavanols, flavadiols, flavonols, and phenolic acids (Hertog et al., 1993). Flavanols are the major component of tea, which are mainly composed of catechins, (-)-epicatechin (EC), (-)-epigallocatechin (EGC), (-)-epicatechin gallate (ECg) and (-)-epigallocatechin gallate (EGCg). Total polyphenol content is

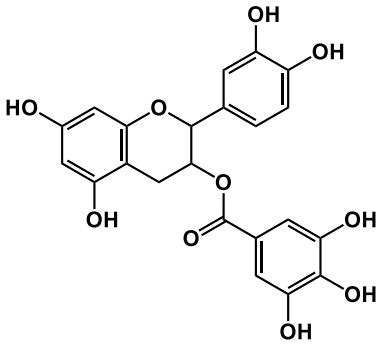
similar in different types of tea, but the composition varies based on the degree of polyphenol oxidation during the manufacturing process. Unlike oolong tea and black tea, the green tea leaves undergo a quick steam or heat treatment after being picked, in order to previously inactivate the oxidative enzymes and retain the polyphenol contents during the following manufacturing processes. Green tea contains mainly catechins, predominantly EGCg which may account for more than 50% of the total catechins (Khan et al., 2006). In the manufacture of black tea, the monomeric flavanols are susceptible to oxidative polymerization resulting in the formation of bisflavanols, theaflavins, thearubigins, and other oligomers during fermentation (Menet et al., 2004; Obanda et al., 2001). Nearly 10-20% of the dry weight of black tea is due to thearubigins, which are more extensively oxidized and polymerized (Lin et al., 1999). Oolong tea contains monomeric catechins, theaflavins, and thearubigins (Dou et al., 2007).



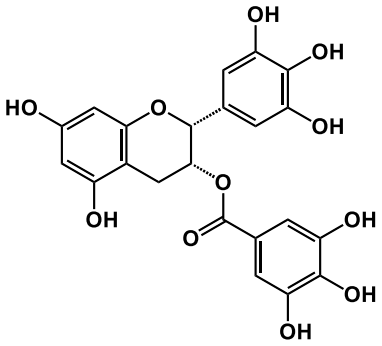
(-)-Epicatechin (EC)



(-)-Epigallocatechin (EGC)



(-)-Epicatechin gallate (ECg)



(-)-Epigallocatechin gallate (EGCg)

Figure 1.1 Chemical structures of four major tea catechins

1.3 Cardiovascular-protective role of tea and tea catechins

In recent years, tea has attracted significant attention due to its numerous health benefits, such as antioxidant, anti-cancer, anti-inflammatory, and neuroprotective effects (Azam et al., 2004; Higdon and Frei, 2003; Kakuda, 2002; Tipoe et al., 2007). The cardiovascular-protective effects of tea consumption have also been intensively studied. Accumulating epidemiological and clinical studies indicated that consumption of green tea is inversely associated with the risk of developing cardiovascular diseases. According to the Ohsaki National Health Insurance Cohort Study, which followed 40,530 Japanese adults aged 40 to 79 years, green tea consumption was associated with reduced mortality due to cardiovascular disease. This inverse association was strongest in women with a relative risk of 0.69 (95% CI, 0.53-0.90) for women who consumed five or more cups per day (Kuriyama et al., 2006). Arab and colleagues found an inverse dose-response association with tea consumption on both incidence and mortality of stroke (Arab et al., 2013). A case-control study, which was conducted in southern China, also demonstrated that long-term tea drinking significantly decreased the risk of ischemic stroke (Liang et al., 2009). Increasing evidence shows that tea consumption has a direct effect on cardiovascular disease risk factors. A meta-analysis of 13 randomized controlled trials examined the effects of green tea consumption on blood pressure. The overall outcome of this meta-analysis suggested that green tea consumption significantly decreases both systolic and diastolic blood pressure level (Peng et al., 2014). Furthermore, inclusion of black tea in a controlled weight-maintaining diet significantly reduces total and LDL cholesterol in mildly hypercholesterolemic adults (Davies et al., 2003).

Tea catechins, the most important bioactive components of tea, are considered to be responsible for a broad variety of health-promoting effects, particularly cardiovascular-protective effects. Based on the data from the Zutphen elderly study, Arts et al., found that catechin intake was inversely associated with ischemic heart disease mortality (Arts et al., 2001). Moreover, in a placebo controlled double-blind study, a beverage containing tea catechins (197.4 mg) was given twice a day to the adult males and females with mild and borderline hypercholesterolemia. A significant decrease on the serum total cholesterol level was observed at 8th week (228 ± 23 mg/dl to 220 ± 21 mg/dl, $p < 0.01$) and maintained until 12 weeks (222 ± 20 mg/dl, $p < 0.05$). The results suggested

the benefits of tea catechins in individuals with mild or borderline hypercholesterolemia (Kajimoto et al., 2003). Similarly, a meta-analysis of randomized controlled trials was conducted and found that green tea and tea catechins significantly reduced the total cholesterol and low-density lipoprotein cholesterol. It also suggests that green tea and its tea catechins might be associated with improved blood pressure, especially in subjects with systolic blood pressure ≥ 130 mmHg (Khalesi et al., 2014).

As mentioned above, increasing observational studies suggest a possible association between the intake of tea or tea catechins and the beneficial effects on cardiovascular disease. Several mechanistic studies have also shown tea and tea catechins display promoting effects on systemic risk factors and direct benefits on the vasculature and platelets which might be responsible for lower cardiovascular risk. Taken together, tea and its catechins can be considered as potential candidates for treatment or adjunctive treatment of cardiovascular disease.

1.4 Angiotensin converting enzyme (ACE)

Angiotensin converting enzyme (ACE) is known as a transmembrane dipeptidase. There are two distinct isoforms of ACE in humans, the somatic and testicular isoforms (Sibony et al., 1993). The somatic ACE is a 170-kDa glycoprotein, which is abundantly expressed in many tissues, such as lung, kidney, liver, and brain (van Sande et al., 1985). The somatic ACE is composed of two independent catalytic domains, termed the N- and C-domains. The two domains are about 60% homologous in both DNA and amino acid sequences. In the regions containing residues involved in catalysis, homology reaches 89% (Araujo et al., 2000; Wei et al., 1991). Both of the two domains contain the zinc coordinating amino acid sequence HEXXH, which is crucial for enzymatic activity. The testis ACE (tACE), a smaller molecular form (90-110 kDa), contains only the C-domain, which is attached to cell membranes. The tACE is exclusively expressed in male germinal cells, and is related with generation (Ehlers et al., 1989; Hagaman et al., 1998).

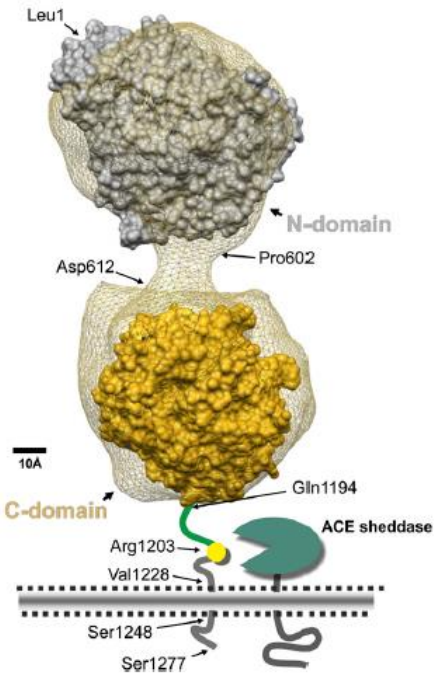


Figure 1.2 Model of human somatic ACE (Bernstein et al., 2013).

ACE was first identified as a key component of the renin-angiotensin system. It plays a crucial role in the regulation of blood pressure and electrolyte homeostasis, by catalyzing angiotensin I to angiotensin II. The binding between angiotensin II and the AT1 receptor leads to vasoconstrictive effects, stimulation of aldosterone secretion from the adrenal gland, retention of salt and water, as well as growth stimulation. It has been already documented that angiotensin II is associated with cardiovascular disease, including hypertension, cardiac failure, coronary heart disease, and post-myocardial infarction. ACE inhibitors, such as captopril, lisinopril, and enalapril, which are capable of lowering the angiotensin II level, are initially introduced as antihypertensive agents. Their effects are greater than expected by their ability to moderate blood pressure alone. It has been reported that ACE inhibitors display clinical efficacy in the treatment of a wide range of cardiovascular diseases, such as heart failure, peripheral artery disease, and acute myocardial infarction (Coppola et al., 2008; Mujib et al., 2013; Shi et al., 2010). Thus, ACE inhibitors are generally used as the first-line drug for the treatment of cardiovascular disease.

1.5 Purpose of this study

Angiotensin converting enzyme is an important member of renin-angiotensin system, whose main function is to regulate the blood pressure, fluid and electrolyte balance. Inhibition of ACE is one of the most effective strategies for the treatment of hypertension. Synthetic ACE inhibitors, such as captopril and enalapril, are also widely used for the therapy of cardiovascular disease. However, certain adverse effects, such as cough and skin rashes, are associated with the clinical use of these artificial ACE inhibitors. Therefore, the investigation of safe and efficient ACE inhibitors could greatly benefit CVD patients. Tea catechins, the major biologically active components of tea, have been reported to inhibit the enzymatic activity of ACE. However, the precise mechanism involved in the inhibition remains to be clarified. The objective of the present study is to investigate the molecular mechanism involved in the ACE inhibition by tea catechins.

CHAPTER 2

Galloylated catechins as potent inhibitors of angiotensin converting enzyme activity

2.1. Abstract

In the present study, I investigated the inhibitory effects of four tea catechins, including (-)-epicatechin (EC), (-)-epigallocatechin (EGC), (-)-epicatechin gallate (ECg) and (-)-epigallocatechin gallate (EGCg), on the activity of purified ACE from rabbit lung 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 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.

2.2. Introduction

Accumulating epidemiological and intervention studies indicated that green tea consumption is conversely associated with the risk of hypertension, which might be largely ascribed to the presence of polyphenols (Arab et al., 2013; Kuriyama, 2008; Nakachi et al., 2000). 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., 2006a; Persson et al., 2006). ACE, a dipeptidyl-carboxypeptidase involved in renin-angiotensin system regulating blood pressure and electrolyte balance, contributes to conversion of angiotensin I (Ang I) into angiotensin II (Ang II), a strong

vasoconstrictor, as well as metabolism of biologically active peptides such as bradykinin.

Like 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 the protein function through the covalent adduct formation and oxidation, respectively (Nakamura and Miyoshi, 2010). Our group previously demonstrated that catalase significantly abolished the cytotoxicity induced by the 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, glyceradehyde-3-phosphate dehydrogenase, and catalase (Mori et al., 2010; Pal et al., 2014; Wang et al., 2003). However, the precise mechanism underlying the ACE inhibition by tea catechins remains to be clarified.

Tea catechins have a flavan-3-ol structure composed of A-, B-, and C-rings with or without a 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, I hypothesized that they would inhibit ACE activity through a common mechanism to the different extent. To test this hypothesis, I 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 complex with coordinate linkage (Mochizuki et al., 2002). The covalent modification of ACE by tea catechins was determined by a redox-cycling staining to confirm its involvement in ACE inhibition. Finally, I employed a Lineweaver-Burk plot to confirm tea catechins as non-competitive inhibitors.

2.3. Materials and methods

2.3.1. Chemicals

ACE from rabbit lung, hippuryl-histidyl-leucine (HHL) and *o*-phthaldialdehyde (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).

2.3.2. ACE activity 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 μl at 37ºC for 30 min. In an inhibitory assay with tea catechins, ACE was pre-incubated with the chemicals at 4ºC for 15 min before the addition of substrate. Enzymatic reaction was terminated by the addition of 1.2 ml of 0.34 M NaOH, followed by a 10-min incubation at room temperature with 100 μl of OPA (20 mg/ml). The solution was acidified with 200 μl of 3 N HCl and centrifuged at 3,000 × g at 4ºC 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 4ºC for 15 min, followed by the addition of enzyme and substrate.

2.3.3. 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 37ºC for 60 min. The reaction between tea catechins and ACE was terminated by the addition of DTT (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 μm, Millipores), and then

EGCg-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 in dark, resulting in a blue-purple stain of quinoproteins bands and no staining of other proteins. The membrane was also stained with Coomassie Brilliant Blue G-250 (CBB) for checking the protein loading.

2.3.4. Statistical analysis

All results are expressed as means ± SD of more than three independent experiments. Statistical significance is denoted as *P < 0.05.

2.4. Results

2.4.1. Inhibitory effect of tea catechins on ACE activity

It has been reported that tea and tea catechins possess ACE inhibitory activity (Actis-Goretta et al., 2006a; Persson et al., 2006). To evaluate the ACE inhibitory activity of four major tea catechins, a fluorimetric assay reported by Santos et al. was used. The maximum absorption and the elimination metabolism of tea catechins occur very fast (Scalbert and Williamson, 2000). Therefore, to compare the inhibitory effect of tea catechins, ACE was incubated with each catechin at the indicated concentrations for 15 min and then the enzymatic activity of each group was determined. Each catechin treatment significantly reduced the ACE activity with the order of potency being EGCg > ECg > EGC = EC (Figure 2.1); EGCg at the concentration of 50 μM completely inhibited the ACE activity, whereas ECg and EGC or EC at the same concentration reduced it by 55% and 15%, respectively. 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 B ring.

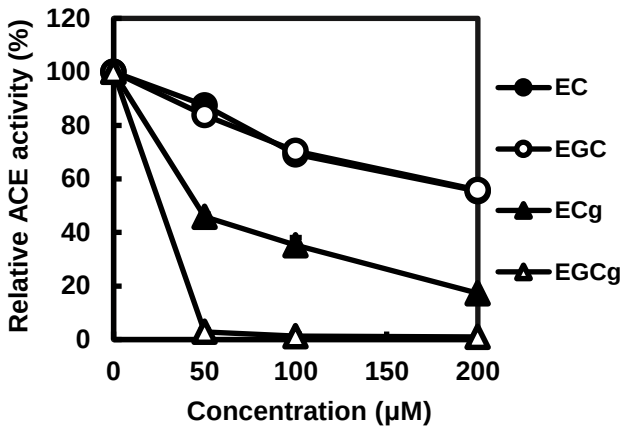


Figure 2.1 Inhibitory Effect of tea catechins on ACE activity. The enzymatic activity of purified ACE (from rabbit lung) was measured in the presence of tea catechins (EC, EGC, ECg, and EGCg) at the indicated concentrations. The relative values represent means ± SD of three independent experiments.

2.4.2. Counteracting effect of borate on the ACE inhibition by tea catechins

To investigate the involvement of *ortho*-dihydroxyl (catechol, pyrogallol or galloyl) groups in tea catechins on their ACE inhibitory activity, borate was used to form the stable complex with these groups at a basic condition (Mochizuki et al., 2002). First, tea catechins were treated with or without 1 mM borate at 4ºC for 15 min. After the addition of purified ACE and HHL, the ACE activity of each group was measured. As shown in Figure 2.2, the presence of 1 mM borate partly but significantly recovered the ACE activities reduced by 100 μM catechins. It should be noted that the influence on the ACE inhibitory activity of the four catechins brought by borate-treatment differ in degree (EGCg > ECg > EGC > EC).

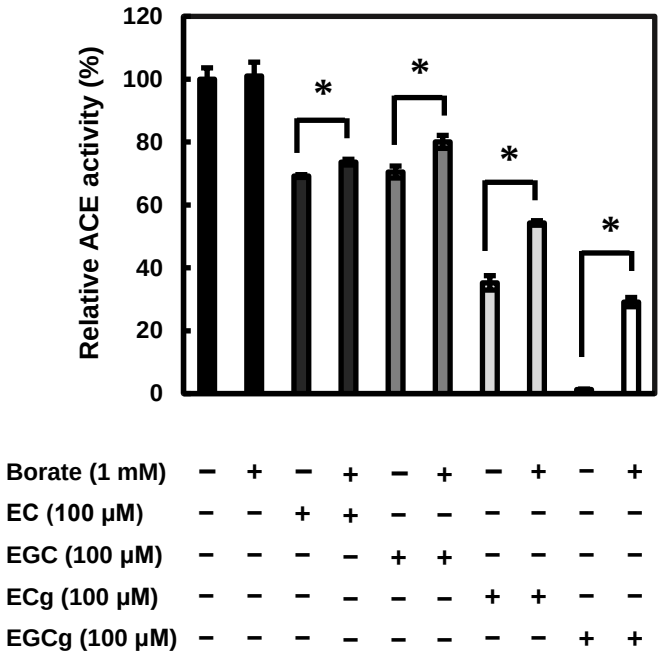


Figure 2.2 Counteracting effect of borate on the ACE inhibition by tea catechins. Tea catechins were pre-incubated with or without 1 mM borate at 4ºC for 15 min. Then the ACE activity of each group was measured. The relative values represent means ± SD of three independent experiments. *P < 0.05 vs. borate (-) group.

2.4.3. Covalent modification of ACE by tea catechins

The covalent modification of ACE by tea catechins was examined by SDS-PAGE/blotting with redox-cycling staining. As shown in Figure 2.3, positive bands at about 50 kDa were observed in the tea catechin-treated ACE groups, suggesting that all the four tea catechins can covalently bind to ACE (N- or C-domain). The order of potency for covalent modification of ACE was EGCg > EGC > ECg > EC, which is consistent with the previous report showing the modification of GAPDH by tea catechins (Mori et al., 2010).

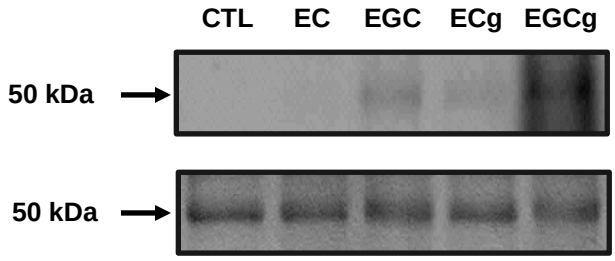


Figure 2.3 Covalent modification of ACE by tea catechins. ACE (15 mU) was treated with tea catechins (2 mM) at 37ºC for 60 min. The catechin-modified ACE was detected by a redox-cycling staining (above) and CBB staining (below), respectively.

2.4.4. Determination of enzyme kinetic parameters of EGC and ECg by Lineweaver-Burk plots

To gain further information about inhibitory mechanism, Lineweaver-Burk plots were employed for the ACE inhibition by EGC and ECg. EGC (100 μM) or ECG (100 μM) was co-incubated with various substrate concentrations (0.1, 0.25, 0.5, 1, and 2 mM) and 1 mU ACE, and the plots are shown in Figure 2.4. In both cases, 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 ACE molecule and produce a dead-end complex, regardless of whether or not a substrate is bound.

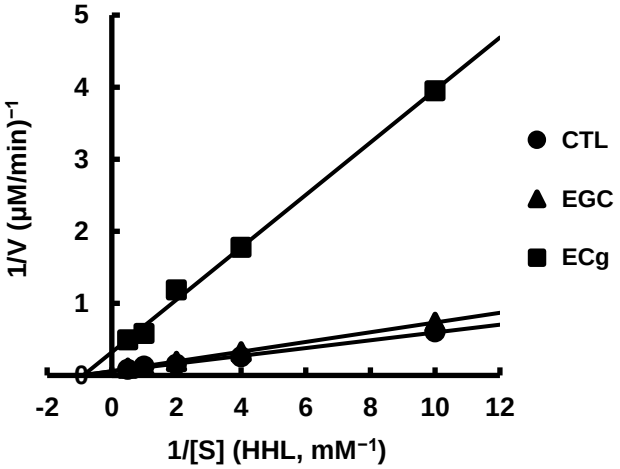


Figure 2.4 Lineweaver-Burk plots of ACE inhibition by EGC and ECg. The ACE activity was measured in the absence (closed circle) or presence of 100 μM EGC (closed triangle) or 100 μM ECg (closed square). 1/V and 1/[S] represent the reciprocal of velocity and substrate concentration, respectively.

2.5. Discussion

Several studies suggested that tea catechins are potential ACE inhibitors. In the present study, I confirmed the ACE inhibitory activity of the four major tea catechins (Figure 2.1). The inhibitory tendency is partly consistent with the previous study using human umbilical vein endothelial cells (Persson et al., 2006), but different from the tendency for GAPDH inhibition by tea catechins (Ishii et al., 2008) and for hydrogen peroxide production (Tang et al., 2014). The ACE inhibitory activity of each tea catechin 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 B ring.

To investigate the involvement of *ortho*-dihydroxyl (catechol, pyrogallol or galloyl) groups in tea catechins on their ACE inhibitory activity, I used borate to form the stable complex with these groups at a basic condition (Mochizuki et al., 2002). The results (Figure 2.2) suggested that hydroxyl groups at 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 EGC (Figure 2.2). This result also suggested that the galloyl ester might play a significant role in the ACE inhibitory activity independently 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 Zn^{2+} ion at the active center of ACE (Loizzo et al., 2007). The structure-activity relationship and protein-ligand docking studies also supported this idea (Guerrero et al., 2012). However, I preliminarily observed that the addition of exogenous Zn^{2+} ion did not influence the inhibitory effect of EGCg on the ACE activity, suggesting little contribution of its 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 (Chen et al., 2011; Ishii et al., 2008; Pal et al., 2014). At alkaline pH, catechins are readily oxidized into their corresponding quinones which are able to react with thiol groups of cysteine residues in proteins or peptides (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). The SDS-PAGE/blotting with redox-cycling experiments revealed that tea catechins were covalently modified the N- or C-domain of ACE (Figure 2.3). The order of potency for covalent modification of ACE was EGCg > EGC > ECg > EC, which is consistent with the previous report showing the modification of GAPDH by tea catechins (Mori et al., 2010). However, there is an inconsistency between the activity inhibition (Figure 2.1) and covalent modification of ACE (Figure 2.4), 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 by the difference in their covalent modification abilities.

Furthermore, Lineweaver-Burk plots suggested that both EGC and ECg were non-competitive inhibitors of ACE. Non-competitive ACE inhibitors are usually to be purified from some food sources (Duan et al., 2014; Ni et al., 2012). However, the inhibition mechanism and the binding site on ACE of these non-competitive inhibitors have not fully been understood. 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, that 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 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 the galloylated catechins such as ECg and EGCg exhibited the more potent inhibitory activity of ACE than EC and EGC with no galloyl group. This tendency is completely matched with 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 specific binding with target proteins through the hydrogen-bonding forces and electrostatic forces. In addition, a comparative study on distribution coefficient of tea catechins suggested that the galloyl moiety would increase the hydrophobicity of the catechin molecule (Hashimoto et al., 1999). Taken together, I speculated that the galloylated catechins inhibit ACE activity in a non-compatitive manner through the 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 an involvement of the galloyl moiety in the catechin-protein interaction. 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.

CHAPTER 3

EGCg inhibits the activity of recombinant human angiotensin-converting enzyme through an autoxidation-dependent mechanism

3.1 Abstract

Although dietary flavonoids, such as (-)-epigallocatechin-3-gallate (EGCg) and luteolin, have been shown to inhibit angiotensin converting enzyme (ACE) activity, the precise mechanisms still remain to be clarified. In this study, I investigated the molecular mechanisms involved in the ACE inhibition by EGCg, a major tea catechin. I first confirmed that the activity of HCT-116 cell lysate and recombinant human ACE (rh-ACE) were inhibited by EGCg in a dose-dependent manner. Co-incubation with Zn^{2+} showed no influence on the ACE inhibition by EGCg, whereas it completely counteracted the inhibitory effect of EDTA, a representative ACE inhibitor chelating Zn^{2+} at the active site of ACE. Although a considerable amount of hydrogen peroxide was produced during the incubation of EGCg, the treatment of ACE with hydrogen peroxide had little effect on the enzymatic activity. On the other hand, the co-incubation of EGCg with inhibitors of catechol oxidation, such as borate or ascorbic acid, significantly diminished the EGCg inhibition. A redox-cycling staining experiment revealed that rh-ACE was covalently modified by EGCg. Furthermore, Lineweaver-Burk plot analysis indicated that EGCg inhibited ACE activity in a non-competitive manner. These results strongly suggested that EGCg might allosterically inhibit the ACE activity through the oxidative conversion into an electrophilic quinone and subsequent binding to the ACE.

3.2 Introduction

Cardiovascular disease (CVD) is the leading cause of mortality worldwide. The majority of CVD is considered preventable by controlling or modifying its risk factors (Bernstein et al., 2013). One of the most powerful risk factors for CVD is high blood pressure (Kannel, 1996), which accounts for an estimated 54% of all strokes and 47% of all ischemic heart disease events (Lawes et al., 2008). Angiotensin converting enzyme (ACE), a dipeptidyl-carboxypeptidase, plays a crucial role in renin-angiotensin system (RAS), which is one of the major control systems for blood pressure,

fluid and electrolyte balance. Within the RAS, ACE catalyzes the conversion of angiotensin I (Ang I) into a potent vasoconstrictor angiotensin II (Ang II), and the metabolism of other peptides such as the vasodilator bradykinin. Therefore, the inhibition of ACE is one of the most promising strategies for prevention of hypertension. Synthetic drugs having ACE inhibitory activity, such as captopril and enalapril, are usually used for the treatment of CVD and for the treatment of heart failure. However, these artificial ACE inhibitors have side effects such as cough, angioneurotic edema and deleterious effects in pregnancy (Hanssens et al., 1991; Israili and Hall, 1992b; Shotan et al., 1994; Simon et al., 1992; Vleeming et al., 1998). Thus, it is desirable for hypertensive patients to investigate and develop a new type of ACE inhibitors without side effects.

Green tea is regarded as one of the most widely consumed beverages worldwide. Accumulating epidemiological and intervention studies indicate that green tea or tea extracts consumption is associated with the beneficial effects on decrease of blood pressure, which might be largely ascribed to the presence of polyphenols, such as flavanols, flavonols, anthocyanidins and phenolic acids (Negishi et al., 2004; Peng et al., 2014; Zaveri, 2006). Among them, flavanols, the collective term for the catechins, is the primary class of polyphenols in green tea; (-)-epicatechin (EC), (-)-epigallocatechin (EGC), (-)-epicatechin gallate (ECg) and (-)-epigallocatechin gallate (EGCg). It has been suggested that the cardioprotective effects of tea catechins are partially associated with its antioxidant and free radical scavenging properties (Babu and Liu, 2008). In addition to the antioxidant action, some studies have demonstrated that tea catechins significantly inhibited ACE activity (Actis-Goretta et al., 2006b; Persson et al., 2006). The well-known artificial ACE inhibitors, such as captopril, enalaprilat and lisinopril, can inhibit ACE activity by directly interacting with Zn^{2+} ions at the active sites of ACE (Natesh et al., 2004; Natesh et al., 2003). Tea catechins are known to have the capacity to chelate metal ions like Zn^{2+}, Fe^{2+} and Cu^{2+} (Esparza et al., 2005; Khokhar and Owusu Apenten, 2003; Mira et al., 2002). The Zn^{2+}-chelating property of tea catechins seems to be one of the possible mechanisms for tea catechins to inhibit ACE activity. Alternatively, autoxidation of polyphenols with a catechol structure leads to the formation of polyphenol quinones and ROS, both of which can modify the protein function through the covalent adduct formation and oxidation, respectively (Ishii et al., 2008; Raza and John, 2007). Although covalent or non-covalent modification with ACE might play a role in the ACE inhibition by tea catechins, the precise

mechanism remains to be clarified.

EGCg, the most abundant catechin in green tea, has been reported to possess higher ACE inhibitory activity than other catechins (Chapter 2). In the present study, the underlying mechanism involved in ACE inhibition by EGCg was investigated. The ACE inhibitory activity of EGCg was attenuated by the combinatory incubation with borate or hydrogen peroxide. Incubation with zinc sulfonate showed no change in the EGCg's effect, whereas it significantly reduced the ACE inhibition by a representative chelating agent, EDTA. These results indicate that Zn chelation and hydrogen peroxide production might be ruled out in the mechanism for ACE inhibition by EGCg. This study provides the evidence showing that EGCg inhibits ACE activity, possibly through the covalent interaction.

3.3 Materials and Methods

3.3.1 Chemicals

Recombinant human ACE/CD143 somatic form was purchased from R&D Systems (Minneapolis, MN, USA). Hippuryl-histidyl-leucine (HHL), histidyl-leucine and *o*-phthaldialdehyde (OPA) were obtained from Sigma (St. Louis, MO, USA). EGCg and $ZnSO_4$ were purchased from Wako Pure Chemical Industries (Osaka, Japan). All other chemicals including EDTA were obtained from Nacalai Tesque (Kyoto, Japan).

3.3.2 Cell culture

Human colon tumor cell lines HCT-116 (carcinoma) was obtained from American Type Culture Collection (Rockville, MD). HCT-116 cells were grown in McCoy's 5a medium supplemented with 10% *v/v* fetal bovine serum (FBS), 50 U/mL penicillin and 50 μg/mL streptomycin (Gibco BRL). Cells were maintained at 37ºC in an incubator under 5% CO_2 and 95% air at constant humidity. After a 3-d preculture, cells were harvested and lysed in 50 mM Tris (pH 7.4) containing 0.5% Nonidet P-40. After centrifugation at 3,000 × g for 10 min at 4ºC, the supernatant was used for determination. The measurement of ACE activity was described below.

3.3.3 ACE activity assay

The proteolytic activity of ACE was determined by a fluorimetric method, which was previously described by Santos et al. with some modifications (Santos et al., 1985). Briefly, a certain amount of recombinant human somatic ACE (rh-ACE) or HCT-116 cell lysate 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 μl at 37ºC for 30 min. In an inhibitory assay with EGCg or EDTA, rh-ACE or cell lysate was pre-incubated with the chemicals at 4ºC for 15 min before the addition of substrate. Enzymatic reaction was stopped by the addition of 1.2 ml of 0.34 M NaOH, followed by a 10-min incubation at room temperature with 100 μl of OPA (20 mg/ml). The solution was acidified with 200 μl of 3 N HCl and centrifuged at 3,000 × g at 4ºC for 10 min. The fluorescence of the OPA-His-Leu adduct was detected by a Hitachi F-2500 fluorescence spectrophotometer (360 nm excitation, 490 nm emission wavelength). All assays were carried out in triplicate.

3.3.4 Influence of external factors on the ACE inhibitory activity of EGCg

The effect of Zn^{2+} ions on the ACE inhibitory activity of EGCg was investigated by measuring the enzymatic activity in the presence of EGCg supplemented with different concentrations (0, 5, 10, and 20 μM) of $ZnSO_4$. ACE activity was also measured in the presence of Zn-EGCg complex which was prepared according to method proposed by Sun et al. (Sun et al., 2008). EDTA was used as a reference zinc chelator, and its ACE inhibitory activity was measured as mentioned above with the addition of varied concentrations (0, 2, 2.5, 3, and 5 μM) of $ZnSO_4$. Borate, H_2O_2, or ascorbic acid was used to interfere the ACE inhibition by EGCg. EGCg was pre-incubated with borate (0, 0.25, 0.5, and 1 mM), or hydrogen peroxide (0, 20, 50, and 100 μM) at 4ºC for 15 min, whereas ascorbic acid (0, 50, 100, and 200 μM) was pre-incubated with EGCg at 37ºC for 15 min. Then the ACE activity assay was initiated by the addition of enzyme and substrate.

3.3.5 Determination of hydrogen peroxide by the FOX assay

Ferrous ion oxidation-xylenol orange method (Qi et al., 2011) was used to determine the change of hydrogen peroxide levels during the ACE activity assay. Different concentrations (0, 5, 10, 50,

and 100 μM) of EGCg was incubated with recombinant human ACE at 37ºC for 30 min. 45 μl of resulted solution was collected and mixed with 5 μl of methanol, and incubated at room temperature. After an incubation period of 30 min, FOX reagent composed of 125 μM xylenol orange, 250 μM ammonium ferrous sulphate and 100 mM sorbitol in 25 mM H_2SO_4 was added and incubated for another 30 min. The solution was then centrifuged at 15,000 rpm for 10 min at room temperature and the absorbance was measured at 560 nm using Bio-Rad Benchmark Plus Multiplate Spectrophotometer (Bio-Rad, Hercules, CA). The concentration of hydrogen peroxide was calculated from standard curve. Stock solution of FOX reagent was previously prepared and was used in the experiments at 10 times dilution.

3.3.6 SDS-PAGE and redox-cycling staining

To detect the covalent modification by EGCg, 2.5 ng of rh-ACE was incubated with different concentrations of EGCg (0, 0.5 mM, and 1 mM) in 50 mM potassium phosphate buffer (pH 7.0) at 37ºC for 60 min. The reaction between EGCg and ACE was terminated by the addition of DTT (50 mM final), following the method of Ishii (Ishii et al., 2008). The reaction mixtures were separated by SDS-PAGE using 10% gels. For redox cycling staining, the gel bands were transferred onto a PVDF membrane (0.45 μm, Millipores), and then EGCg-modified proteins were detected by staining with NBT (0.2 mg/ml in 2 M potassium glycinate, pH 10). The blotting membrane was immersed in the glycinate/NBT solution for approximately 3 h in the dark, resulting in a blue-purple stain of quinoproteins bands and no staining of other proteins. After redox-cycling staining, the proteins were stained with Coomassie Brilliant Blue G-250 (CBB).

3.4 Results

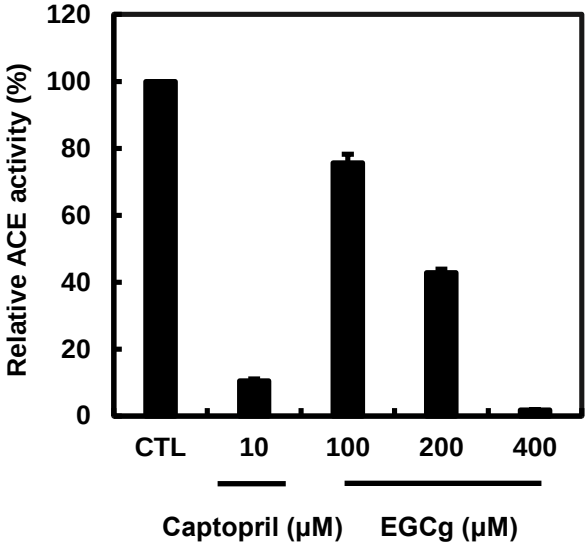
3.4.1 Inhibitory effects of EGCg on the ACE activity in vitro and cellular systems

To confirm the inhibitory effect of EGCg on the enzymatic activity of ACE, a modified fluorimetric assay was applied to measure the ACE activity of HCT-116 cell lysate in the presence or absence of EGCg. An aliquot of lysate was pre-incubated with different concentrations of EGCg (0, 1, 5, and 10 μM) at 4ºC for 15 min, then the enzyme activity assay of each group was initiated

by the addition of artificial tripeptide substrate, HHL. The cell lysate treated with captopril, a commercial available ACE inhibitor, was used as a positive control. As shown in Figure 3.1A, captopril (10 μM) treatment decreased the original ACE activity to 10.6%. The ACE inhibitory activity of 100, 200, and 400 μM of EGCg was 75.6%, 42.9%, and 1.8%, respectively. Although EGCg was weaker than captopril as ACE inhibitor, the treatment with EGCg could effectively and dose-dependently inhibit the human ACE activity.

To avoid influences coming from the contaminants in the cell lysate, recombinant human ACE (rh-ACE) was used in the following experiments. I first examined the inhibitory effect of EGCg on the enzymatic activity of rh-ACE. As shown in Figure 3.1B, treatment with EGCg dose-dependently inhibited the rh-ACE activity. Residual ACE activity after pretreatment of 1, 5 or, 10 μM EGCg was 68.5%, 19.8%, or 5.1%, respectively. These results strongly suggested that EGCg is a potential ACE inhibitor.

(A)



(B)

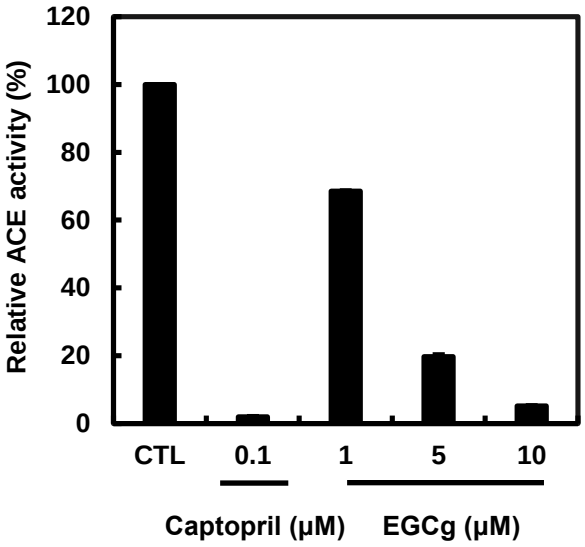


Figure 3.1 Inhibitory activity EGCg on the enzymatic activity of ACE. The enzymatic activity of HCT-116 cell lysate (A) and recombinant human ACE (B) were measured in the presence of EGCg at the indicated concentrations. The relative values represent means ± SD of three independent experiments.

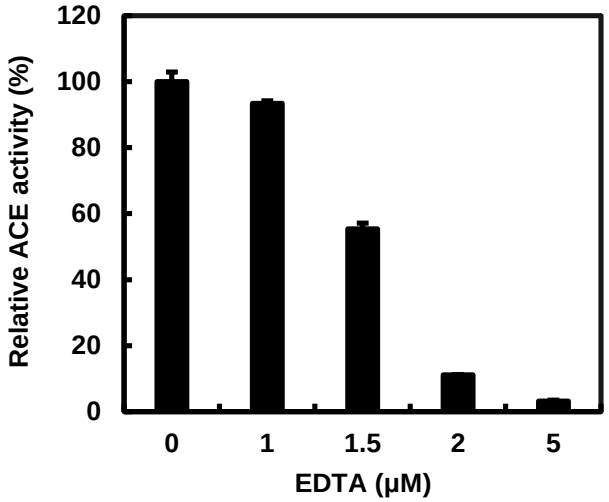
3.4.2 The Zn^{2+} chelation mechanism should be ruled out in the ACE inhibition by EGCg

ACE has an absolute requirement of Zn^{2+} for its proteolytic activity. EDTA is a well-known ACE inhibitor by chelating Zn^{2+} in the active sites of ACE (Buttery and Stuart, 1993). The rh-ACE activity was actually suppressed by EDTA in a dose-dependent fashion (Figure 3.2A), which was consistent with previous a report (Kasahara and Ashihara, 1981). EDTA (5 μM) decreased the rh-ACE activity to 3.2%, whereas exogenously added Zn^{2+} competitively counteracted this inhibition by EDTA (Figure 3.2B). These results further supported the idea that EDTA act as a Zn^{2+} chelator to inhibit the ACE activity.

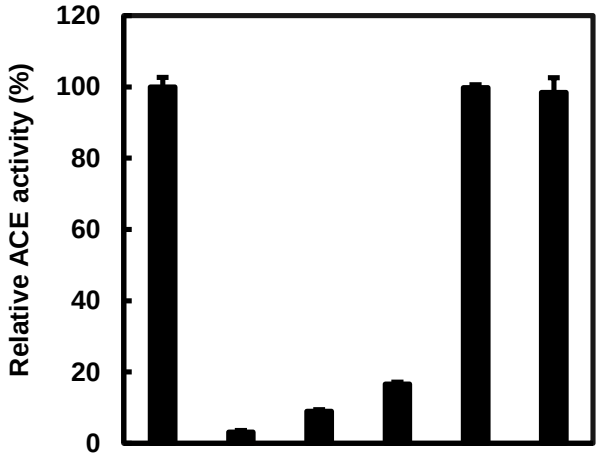
EGCg, which is capable of chelating metal ions (such as Fe^{2+}, Cu^{2+}, and Zn^{2+}), is believed to inhibit ACE activity by coordinating Zn^{2+} in the active site (Kumamoto et al., 2001; Chen et al., 2007). To investigate the involvement of Zn chelation in ACE inhibition by EGCg, ACE inhibitory effect of EGCg was measured in the presence of Zn^{2+} ions. As shown in Figure 3.2C, the rh-ACE activity was decreased to 7.4% in the presence of 10 μM of EGCg. However, no significant changes in the ACE activity had been observed when 5, 10, or 20 μM of Zn^{2+} was supplemented.

I next prepared an already-formed complex of EGCg with Zn^{2+} (Zn-EGCg complex) according to Sun et al (Sun et al., 2008). As shown in Figure 3.2D, the UV-vis absorption spectra of EGCg with or without Zn^{2+} were completely matched, whereas that of Zn-EGCg showed a disappearance of maximum absorption at 322 nm. The UV-vis absorption spectra indicated that Zn-EGCg complex is less likely to be formed in the potassium phosphate buffer (pH 8.3). Furthermore, Zn-EGCg complex exhibited much less inhibitory effect on ACE activity than EGCg (Figure 3.2E). These results suggested that ACE inhibition induced by EGCg might not be attributed to the metal ions-chelating property.

(A)

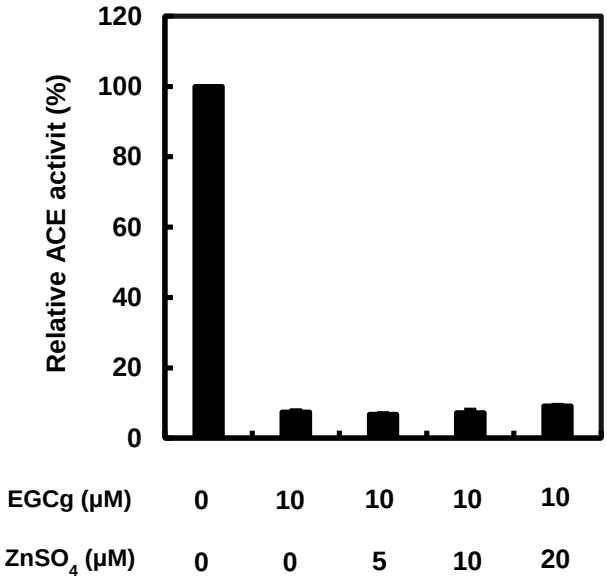


(B)

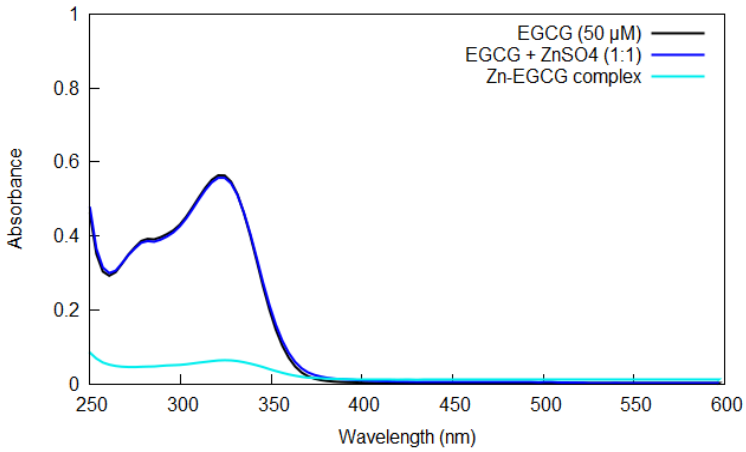


EDTA (μM)	0	5	5	5	5	5
$ZnSO_4$ (μM)	–	–	2	2.5	3	5

(C)


Relative ACE activit (%)
120
100
80
60
40
20
0
EGCg (μM) 0 10 10 10 10
ZnSO4 (μM) 0 0 5 10 20

(D)


EGCG (50 μM)
EGCG + ZnSO4 (1:1)
Zn-EGCG complex
Absorbance
1
0.8
0.6
0.4
0.2
0
250
300
350
400
450
500
550
600
Wavelength (nm)

(E)

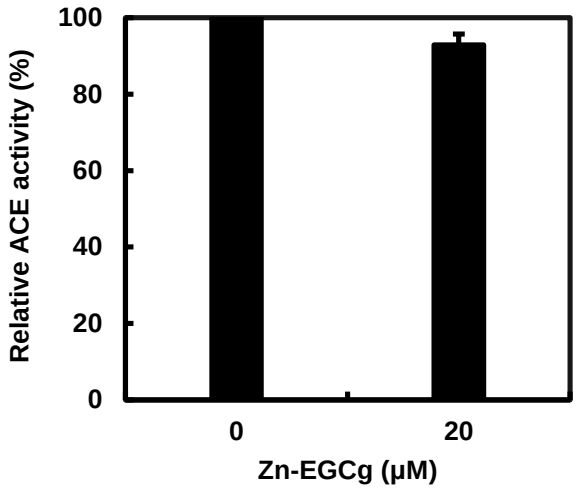
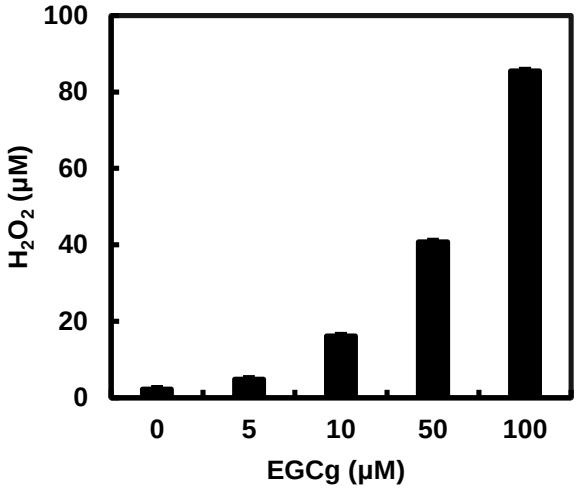


Figure 3.2 The involvement of Zn^{2+} chelation in the ACE inhibition induced by EDTA or EGCg. (A) ACE inhibitory activity of EDTA. (B) Effect of Zn^{2+} on the ACE inhibitory activity of EDTA. (C) Effect of Zn^{2+} on the ACE inhibitory activity of EGCg. (D) UV-vis absorption spectra of EGCg alone, zinc-EGCg complex and Zn^{2+}/EGCg mixture. (E) ACE inhibitory activity of Zn-EGCg complex.

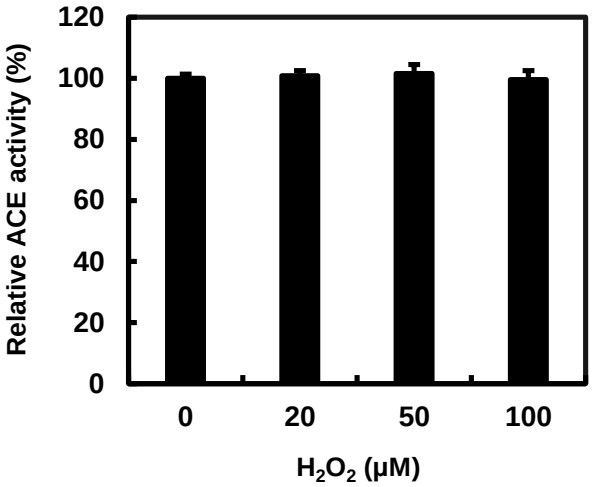
3.4.3 Generated hydrogen peroxide might not be involved in the ACE inhibition by EGCg

EGCg has been reported to easily undergoes autoxidation at alkaline pH, which is accompanied by the generation of hydrogen peroxide (Mochizuki et al., 2002; Nakayama et al., 2002). The formation of hydrogen peroxide is considered to contribute to the anti-cancer properties of EGCg against certain tumor cell lines. The net increase in hydrogen peroxide level triggers several signaling cascades, which will lead to apoptotic cell death (Nakagawa et al., 2004; Ranzato et al., 2012; Satoh et al., 2013). Thus, the involvement of hydrogen peroxide generation in the ACE inhibition by EGCg was investigated. After the incubation of EGCg in potassium phosphate buffer (pH 8.3) at 4ºC for 15 min, the FOX assay was used to monitor the changes in hydrogen peroxide levels. As shown in Figure 3.3A, the hydrogen peroxide amount was elevated as the concentration of EGCg increased (0, 5, 10, 50, and 100 μM). Further incubation of EGCg with ACE and HHL at 37ºC for 30 min showed no significant change in hydrogen peroxide level (data not shown). EGCg at 10 μM, which could completely inhibit the enzymatic activity of ACE, produced 16.1 μM of hydrogen peroxide during the 30-min incubation in assay buffer (Figure 3.3A). In fact, 20 μM of exogenous hydrogen peroxide had no inhibitory effect on the ACE activity (Figure 3.3B). Furthermore, co-incubation of hydrogen peroxide with EGCg did not enhance but rather inhibit the EGCg-dependent effect on the ACE activity. These results indicated that hydrogen peroxide produced by the autoxidation of EGCg might not be responsible for the ACE inhibition by EGCg.

(A)


100
80
60
40
20
0
H_2O_2 (μM)
0
5
10
50
100
EGCg (μM)

(B)


120
100
80
60
40
20
0
Relative ACE activity (%)
0
20
50
100
H_2O_2 (μM)

(C)

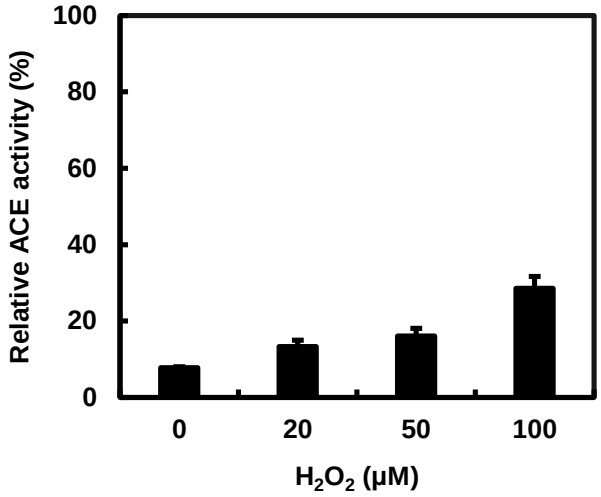


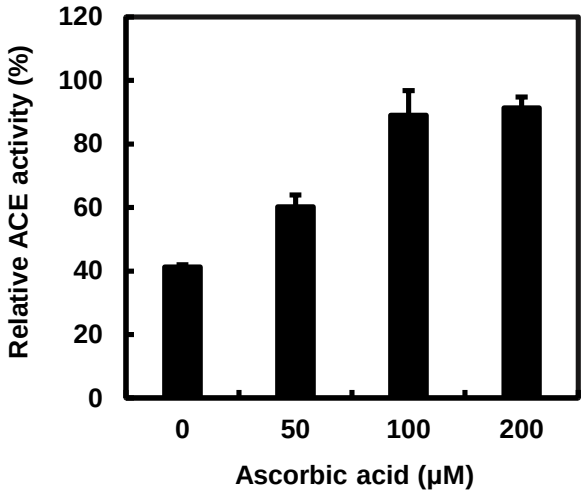
Figure 3.3 The involvement of the hydrogen peroxide formation in the ACE inhibition by EGCg. (A) Hydrogen peroxide formation by EGCg in potassium phosphate buffer (pH 8.3). Different concentrations (0, 5, 10, 50, and 100 μM) of EGCg were added to potassium phosphate buffer (pH 8.3), and incubated at 4ºC for 15 min. The hydrogen peroxide concentration was measured by FOX assay. (B) Effect of exogenous hydrogen peroxide on the enzymatic activity of ACE. Different concentrations (0, 20, 50, and 100 μM) of hydrogen peroxide was mixed with rh-ACE, and co-incubated at 4ºC for 15 min before the ACE activity assay. (C) Effect of exogenous hydrogen peroxide on the ACE inhibitory activity of EGCg. EGCg (10 μM) was pre-incubated with hydrogen peroxide at the indicated concentrations at 4ºC. After 15 min, ACE activity of each group was measured.

3.4.4 EGCg inhibited the ACE activity through an autoxidation-dependent manner

Next, I attempted to investigate whether autoxidation is involved in the ACE inhibition by EGCg. It has been reported that several reducing agents, such as ascorbic acid, are able to prevent EGCg oxidation (Chen et al., 1998). To examine the effect of ascorbic acid on the ACE inhibitory effect of EGCg, EGCg was pre-incubated in potassium phosphate buffer (pH 8.3) in the presence of different concentrations of ascorbic acid (0, 50, 100, and 200 μM) at 37ºC for 15 min. As shown in Figure 3.4A, the ACE activity was dose-dependently recovered by the addition of ascorbic acid. With 200 μM of ascorbic acid, the ACE inhibition by EGCg was completely abolished. These results suggested that autoxidation of EGCg plays an important role in the inhibitory effect on the ACE activity.

Numerous evidence reveals that oxidation of EGCg leads to the formation of semiquinone radicals which electrophilically attacks the thiol groups in proteins to form covalent adducts (Ishii et al., 2008; Chen et al., 2010). To determine whether EGCg covalently binds to ACE, ACE (2.5 ng) was co-incubated with varied concentrations of EGCg (0, 0.5, and 1 mM) in potassium phosphate buffer (pH 7.4) at 37ºC for 60 min, then the reaction mixtures were analyzed by SDS-PAGE/blotting with redox-cycling staining. As shown in Figure 3.4B, the positive bands around 160 kDa were detected in the EGCg-treated group, indicating that EGCg covalently binds to ACE.

(A)



(B)

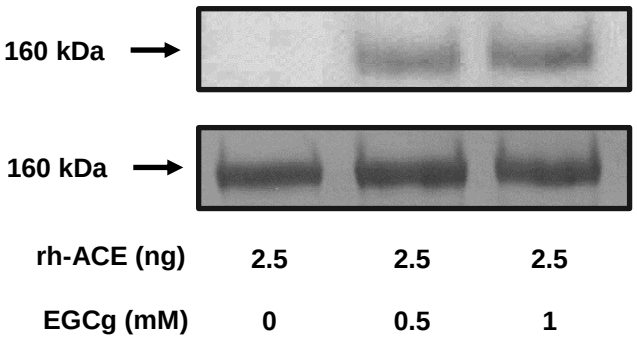


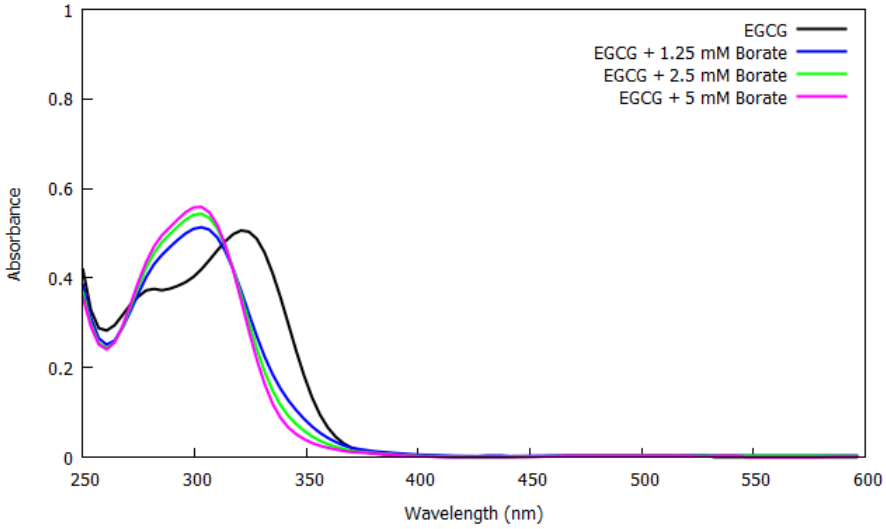
Figure 3.4 Involvement of autoxidation in ACE inhibition by EGCg. (A) Effect of ascorbic acid on the ACE inhibitory activity of EGCg. EGCg (10 μM) was co-incubated with the indicated concentrations of ascorbic acid (0, 50, 100, and 200 μM) in potassium phosphate buffer at 37ºC for 15 min. (B) ACE (2.5 ng) was treated with different concentrations of EGCg (0, 0.5, and 1 mM) at 37ºC for 60 min. The EGCg-bound ACE was detected by redox-cycling staining (above) and CBB staining (below), respectively.

3.4.5 ACE inhibitory activity of EGCg was suppressed by the formation of complex with borate

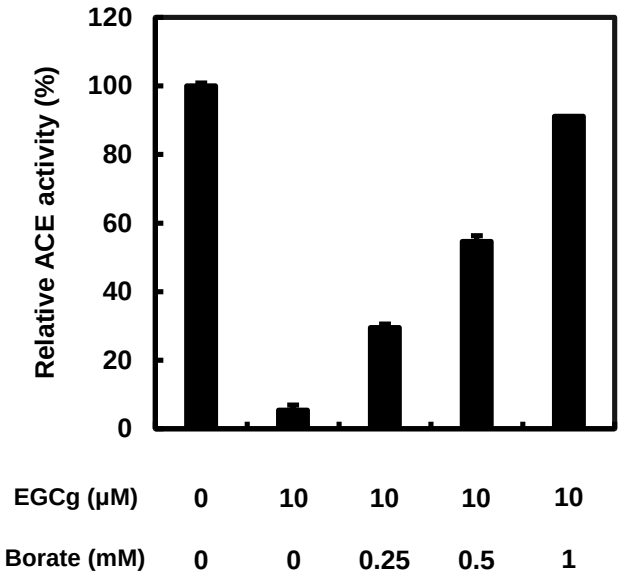
To further confirm the involvement of *o*-dihydroxyl groups in the ACE inhibition by EGCg, the effect of borate on the ACE inhibitory activity of EGCg was investigated. It is well-known that EGCg is capable of chelating with borate ion, and leads to the formation of EGCg-borate complex. As shown in Figure 3.5A, the maximum absorption at 322 nm of EGCg was hypsochromically shifted to 301 nm in the presence of borate. The concentration-dependent increase of the absorption at 301 nm clearly supported the formation of the EGCg-borate complex.

Next, the effect of borate on the ACE inhibition by EGCg was examined. The co-incubation of EGCg and borate at 4ºC for 15 min significantly dose-dependently diminished the ACE inhibitory activity of EGCg (Figure 3.5B). Furthermore, no EGCg-modified ACE was detected in the presence of borate, whereas the positive band was observed in the absence of borate. These results suggested that the generation of EGCg-modified ACE is one of the important mechanism for the ACE inhibition by EGCg.

(A)


EGCG
EGCG + 1.25 mM Borate
EGCG + 2.5 mM Borate
EGCG + 5 mM Borate
Absorbance
Wavelength (nm)
1
0.8
0.6
0.4
0.2
0
250
300
350
400
450
500
550
600

(B)


Relative ACE activity (%)
120
100
80
60
40
20
0
EGCg (μM) 0 10 10 10 10
Borate (mM) 0 0 0.25 0.5 1

(C)

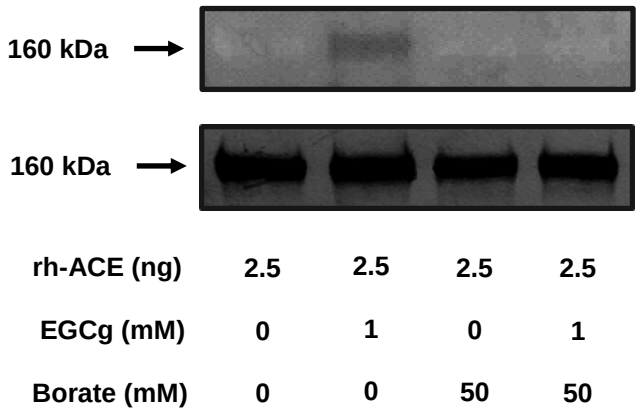


Figure 3.5 Effect of borate on the ACE inhibitory activity of EGCg. (A) UV-vis spectrum change of 50 μM EGCg in the presence or absence of borate. (B) Effect of borate on the ACE inhibition by EGCg. EGCg was co-incubated with the indicated concentrations of borate. (C) Effect of borate on the covalent modification of ACE by EGCg. ACE (2.5 ng) was treated incubated with 1 mM EGCg in the presence or absence of 50 mM borate at 37ºC for 60 min. Proteins were applied to SDS-PAGE/blotting with redox-cycling staining (above) and CBB staining (below).

3.4.6 A Lineweaver-Burk plot

To determine the inhibition pattern of EGCg on the rh-ACE activity, I employed a Lineweaver-Burk plot using various concentrations of the substrate and EGCg. As shown in Figure 3.6, two straight lines intersected at one point on the 1/[S] axis, indicating that the inhibition of ACE by EGCg is of non-competitive type. This result suggested that EGCg could bind to a non-catalytic but specific site of ACE molecule and produce a dead-end complex, regardless of whether or not a substrate is bound.

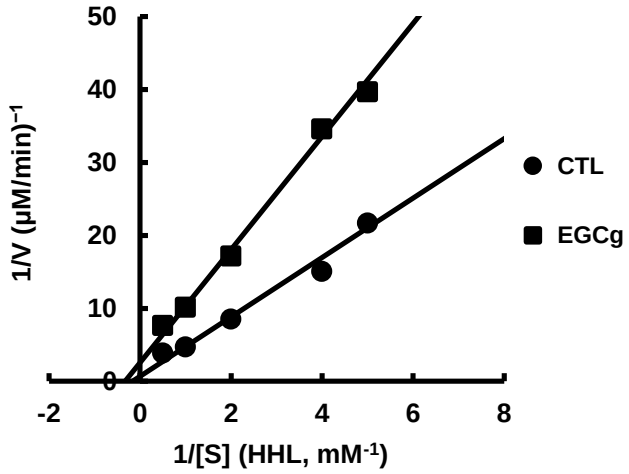


Figure 3.6 A Lineweaver-Burk plot of ACE inhibition by EGCg. The ACE activity was measured in the absence (closed circle) or presence of 2.5 μM EGCg (closed square). 1/V and 1/[S] represent the reciprocal of velocity and substrate concentration, respectively.

3.5 Discussion

ACE is a widely distributed dipeptidyl carboxypeptidase in mammalian tissues and can be expressed in many cell types, such as endothelial cells, and epithelial cells (Bruneval et al., 1986; Caldwell et al., 1976; Defending et al., 1983; van Sande et al., 1985). In the preliminary study, I found that the lysate of HCT-116 cells exhibited ACE activity, which could be inhibited by captopril and EGCg (Figure 3.1A). In addition, the enzymatic activity of rh-ACE was also suppressed by EGCg (Figure 3.1B). These results clearly supported that EGCg is effective to inhibit ACE activity, which is consistent with the previous research (Persson et al., 2006).

EGCg is able to chelate metal ions, such as Zn^{2+}, Fe^{2+} and Cu^{2+} (Esparza et al., 2005; Khokhar and Owusu Apenten, 2003; Mira et al., 2002). EGCg coordinates to the Zn^{2+} complexes in a 1:1 molar ratio (Esparza et al., 2005; Sun et al., 2008). This metal-chelating property has been reported to be associated with several biological activities, including the inhibition of enzyme activity, tumor invasion and angiogenesis, as well as the misfolding or aggregation of amyloid β-sheet (Ghosh et al., 2006; Jung and Ellis, 2001; Zhang et al., 2012). ACE is known as a zinc-dependent peptidase whose main function is to regulate arterial blood pressure as well as electrolyte balance through the RAS. It is well-known that the pharmacological mechanism of captopril, enalaprilat, as well as lisinopril, which are applied as the first-line drug therapy for hypertension, is the ability to directly interact with the Zn^{2+} ion at the active site of the enzyme. The ability of EGCg to chelate Zn^{2+} is widely regarded as one of the possible explanations for its ACE inhibitory activity (Guerrero et al., 2012; Persson et al., 2006). However, Zn^{2+} supplementation did not change the ACE inhibitory activity of EGCg (Figure 3.2C), whereas the ACE inhibition by EDTA was suppressed in the presence of Zn^{2+} (Figure 3.2B). Moreover, UV-vis absorption spectra indicated that Zn-EGCg complex is less likely to be formed in the potassium phosphate buffer (Figure 3.2D). All these findings suggest that ACE inhibition induced by EGCg might not be attributed to metal ions-chelating property of tea catechins as EDTA did.

Hydrogen peroxide generated during the oxidation of EGCg, is known to be responsible for several biological properties, such as bactericidal action and anti-tumor effect (Arakawa et al., 2004; Ranzato et al., 2012). Hydrogen peroxide has been shown to directly react with thiol groups in

bioactive peptides or proteins and lead to the transduction of cellular signals (Winterbourn and Hampton, 2008; Winterbourn and Metodiewa, 1999). Moreover, it has been found that hydrogen peroxide is able to oxidize the cysteine residues of certain enzymes causing reversible inactivation (Bogumil et al., 2000; Denu and Tanner, 1998). In the present study, I found that exogenous hydrogen peroxide had no effect on the enzymatic activity of rh-ACE (Figure 3.3B). Furthermore, the addition of hydrogen peroxide showed a slightly counteractive effect on the ACE inhibition by EGCg (Figure 3.3C). These data suggested that the inhibition of rh-ACE activity by EGCg might be independent of hydrogen peroxide-related mechanisms.

EGCg is extremely unstable in alkaline solutions and easily undergoes autoxidation. The generation of hydrogen peroxide provides evidence that EGCg has been autoxidized during the incubation in potassium phosphate buffer. Ascorbic acid is reported to stabilize EGCg and is able to reduce the oxidized EGCg (Chen et al., 1998; Sang et al., 2007). As shown in Figure 3.4A, ACE inhibitory effect of EGCg was weakened by the addition of ascorbic acid, suggesting that EGCg might inhibit the enzymatic activity of ACE through an autoxidation-dependent way. In recent years, accumulating evidence suggests that catechol type polyphenol has potential to covalently bind to thiol groups of proteins through autoxidation (Chen et al., 2011; Ishii et al., 2009; Ishii et al., 2008). EGCg has been reported to covalently bind to and inhibit several cellular key enzymes, such as catalase and fatty-acid synthase (Pal et al., 2014; Wang et al., 2003). Redox-cycling staining, which can specifically detect the presence of quinoproteins, provides evidence that EGCg covalently binds to ACE (Figure 3.4B).

To investigate the relation between the ACE inhibition and the covalent modification of ACE by EGCg, borate was used to form a stable complex with *ortho*-dihydroxyl groups in EGCg. As shown in Figure 3.5A, UV-vis absorption spectra support the complex formation between EGCg and borate. The formation of borate-EGCg complex diminished the ACE inhibitory activity of EGCg, and prevented EGCg to covalent bind to ACE (Figure 3.5B and 3.5C). These data indicate that the covalent modification of ACE by EGCG would lead to the enzyme inhibition. Additionally, the Lineweaver-Burk plot (Figure 3.6) reveals that EGCg is a non-competitive inhibitor of ACE, which is consistent with previous study. (Liu et al., 2003). Non-competitive inhibition means that

the ACE inhibitory activity of EGCg is not affected, whether a substrate molecule is bound to the active site of the enzyme or not. It also indicates that EGCg can bind to a specific site but not to the active site of ACE.

In conclusion, I provide evidence that the ACE inhibition by EGCg might be independent of Zn chelation and EGCg-induced hydrogen peroxide generation. Moreover, the study strongly suggested that EGCg allosterically inhibits the ACE activity through the oxidative conversion into an electrophilic quinone and subsequent binding to ACE. Further study is encouraged to explore the precise binding site of ACE by EGCg utilizing proteomic and reverse genetic approaches.

CHAPTER 4

Detection of ACE by a novel tag-free probe

4.1 Abstract

3,4-Dihydoxyphenylacetic acid (DOPAC), is one of the colonic microflora-produced catabolites of quercetin 4′-glucoside (Q4′G). It has been reported that the oxidation of DOPAC leads to the formation of *o*-quinone and the consequently covalent modification of proteins. In this study, the effect of DOPAC on ACE activity and the involvement of their interaction were investigated. Like other polyphenols, DOPAC inhibited the enzymatic activity of ACE in a dose-dependent manner. The inhibitory activity of DOPAC to inhibit ACE was quite similar to that of a tea catechin, EC. The covalent binding between DOPAC and ACE was confirmed by both redox-cycling staining and a tag-free DOPAC probe. These findings suggested that (1) the catechol structure might be associated with a potential ACE inhibitory activity; (2) ACE might be considered as one cellular target of DOPAC.

4.2 Introduction

Renin-angiotensin system (RAS) is one of the major control systems for blood pressure, fluid and electrolyte balance. Angiotensin converting enzyme (ACE), a dipeptidyl-carboxypeptidase, plays a crucial role in RAS by catalyzing the conversion of angiotensin I (Ang I) into vasoconstrictive angiotensin II (Ang II), and the metabolism of other peptides such as the vasodilator bradykinin. Inhibition of ACE activity is considered to be one of the effective strategies of the therapy of hypertension. Artificial ACE inhibitors, such as captopril and lisinopril, are the first-line drugs for the treatment of hypertension. Additionally, the ACE inhibitors are associated with the improvement of the cardiovascular disease, such as heart attack and stroke (Mancini et al., 1996; McFarlane et al., 2003; Wald and Law, 2003). However, certain side effects, including cough and angioneurotic edema, have been reported due to the administration of these synthetic ACE inhibitors (Israili and Hall, 1992a; Simon et al., 1992). Thus, there is an urgent requirement for the exploration and development of a new type of ACE inhibitor without adverse effects.

Recent years, it has been widely reported that the oxidation of dietary polyphenols with a catechol structure leads to the formation of a corresponding *o*-quinone, which electrophilically reacts with thiol groups in proteins to form covalent thiol-flavonoid adducts (Ishii et al., 2009; Mori et al., 2010). The covalent protein-binding ability of polyphenols is associated with the inhibition of several key cellular enzymes including fatty acid synthase, glyceradehyde-3-phosphate dehydrogenase (GAPDH), and catalase (Mori et al., 2010; Pal et al., 2014; Wang et al., 2003). Tea catechins, the major polyphenols in tea, are found to be potential ACE inhibitors (Persson et al., 2006). Our previous research indicated that the ACE inhibition by tea catechins might be attributed to the covalent modification of ACE by tea catechins. Additionally, galloylated catechins were found to be potent inhibitor of ACE. However, the role of catechol-like structures needs further investigation.

3,4-Dihydroxyphenylacetic acid (DOPAC), a catechol-like substance, is one of the colonic microflora-produced catabolites of quercetin 4′-glucoside (Q4′G). It has been reported that the catechol structure in DOPAC can be oxidized to *o*-quinone, then electrophilically reacts with the thiol groups in peptides or proteins (Ishii et al., 2009). Although the interaction between DOPAC and cellular proteins might be contributed to its biological activity, its targeted proteins have not been fully identified. A novel tag-free DOPAC probe, designed by Nakashima et al., was confirmed to be effective to investigate its molecular target for induction of several phase II enzymes (Nakashima et al., 2016).

In the present study, the ACE inhibitory activity of DOPAC was determined. I also confirmed that Zn chelation was not involved in the ACE inhibition by DOPAC. Redox-cycling staining was applied to detect the covalent binding of DOPAC to ACE. Finally, the efficiency of the tag-free DOPAC probe toward ACE was examined.

4.3 Materials and methods

4.3.1 Chemicals

ACE from rabbit lung, hippuryl-histidyl-leucine (HHL) and *o*-phthaldialdehyde (OPA),

DOPAC, tyrosinase from mushroom, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), tris[(1-benzyl-1H-1,2,3-triazol-4-yl)methyl]amine (TBTMA), bis(4-nitrophenyl) phosphate (BNPP) and azide-PEG3-biotin conjugate were purchased from Sigma (St. Louis, MO, USA). Nitro blue tetrazolium (NBT) and 2-Propyl-1-ol were from Tokyo Chemical Industry (Tokyo, Japan). Streptavidin, HRP conjugate was purchased from Funakoshi (Tokyo, Japan). Anti-actin antibody and horseradish peroxidase-linked anti mouse IgG were purchased from Santa Cruz Biotechnology (Santa Cruz, USA). All other chemicals were obtained from Nacalai Tesque (Kyoto, Japan). All other chemicals such as benzyl azide were purchased from Wako Pure Chemical Industries (Osaka, Japan).

4.3.2 ACE activity 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 μl at 37ºC for 30 min. In an inhibitory assay, ACE was pre-incubated with DOPAC at 4ºC for 15 min before the addition of substrate. Enzymatic reaction was terminated by the addition of 1.2 ml of 0.34 M NaOH, followed by a 10-min incubation at room temperature with 100 μl of OPA (20 mg/ml). The solution was acidified with 200 μl of 3 N HCl and centrifuged at 3,000 × g at 4ºC 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.

4.3.3 Effects of Zn^{2+} on the ACE inhibitory activity of DOPAC

The effect of Zn^{2+} on the ACE inhibitory activity of DOPAC was investigated by measuring the enzymatic activity in the presence of 400 μM DOPAC supplemented with different concentrations (0, 200, 400, and 800 μM) of $ZnSO_4$. After 15 min incubation at 4ºC, the ACE activity assay was initiated by the addition of ACE and HHL to each group.

4.3.4 SDS-PAGE and redox-cycling staining

ACE (15 mU) was incubated with 2 mM (or 4 mM) of DOPAC in 50 mM potassium phosphate buffer (pH 7.4) at 37ºC for 60 min. The reaction between tea catechins and ACE was terminated by the addition of DTT (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 μm, Millipores), and then DOPAC-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 in dark, resulting in a blue-purple stain of quinoproteins bands and no staining of other proteins. The membrane was also stained with Coomassie Brilliant Blue G-250 (CBB) for checking the protein loading.

4.3.5 Synthesis of DPE via Fisher esterification

DPE was synthesized as previously reported (Nakashima et al., 2016). Briefly, DOPAC (30 mg, 0.18 mmol) and PTSA (6 mg, 0.03 mmol) were dissolved in the solution of dehydrated toluene (20 ml) and 2-propyl-1-ol (1 ml, 17 mmol), and stirred for 6 h at 40ºC, after cooling to room temperature. The reaction mixture was washed with 5% $NaHCO_3$ solution and water twice. The aqueous phase was extracted with 20 ml ethyl acetate and combined with the organic phase. The combined extract was dried over anhydrous $MgSO_4$, filtered, and evaporated to dryness. The product was purified by preparative thin-layer chromatography ($CHCl_3$-MeOH (9:1)) to afford 0.34 mg (0.07 mmol) of 3,4-dihydroxyphenylacetic acid propargyl ester (DPE). Its structure and purity was confirmed by NMR and mass spectrometry.

4.3.6 Click chemistry (CuAAC reaction)

CuAAC reaction with DPE and benzyl azide was performed as previously reported (Nakashima et al., 2016). ACE was incubated with of 100 μM DPE in 50 mM potassium phosphate buffer (pH 7.4) at 37ºC for 1 h. Subsequently, the solution was mixed with 1 mM copper (II) sulphate pentahydrate, 1 mM sodium ascorbate, 0.1 mM TBTMA, and 20 μM azide-PEG3-biotin. After incubation in the dark for 1 h at room temperature, the protein samples were used to subject to SDS-

PAGE/blotting followed by the treatment with HRP-conjugated streptavidin for 1 h. The generated streptavidin-biotin complex was visualized by Chemi-Lumi One Super.

4.3.7 Statistical analysis

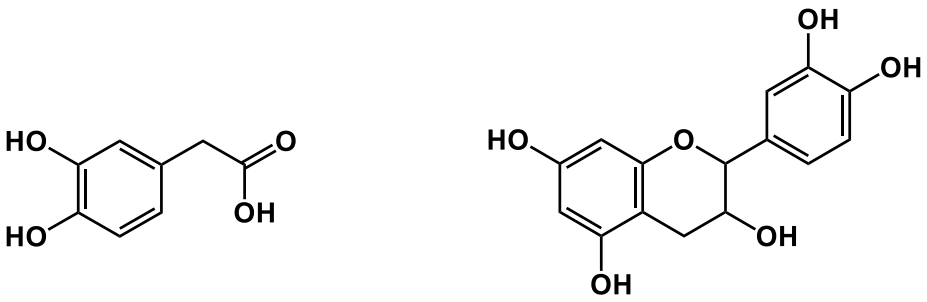
All results are expressed as means ± SD of more than three independent experiments.

4.4 Results

4.4.1 Inhibitory effect of DOPAC on ACE activity.

To investigate the ACE inhibitory effect of DOPAC, purified ACE (from rabbit lung) was incubated with various concentrations of DOPAC (0, 50, 100, 200, and 400 μM) at 4ºC for 15 min and then the ACE activity of each group was measured. As shown in Figure 4.1C, ACE activity was dose-dependently inhibited by DOPAC. DOPAC also showed a similar ACE inhibitory activity to that of a tea catechin, EC.

(A) **(B)**



(C)

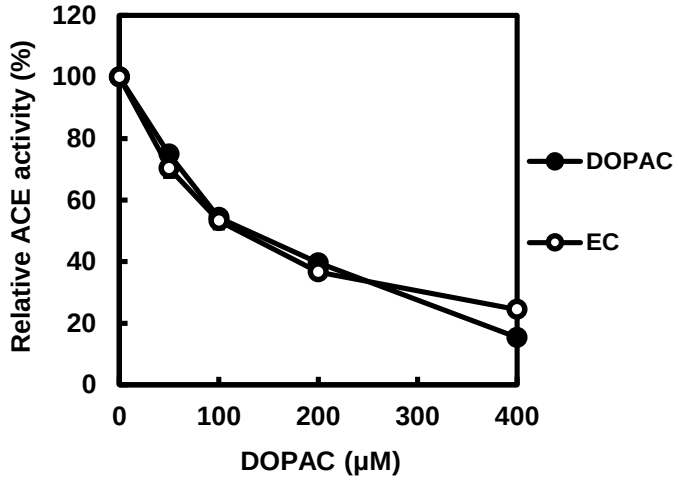


Figure 4.1 Inhibitory effect of DOPAC on the enzymatic activity of ACE. Chemical structures of DOPAC (A) and EC (B). (C) The enzymatic activity of purified ACE (from rabbit lung) was measured in the presence of DOPAC (closed circle) or EC (open circle) at indicated concentrations. The relative values represent means ± SD of three independent experiments.

4.4.2 Effect of Zn^{2+} on the ACE inhibitory activity of DOPAC

To determine whether ACE inhibition by DOPAC is due to its zinc-chelating effect, ACE inhibitory activity of DOPAC (400 μM) was measured in the presence of different concentrations of $ZnSO_4$ (0, 200, 400, and 800 μM). As shown in Figure 4.2, no significant changes in the ACE inhibitory activity of DOPAC had been observed when 200, 400, and 800 μM of Zn^{2+} was supplemented. These results indicated that ACE inhibition by DOPAC might not be attributed to its zinc-chelating property.

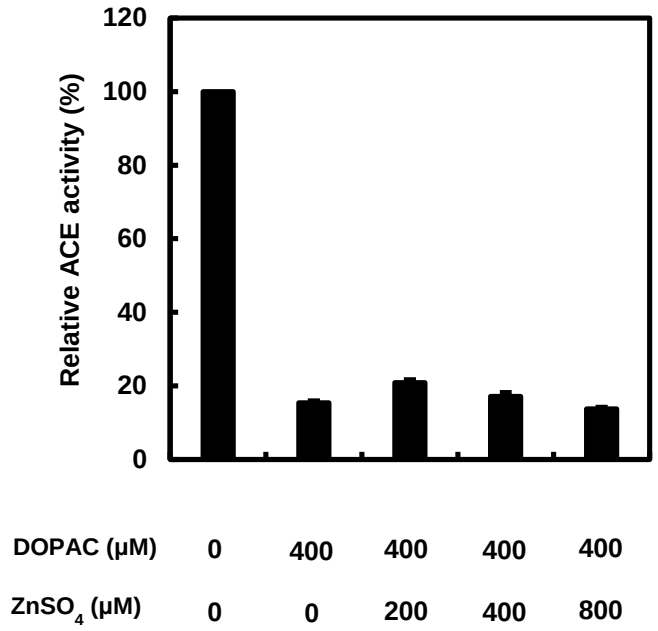


Figure 4.2 Effect of Zn^{2+} on the ACE inhibitory activity of DOPAC. The ACE inhibitory activity of 400 μM DOPAC was measured in the presence or absence of $ZnSO_4$. The relative values represent means ± SD of three independent experiments.

4.4.3 Detection of DOPAC-bound ACE by SDS-PAGE/blotting with redox-cycling staining

To examine whether DOPAC could covalently bind to ACE, ACE was co-incubated with 2 mM (or 4 mM) of DOPAC in potassium phosphate buffer (pH 7.4) at 37ºC for 60 min, then DOPAC-modified ACE was detected by SDS-PAGE/blotting with redox-cycling staining. As shown in Figure 4.3, the DOPAC-treated ACE generated positive bands around 50 kDa, indicating that DOPAC covalently binds to ACE.

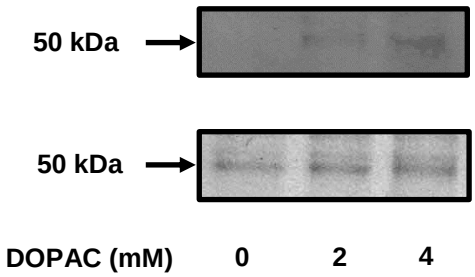


Figure 4.3 Covalent modification of ACE by DOPAC. ACE (15 mU) was treated with different concentrations of DOPAC (0, 2 or 4 mM) at 37ºC for 60 min. The DOPAC-modified ACE was detected by a redox-cycling staining (above) and CBB staining (below), respectively.

4.4.4 Detection of ACE by click chemistry

Redox-cycling staining suggested that DOPAC can covalently bind to ACE. Therefore, to get further evidence for covalent binding of DOPAC with ACE, a tag-free DOPAC probe with the azide labeled biotin and a horseradish peroxidase (HRP)-streptavidin complex was used. DPE was incubated with ACE in the presence of laccase, followed by the CuAAC reaction with the azide-labelled biotin and identification using the HRP-conjugated streptavidin. Several positive bands were observed in the DPE-treated groups, whereas no bands had been detected in the absence of DPE (Figure 4.4). This result further supported the idea that DPE can covalently bind to ACE. It also suggested that the tag-free probe using click chemistry is extremely effective for the detection of the covalently modified ACE.

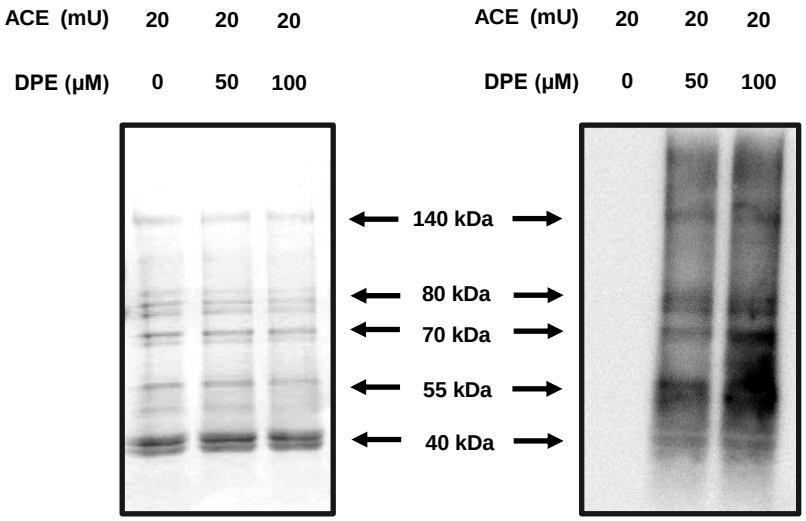


Figure 4.4 Detection of ACE by click chemistry. Detection of ACE tagged by CuAAC reaction with DPE and the azide-labelled biotin. Purified ACE (20 mU) was incubated with or without DPE in the presence of 30 U tyrosinase in 50 mM potassium phosphate buffer (pH 7.4) for 1 h at 37ºC. DPE-tagged ACE was detected by CBB staining (left panel) and HRP-streptavidin (right panel).

4.5 Discussion

Flavonoids are polyphenolic compounds which are widely distributed in plants, such as fruits, vegetables, tea, and wine (Harnly et al., 2006). Accumulating evidence suggests that certain flavonoids are able to inhibit ACE activity, which plays a crucial role in the regulation of blood pressure (Kameda et al., 1987; Loizzo et al., 2007; Oh et al., 2004). Structure-activity relationship studies have found that the catechol group in flavonoids is important for the extent of ACE inhibition (Guerrero et al., 2012). DOPAC (Figure 4.1A), one of the colonic microflora-produced catabolites of quercetin 4'-glucoside, exhibited inhibitory effect on the ACE activity (Figure 4.1C). Additionally, the ACE inhibitory potency of DOPAC was extremely similar as that of EC. It indicates that the catechol group presented in DOPAC or EC might contribute, at least in part, to their ACE inhibitory activity. Here, I speculated that both DOPAC and EC would inhibit ACE through a similar mechanism.

ACE is a zinc-dependent metalloproteinase and has an absolute zinc requirement for its enzymatic activity (Bünning and Riordan, 1981; Reeves and O'Dell, 1985). Numerous studies suggest that certain health-promoting effects of flavonoids, including anti-oxidation, anti-Alzheimer's activity, might be ascribed to their metal ion chelating properties (Fernandez et al., 2002; Mira et al., 2002). It has been revealed that the metal chelating ability of flavonoids depends largely on the number of hydroxyl groups associated to the aromatic ring (Andjelković et al., 2006; Khokhar and Owusu Apenten, 2003). Thus, zinc-chelating property of catechol or galloyl group was usually proposed to explain the ACE inhibition by certain flavonoids (Guerrero et al., 2012). However, ACE inhibition by DOPAC was independent of zinc chelation (Figure 4.2). In addition, my previous work shows that ACE inhibitory activity of EGCg might not due to zinc chelation. These findings suggested that although the presence of catechol or galloyl group might be associated with the ACE inhibition by certain polyphenols, their metal-chelating properties might be ruled out in the mechanism for the ACE inhibition.

Because of the presence of catechol structure, DOPAC is oxidized to form *o*-quinone, which is able to covalently bind to thiol groups in GSH or proteins (Ishii et al., 2009). This covalent modification of protein is usually associated with the protein function change. To confirm whether

ACE inhibition by DOPAC is due to the covalent binding, redox-cycling staining was applied. The result indicated that DOPAC could covalently bind to ACE (Figure 4.3). These findings suggested that DOPAC was initially autoxidized into its quinone form, then the corresponding quinone electrophilically attacked the thiol groups of ACE. The results also indicated that ACE might be a potential intracellular target of DOPAC.

In conclusion, it is interesting to note that both DOPAC and tea catechins could covalently bind to ACE, and inhibit the enzymatic activity. It might suggest that the presence of catechol (or pyrogallol) group could inhibit the ACE activity through oxidative conversion into an electrophilic quinone and subsequent binding to ACE. Further study is needed to gain structural insight into the autoxidation-dependent interaction between catechol (or pyrogallol) group and ACE.

SUMMARY

Renin-angiotensin system (RAS) is one of the major control systems for blood pressure, fluid and electrolyte balance. Angiotensin converting enzyme (ACE) plays a crucial role in the RAS by converting angiotensin I to angiotensin II, a potent vasoconstrictor. The inhibition of ACE is one of the most promising strategies for treatment of hypertension. Synthetic ACE inhibitors, such as captopril and enalapril, are also used as the first-line drugs for the therapy of cardiovascular diseases. However, these artificial ACE inhibitors are believed to have certain side effects such as cough, skin rashes, and angioneurotic edema. Thus, there is an urgent requirement for the exploration and development of a new type of ACE inhibitors without adverse effects. Accumulating epidemiological and intervention studies indicated that green tea consumption is inversely associated with the risk of cardiovascular disease, which might be largely attributed to the presence of polyphenols. Tea catechins, including (-)-epicatechin (EC), (-)-epigallocatechin (EGC), (-)-epicatechin gallate (ECg) and (-)-epigallocatechin gallate (EGCg), are the primary class of polyphenols in green tea. Although green tea and tea catechins has been shown to be promising as potential ACE inhibitors, the precise mechanisms underlying ACE inhibition by catechins remain to be clarified. In this thesis, the molecular mechanisms involved in the ACE inhibition by tea catechins are investigated.

In Chapter 2, the inhibitory effects of four tea catechins, including (-)-epicatechin (EC), (-)-epigallocatechin (EGC), (-)-epicatechin gallate (ECg) and (-)-epigallocatechin gallate (EGCg), on the enzymatic activity of ACE (purified from rabbit lung) were compared. 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 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 hydrophobic and hydrogen-bonding interaction of the galloyl moiety with the non-catalytic site of ACE.

In Chapter 3, the molecular mechanisms involved in the ACE inhibition by EGCg, a major tea catechin, were further investigated. The enzyme activity of recombinant human ACE was inhibited by EGCg in a dose-dependent manner. Co-incubation with Zn^{2+} ion showed no influence on the ACE inhibition by EGCg, whereas it completely counteracted the inhibitory effect of EDTA, a representative ACE inhibitor chelating Zn^{2+} at the active site of ACE. Although a considerable amount of hydrogen peroxide was produced during the incubation of EGCg, the treatment of ACE with hydrogen peroxide showed little effect on its enzymatic activity. On the other hand, the co-incubation of EGCg with inhibitors of catechol oxidation, such as borate or ascorbic acid, significantly diminished the EGCg inhibition. A redox-cycling staining experiment revealed that ACE was covalently modified by EGCg. Furthermore, a Lineweaver-Burk plot analysis indicated that EGCg also inhibited the ACE activity in a non-competitive manner. These results strongly suggested that EGCg allosterically inhibits the ACE activity through the oxidative conversion into an electrophilic quinone and subsequent binding to the ACE.

3,4-Dihydoxyphenylacetic acid (DOPAC), having a catechol group, is one of the colonic microflora-produced catabolites of quercetin 4´-glucoside. A previous study indicated that DOPAC is oxidized to form *o*-quinone, then covalently binds to sulfhydryls in GSH or proteins due to its catechol structure. In Chapter 4, the modulating effect of DOPAC on ACE activity was also studied. The co-incubation of DOPAC significantly inhibited the enzymatic activity of ACE in a dose-dependent manner. Among the four tea catechins, DOPAC has the similar potency to inhibit ACE to that of EC. In addition, the covalent binding of DOPAC with ACE was detected by a tag-free DOPAC probe with the azide labeled biotin and a horseradish peroxidase (HRP)-streptavidin complex. These findings suggested that (1) the catechol structure and its oxidative conversion into electrophilic species are involved in the potential ACE inhibitory activity; (2) ACE is one of the potential cellular targets of DOPAC.

In conclusion, this study indicates that: (1) tea catechins and DOPAC, both of which are derived from food stuffs, are potential ACE inhibitors; (2) Zn^{2+} chelation as well as hydrogen peroxide-dependent mechanism are ruled out in the mechanisms of the ACE inhibition by tea catechins; (3) the galloylated catechins (ECg and EGCg) allosterically inhibit the ACE activity through the

oxidative conversion into an electrophilic quinone and subsequent binding to the ACE; (4) the presence of galloyl moiety might play an important role in the hydrophobic and hydrogen-bonding interaction with the non-catalytic site of ACE, associated with more potent ACE inhibitory activity. 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. It is likely that basic knowledge of the ACE-binding site of tea catechins will provide a platform for the rational design of new domain-selective ACE inhibitors with improved efficacy and pharmacological profiles. Taken together, this study suggests that the galloylated catechins could be potential candidates to develop nutraceuticals against hypertension and its related disease.

ACKNOWLEDGEMENTS

Three years ago, I was extremely grateful and honored that Professor Yoshimasa Nakamura provided me the opportunity to come to Japan and study as a doctoral student in his lab. I would like to express the deepest appreciation to him for his professional advice, valuable suggestions and continuous encouragement I received throughout the course of this study. Without his guidance and persistent help, it would not have been possible to finish this dissertation.

I would like to thank Professor Yoshiyuki Murata for his amazing ideas and academic suggestions. Special thanks to him for all the efforts he did throughout my research.

Thanks go to Shintaro Munemasa and Toshiyuki Nakamura for their valuable suggestions and meaningful academic discussions.

Special thanks go to Yue Tang, Yinye Li, Wenxiu Ye, Xiaoyang Liu, Yujia Liu, Ying Liang, Wensi Xu, Qifu Yang, as well as other foreign students in this lab. Their company and supporting makes me feel less homesick and more concentrated on my research.

Thanks go to all the Japanese students here for their sincere help no matter in my research work or in my daily life. I really feel appreciated!

Finally, I would love to give my special thanks to my wonderful parents for their love and continuous support. I hope I can make my parents proud of me.

REFERENCES

Actis-Goretta, L., J.I. Ottaviani, and C.G. Fraga. 2006a. Inhibition of angiotensin converting enzyme activity by flavanol-rich foods. *J Agric Food Chem*. 54:229-234.

Actis-Goretta, L., J.I. Ottaviani, and C.G. Fraga. 2006b. Inhibition of Angiotensin Converting Enzyme Activity by Flavanol-Rich Foods. *Journal of Agricultural and Food Chemistry*. 54:229-234.

Andjelković, M., J. Van Camp, B. De Meulenaer, G. Depaemelaere, C. Socaciu, M. Verloo, and R. Verhe. 2006. Iron-chelation properties of phenolic acids bearing catechol and galloyl groups. *Food Chemistry*. 98:23-31.

Arab, L., F. Khan, and H. Lam. 2013. Tea consumption and cardiovascular disease risk. *Am J Clin Nutr*. 98:1651S-1659S.

Arakawa, H., M. Maeda, S. Okubo, and T. Shimamura. 2004. Role of hydrogen peroxide in bactericidal action of catechin. *Biol Pharm Bull*. 27:277-281.

Araujo, M.C., R.L. Melo, M.H. Cesari, M.A. Juliano, L. Juliano, and A.K. Carmona. 2000. Peptidase specificity characterization of C- and N-terminal catalytic sites of angiotensin I-converting enzyme. *Biochemistry*. 39:8519-8525.

Arts, I.C., P.C. Hollman, E.J. Feskens, H.B. Bueno de Mesquita, and D. Kromhout. 2001. Catechin intake might explain the inverse relation between tea consumption and ischemic heart disease: the Zutphen Elderly Study. *Am J Clin Nutr*. 74:227-232.

Azam, S., N. Hadi, N.U. Khan, and S.M. Hadi. 2004. Prooxidant property of green tea polyphenols epicatechin and epigallocatechin-3-gallate: implications for anticancer properties. *Toxicol In Vitro*. 18:555-561.

Bünning, P., and J.F. Riordan. 1981. The Role of Zinc in Angiotensin Converting Enzyme. *Israel Journal of Chemistry*. 21:43-47.

Babu, P.V., and D. Liu. 2008. Green tea catechins and cardiovascular health: an update. *Curr Med Chem*. 15:1840-1850.

Bae, M.J., T. Ishii, K. Minoda, Y. Kawada, T. Ichikawa, T. Mori, M. Kamihira, and T. Nakayama. 2009. Albumin stabilizes (-)-epigallocatechin gallate in human serum: binding capacity and antioxidant property. *Mol Nutr Food Res*. 53:709-715.

Bernstein, K.E., F.S. Ong, W.L. Blackwell, K.H. Shah, J.F. Giani, R.A. Gonzalez-Villalobos, X.Z. Shen,

S. Fuchs, and R.M. Touyz. 2013. A modern understanding of the traditional and nontraditional biological functions of angiotensin-converting enzyme. *Pharmacol Rev*. 65:1-46.

Bogumil, R., D. Namgaladze, D. Schaarschmidt, T. Schmachtel, S. Hellstern, R. Mutzel, and V. Ullrich. 2000. Inactivation of calcineurin by hydrogen peroxide and phenylarsine oxide. Evidence for a dithiol-disulfide equilibrium and implications for redox regulation. *Eur J Biochem*. 267:1407-1415.

Bruneval, P., N. Hinglais, F. Alhenc-Gelas, V. Tricottet, P. Corvol, J. Menard, J.P. Camilleri, and J. Bariety. 1986. Angiotensin I converting enzyme in human intestine and kidney. *Histochemistry*. 85:73-80.

Buttery, J.E., and S. Stuart. 1993. Studies on zinc and angiotensin-converting enzyme. *Clin Chim Acta*. 217:213-216.

Caldwell, P.R., B.C. Seegal, K.C. Hsu, M. Das, and R.L. Soffer. 1976. Angiotensin-converting enzyme: vascular endothelial localization. *Science*. 191:1050-1051.

Chen, R., J.B. Wang, X.Q. Zhang, J. Ren, and C.M. Zeng. 2011. Green tea polyphenol epigallocatechin-3-gallate (EGCG) induced intermolecular cross-linking of membrane proteins. *Arch Biochem Biophys*. 507:343-349.

Chen, Z.-Y., Q.Y. Zhu, Y.F. Wong, Z. Zhang, and H.Y. Chung. 1998. Stabilizing Effect of Ascorbic Acid on Green Tea Catechins. *Journal of Agricultural and Food Chemistry*. 46:2512-2516.

Coppola, G., G. Romano, E. Corrado, R.M. Grisanti, and S. Novo. 2008. Peripheral artery disease: potential role of ACE-inhibitor therapy. *Vasc Health Risk Manag*. 4:1179-1187.

Davies, M.J., J.T. Judd, D.J. Baer, B.A. Clevidence, D.R. Paul, A.J. Edwards, S.A. Wiseman, R.A. Muesing, and S.C. Chen. 2003. Black tea consumption reduces total and LDL cholesterol in mildly hypercholesterolemic adults. *J Nutr*. 133:3298S-3302S.

Defending, R., E.A. Zimmerman, J.A. Weare, F. Alhenc-Gelas, and E.G. Erdös. 1983. Angiotensin-Converting Enzyme in Epithelial and Neuroepithelial Cells. *Neuroendocrinology*. 37:32-40.

Denu, J.M., and K.G. Tanner. 1998. Specific and reversible inactivation of protein tyrosine phosphatases by hydrogen peroxide: evidence for a sulfenic acid intermediate and implications for redox regulation. *Biochemistry*. 37:5633-5642.

Dou, J., V.S. Lee, J.T. Tzen, and M.R. Lee. 2007. Identification and comparison of phenolic compounds in the preparation of oolong tea manufactured by semifermentation and drying processes. *J Agric Food Chem*. 55:7462-7468.

Dragsted. 2003. Antioxidant Actions of Polyphenols in Humans. *International Journal for Vitamin and*

Nutrition Research. 73:112-119.

Duan, X., F. Wu, M. Li, N. Yang, C. Wu, Y. Jin, J. Yang, Z. Jin, and X. Xu. 2014. Naturally occurring angiotensin I-converting enzyme inhibitory peptide from a fertilized egg and its inhibitory mechanism. *J Agric Food Chem*. 62:5500-5506.

Ehlers, M.R., E.A. Fox, D.J. Strydom, and J.F. Riordan. 1989. Molecular cloning of human testicular angiotensin-converting enzyme: the testis isozyme is identical to the C-terminal half of endothelial angiotensin-converting enzyme. *Proc Natl Acad Sci U S A*. 86:7741-7745.

Esparza, I., Í. Salinas, C. Santamaría, J.M. García-Mina, and J.M. Fernández. 2005. Electrochemical and theoretical complexation studies for Zn and Cu with individual polyphenols. *Analytica Chimica Acta*. 543:267-274.

Fernandez, M.T., M.L. Mira, M.H. Florencio, and K.R. Jennings. 2002. Iron and copper chelation by flavonoids: an electrospray mass spectrometry study. *J Inorg Biochem*. 92:105-111.

Gentilucci, L., A. Tolomelli, R. De Marco, and R. Artali. 2012. Molecular docking of opiates and opioid peptides, a tool for the design of selective agonists and antagonists, and for the investigation of atypical ligand-receptor interactions. *Curr Med Chem*. 19:1587-1601.

Ghosh, K.S., T.K. Maiti, A. Mandal, and S. Dasgupta. 2006. Copper complexes of (-)-epicatechin gallate and (-)-epigallocatechin gallate act as inhibitors of Ribonuclease A. *FEBS Lett*. 580:4703-4708.

Guerrero, L., J. Castillo, M. Quinones, S. Garcia-Vallve, L. Arola, G. Pujadas, and B. Muguerza. 2012. Inhibition of angiotensin-converting enzyme activity by flavonoids: structure-activity relationship studies. *PLoS One*. 7:e49493.

Hagaman, J.R., J.S. Moyer, E.S. Bachman, M. Sibony, P.L. Magyar, J.E. Welch, O. Smithies, J.H. Krege, and D.A. O'Brien. 1998. Angiotensin-converting enzyme and male fertility. *Proc Natl Acad Sci U S A*. 95:2552-2557.

Hanssens, M., M.J. Keirse, F. Vankelecom, and F.A. Van Assche. 1991. Fetal and neonatal effects of treatment with angiotensin-converting enzyme inhibitors in pregnancy. *Obstetrics & Gynecology*. 78:128-135.

Harnly, J.M., R.F. Doherty, G.R. Beecher, J.M. Holden, D.B. Haytowitz, S. Bhagwat, and S. Gebhardt. 2006. Flavonoid content of U.S. fruits, vegetables, and nuts. *J Agric Food Chem*. 54:9966-9977.

Hashimoto, T., S. Kumazawa, F. Nanjo, Y. Hara, and T. Nakayama. 1999. Interaction of tea catechins with lipid bilayers investigated with liposome systems. *Biosci Biotechnol Biochem*. 63:2252-2255.

Hayakawa, F., T. Kimura, N. Hoshino, and T. Ando. 1999. DNA cleavage activities of (-)-epigallocatechin, (-)-epicatechin, (+)-catechin, and (-)-epigallocatechin gallate with various kinds of metal ions. *Biosci Biotechnol Biochem*. 63:1654-1656.

Hertog, M.G.L., P.C.H. Hollman, and B. van de Putte. 1993. Content of potentially anticarcinogenic flavonoids of tea infusions, wines, and fruit juices. *Journal of Agricultural and Food Chemistry*. 41:1242-1246.

Higdon, J.V., and B. Frei. 2003. Tea catechins and polyphenols: health effects, metabolism, and antioxidant functions. *Crit Rev Food Sci Nutr*. 43:89-143.

Ishii, T., M. Ishikawa, N. Miyoshi, M. Yasunaga, M. Akagawa, K. Uchida, and Y. Nakamura. 2009. Catechol type polyphenol is a potential modifier of protein sulfhydryls: development and application of a new probe for understanding the dietary polyphenol actions. *Chem Res Toxicol*. 22:1689-1698.

Ishii, T., T. Mori, T. Tanaka, D. Mizuno, R. Yamaji, S. Kumazawa, T. Nakayama, and M. Akagawa. 2008. Covalent modification of proteins by green tea polyphenol (-)-epigallocatechin-3-gallate through autoxidation. *Free Radic Biol Med*. 45:1384-1394.

Israili, Z.H., and W.D. Hall. 1992a. Cough and angioneurotic edema associated with angiotensin-converting enzyme inhibitor therapy. A review of the literature and pathophysiology. *Ann Intern Med*. 117:234-242.

Israili, Z.H., and W.D. Hall. 1992b. Cough and Angioneurotic Edema Associated with Angiotensin-Converting Enzyme Inhibitor TherapyA Review of the Literature and Pathophysiology. *Annals of Internal Medicine*. 117:234-242.

Jung, Y.D., and L.M. Ellis. 2001. Inhibition of tumour invasion and angiogenesis by epigallocatechin gallate (EGCG), a major component of green tea. *Int J Exp Pathol*. 82:309-316.

Kajimoto, O., Y. Kajimoto, M. Yabune, A. Nozawa, K. Nagata, and T. Kakuda. 2003. Tea Catechins Reduce Serum Cholesterol Levels in Mild and Borderline Hypercholesterolemia Patients. *Journal of Clinical Biochemistry and Nutrition*. 33:101-111.

Kakuda, T. 2002. Neuroprotective effects of the green tea components theanine and catechins. *Biol Pharm Bull*. 25:1513-1518.

Kameda, K., T. Takaku, H. Okuda, Y. Kimura, T. Okuda, T. Hatano, I. Agata, and S. Arichi. 1987. Inhibitory effects of various flavonoids isolated from leaves of persimmon on angiotensin-converting enzyme activity. *J Nat Prod*. 50:680-683.

Kannel, W.B. 1996. Blood pressure as a cardiovascular risk factor: prevention and treatment. *JAMA*. 275:1571-1576.

Kasahara, Y., and Y. Ashihara. 1981. Colorimetry of angiotensin-I converting enzyme activity in serum. *Clin Chem*. 27:1922-1925.

Khalesi, S., J. Sun, N. Buys, A. Jamshidi, E. Nikbakht-Nasrabadi, and H. Khosravi-Boroujeni. 2014. Green tea catechins and blood pressure: a systematic review and meta-analysis of randomised controlled trials. *Eur J Nutr*. 53:1299-1311.

Khan, N., F. Afaq, M. Saleem, N. Ahmad, and H. Mukhtar. 2006. Targeting multiple signaling pathways by green tea polyphenol (-)-epigallocatechin-3-gallate. *Cancer Res*. 66:2500-2505.

Khokhar, S., and R.K. Owusu Apenten. 2003. Iron binding characteristics of phenolic compounds: some tentative structure–activity relations. *Food Chemistry*. 81:133-140.

Kuriyama, S. 2008. The relation between green tea consumption and cardiovascular disease as evidenced by epidemiological studies. *J Nutr*. 138:1548S-1553S.

Kuriyama, S., T. Shimazu, K. Ohmori, N. Kikuchi, N. Nakaya, Y. Nishino, Y. Tsubono, and I. Tsuji. 2006. Green tea consumption and mortality due to cardiovascular disease, cancer, and all causes in Japan: the Ohsaki study. *JAMA*. 296:1255-1265.

Lawes, C.M., S. Vander Hoorn, A. Rodgers, and H. International Society of. 2008. Global burden of blood-pressure-related disease, 2001. *Lancet*. 371:1513-1518.

Liang, W., A.H. Lee, C.W. Binns, R. Huang, D. Hu, and Q. Zhou. 2009. Tea consumption and ischemic stroke risk: a case-control study in southern China. *Stroke*. 40:2480-2485.

Lin, Y.L., S.H. Tsai, S.Y. Lin-Shiau, C.T. Ho, and J.K. Lin. 1999. Theaflavin-3,3'-digallate from black tea blocks the nitric oxide synthase by down-regulating the activation of NF-kappaB in macrophages. *Eur J Pharmacol*. 367:379-388.

Liu, J.-C., F.-L. Hsu, J.-C. Tsai, P. Chan, J.Y.-H. Liu, G.N. Thomas, B. Tomlinson, M.-Y. Lo, and J.-Y. Lin. 2003. Antihypertensive effects of tannins isolated from traditional Chinese herbs as non-specific inhibitors of angiontensin converting enzyme. *Life Sciences*. 73:1543-1555.

Loizzo, M.R., A. Said, R. Tundis, K. Rashed, G.A. Statti, A. Hufner, and F. Menichini. 2007. Inhibition of angiotensin converting enzyme (ACE) by flavonoids isolated from Ailanthus excelsa (Roxb) (Simaroubaceae). *Phytother Res*. 21:32-36.

Mancini, G.B., G.C. Henry, C. Macaya, B.J. O'Neill, A.L. Pucillo, R.G. Carere, T.J. Wargovich, H. Mudra,

T.F. Luscher, M.I. Klibaner, H.E. Haber, A.C. Uprichard, C.J. Pepine, and B. Pitt. 1996. Angiotensin-converting enzyme inhibition with quinapril improves endothelial vasomotor dysfunction in patients with coronary artery disease. The TREND (Trial on Reversing ENdothelial Dysfunction) Study. *Circulation*. 94:258-265.

McFarlane, S.I., A. Kumar, and J.R. Sowers. 2003. Mechanisms by which angiotensin-converting enzyme inhibitors prevent diabetes and cardiovascular disease. *Am J Cardiol*. 91:30H-37H.

Menet, M.C., S. Sang, C.S. Yang, C.T. Ho, and R.T. Rosen. 2004. Analysis of theaflavins and thearubigins from black tea extract by MALDI-TOF mass spectrometry. *J Agric Food Chem*. 52:2455-2461.

Mira, L., M.T. Fernandez, M. Santos, R. Rocha, M.H. Florencio, and K.R. Jennings. 2002. Interactions of flavonoids with iron and copper ions: a mechanism for their antioxidant activity. *Free Radic Res*. 36:1199-1208.

Mochizuki, M., S. Yamazaki, K. Kano, and T. Ikeda. 2002. Kinetic analysis and mechanistic aspects of autoxidation of catechins. *Biochim Biophys Acta*. 1569:35-44.

Mori, T., T. Ishii, M. Akagawa, Y. Nakamura, and T. Nakayama. 2010. Covalent binding of tea catechins to protein thiols: the relationship between stability and electrophilic reactivity. *Biosci Biotechnol Biochem*. 74:2451-2456.

Mujib, M., K. Patel, G.C. Fonarow, D.W. Kitzman, Y. Zhang, I.B. Aban, O.J. Ekundayo, T.E. Love, M.L. Kilgore, R.M. Allman, M. Gheorghiade, and A. Ahmed. 2013. Angiotensin-converting enzyme inhibitors and outcomes in heart failure and preserved ejection fraction. *Am J Med*. 126:401-410.

Nakachi, K., S. Matsuyama, S. Miyake, M. Suganuma, and K. Imai. 2000. Preventive effects of drinking green tea on cancer and cardiovascular disease: epidemiological evidence for multiple targeting prevention. *Biofactors*. 13:49-54.

Nakagawa, H., K. Hasumi, J.T. Woo, K. Nagai, and M. Wachi. 2004. Generation of hydrogen peroxide primarily contributes to the induction of Fe(II)-dependent apoptosis in Jurkat cells by (-)-epigallocatechin gallate. *Carcinogenesis*. 25:1567-1574.

Nakamura, Y., and N. Miyoshi. 2010. Electrophiles in foods: the current status of isothiocyanates and their chemical biology. *Biosci Biotechnol Biochem*. 74:242-255.

Nakashima, S., Z. Liu, Y. Yamaguchi, S. Saiki, S. Munemasa, T. Nakamura, Y. Murata, and Y. Nakamura. 2016. A novel tag-free probe for targeting molecules interacting with a flavonoid catabolite. *Biochemistry*

and Biophysics Reports. 7:240-245.

Nakayama, T., M. Ichiba, M. Kuwabara, K. Kajiya, and S. Kumazawa. 2002. Mechanisms and Structural Specificity of Hydrogen Peroxide Formation during Oxidation of Catechins. *Food Science and Technology Research*. 8:261-267.

Natesh, R., S.L. Schwager, H.R. Evans, E.D. Sturrock, and K.R. Acharya. 2004. Structural details on the binding of antihypertensive drugs captopril and enalaprilat to human testicular angiotensin I-converting enzyme. *Biochemistry*. 43:8718-8724.

Natesh, R., S.L. Schwager, E.D. Sturrock, and K.R. Acharya. 2003. Crystal structure of the human angiotensin-converting enzyme-lisinopril complex. *Nature*. 421:551-554.

Negishi, H., J.W. Xu, K. Ikeda, M. Njelekela, Y. Nara, and Y. Yamori. 2004. Black and green tea polyphenols attenuate blood pressure increases in stroke-prone spontaneously hypertensive rats. *J Nutr*. 134:38-42.

Ni, H., L. Li, G. Liu, and S.Q. Hu. 2012. Inhibition mechanism and model of an angiotensin I-converting enzyme (ACE)-inhibitory hexapeptide from yeast (Saccharomyces cerevisiae). *PLoS One*. 7:e37077.

Obanda, M., P. Okinda Owuor, and R. Mang'oka. 2001. Changes in the chemical and sensory quality parameters of black tea due to variations of fermentation time and temperature. *Food Chemistry*. 75:395-404.

Oh, H., D.G. Kang, J.W. Kwon, T.O. Kwon, S.Y. Lee, D.B. Lee, and H.S. Lee. 2004. Isolation of angiotensin converting enzyme (ACE) inhibitory flavonoids from Sedum sarmentosum. *Biol Pharm Bull*. 27:2035-2037.

Pal, S., S.K. Dey, and C. Saha. 2014. Inhibition of catalase by tea catechins in free and cellular state: a biophysical approach. *PLoS One*. 9:e102460.

Peng, X., R. Zhou, B. Wang, X. Yu, X. Yang, K. Liu, and M. Mi. 2014. Effect of green tea consumption on blood pressure: a meta-analysis of 13 randomized controlled trials. *Sci Rep*. 4:6251.

Persson, I.A., M. Josefsson, K. Persson, and R.G. Andersson. 2006. Tea flavanols inhibit angiotensin-converting enzyme activity and increase nitric oxide production in human endothelial cells. *J Pharm Pharmacol*. 58:1139-1144.

Qi, H., H. Takano, Y. Kato, Q. Wu, C. Ogata, B. Zhu, Y. Murata, and Y. Nakamura. 2011. Hydrogen [corrected] peroxide-dependent photocytotoxicity by phloxine B, a xanthene-type food colorant. *Biochim Biophys Acta*. 1810:704-712.

Ranzato, E., S. Martinotti, V. Magnelli, B. Murer, S. Biffo, L. Mutti, and B. Burlando. 2012. Epigallocatechin-3-gallate induces mesothelioma cell death via H2 O2 -dependent T-type Ca2+ channel opening. *J Cell Mol Med*. 16:2667-2678.

Raza, H., and A. John. 2007. In vitro protection of reactive oxygen species-induced degradation of lipids, proteins and 2-deoxyribose by tea catechins. *Food Chem Toxicol*. 45:1814-1820.

Reeves, P.G., and B.L. O'Dell. 1985. An experimental study of the effect of zinc on the activity of angiotensin converting enzyme in serum. *Clin Chem*. 31:581-584.

Sang, S., I. Yang, B. Buckley, C.T. Ho, and C.S. Yang. 2007. Autoxidative quinone formation in vitro and metabolite formation in vivo from tea polyphenol (-)-epigallocatechin-3-gallate: studied by real-time mass spectrometry combined with tandem mass ion mapping. *Free Radic Biol Med*. 43:362-371.

Santos, R.A., E.M. Krieger, and L.J. Greene. 1985. An improved fluorometric assay of rat serum and plasma converting enzyme. *Hypertension*. 7:244-252.

Satoh, M., Y. Takemura, H. Hamada, Y. Sekido, and S. Kubota. 2013. EGCG induces human mesothelioma cell death by inducing reactive oxygen species and autophagy. *Cancer Cell Int*. 13:19.

Scalbert, A., and G. Williamson. 2000. Dietary Intake and Bioavailability of Polyphenols. *The Journal of Nutrition*. 130:2073S-2085S.

Shi, L., C. Mao, Z. Xu, and L. Zhang. 2010. Angiotensin-converting enzymes and drug discovery in cardiovascular diseases. *Drug Discovery Today*. 15:332-341.

Shotan, A., J. Widerhorn, A. Hurst, and U. Elkayam. 1994. Risks of angiotensin-converting enzyme inhibition during pregnancy: experimental and clinical evidence, potential mechanisms, and recommendations for use. *Am J Med*. 96:451-456.

Sibony, M., J.M. Gasc, F. Soubrier, F. Alhenc-Gelas, and P. Corvol. 1993. Gene expression and tissue localization of the two isoforms of angiotensin I converting enzyme. *Hypertension*. 21:827-835.

Simon, S.R., H.R. Black, M. Moser, and W.E. Berland. 1992. Cough and ACE inhibitors. *Arch Intern Med*. 152:1698-1700.

Sun, S.L., G.Q. He, H.N. Yu, J.G. Yang, D. Borthakur, L.C. Zhang, S.R. Shen, and U.N. Das. 2008. Free Zn(2+) enhances inhibitory effects of EGCG on the growth of PC-3 cells. *Mol Nutr Food Res*. 52:465-471.

Tang, Y., N. Abe, H. Qi, B. Zhu, Y. Murata, and Y. Nakamura. 2014. Tea Catechins Inhibit Cell Proliferation Through Hydrogen Peroxide-Dependent and -Independent Pathways in Human T

lymphocytic Leukemia Jurkat Cells. *Food Science and Technology Research*. 20:1245-1249.

Tipoe, G.L., T.M. Leung, M.W. Hung, and M.L. Fung. 2007. Green tea polyphenols as an anti-oxidant and anti-inflammatory agent for cardiovascular protection. *Cardiovasc Hematol Disord Drug Targets*. 7:135-144.

van Sande, M.E., S.L. Scharpe, H.M. Neels, and K.O. Van Camp. 1985. Distribution of angiotensin converting enzyme in human tissues. *Clin Chim Acta*. 147:255-260.

Vleeming, W., J.G. van Amsterdam, B.H. Stricker, and D.J. de Wildt. 1998. ACE inhibitor-induced angioedema. Incidence, prevention and management. *Drug Saf*. 18:171-188.

Wald, N.J., and M.R. Law. 2003. A strategy to reduce cardiovascular disease by more than 80%. *BMJ*. 326:1419.

Wang, X., K.-S. Song, Q.-X. Guo, and W.-X. Tian. 2003. The galloyl moiety of green tea catechins is the critical structural feature to inhibit fatty-acid synthase. *Biochemical Pharmacology*. 66:2039-2047.

Wei, L., F. Alhenc-Gelas, P. Corvol, and E. Clauser. 1991. The two homologous domains of human angiotensin I-converting enzyme are both catalytically active. *J Biol Chem*. 266:9002-9008.

Winterbourn, C.C., and M.B. Hampton. 2008. Thiol chemistry and specificity in redox signaling. *Free Radic Biol Med*. 45:549-561.

Winterbourn, C.C., and D. Metodiewa. 1999. Reactivity of biologically important thiol compounds with superoxide and hydrogen peroxide. *Free Radic Biol Med*. 27:322-328.

Zaveri, N.T. 2006. Green tea and its polyphenolic catechins: medicinal uses in cancer and noncancer applications. *Life Sci*. 78:2073-2080.

Zhang, Y., T. Jiang, Y. Zheng, and P. Zhou. 2012. Interference of EGCG on the Zn(ii)-induced conformational transition of silk fibroin as a model protein related to neurodegenerative diseases. *Soft Matter*. 8:5543-5549.