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Full Length Research Paper

Inhibiting effects of epigallocatechin gallate (EGCG) on the formation of age pigment *in vitro* and *in vivo*

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Lipofuscin age pigment is often considered as a hallmark of aging, which shows an accumulation rate inversely correlated with longevity and it is primarily composed of carbonyl-amino cross linked protein residues. The purpose of this study is to determine whether epigallocatechin gallate (EGCG) would inhibit the formation of age pigment in both MDA-modified human serum albumin and D-galactose-induced aging mice models by using spectrofluorometry, HPLC-PDA, FTIR spectrometer, SEM and TEM observation. Results showed that EGCG could inhibit the formation of age pigment *in vitro* and *in vivo*. The structure-activity relationship suggested that besides anti-oxidative stress and trapping the reactive unsaturated aldehydes, the inhibiting effects were correlated with the galloyl D-ring of tea catechins on neutralizing the amino-carbonyl cross-linking reaction.

Key words: Epigallocatechin gallate (EGCG), age pigment, galloyl group, amino-carbonyl cross-linking products.

INTRODUCTION

Lipofuscin, also called age pigment, is a brown-yellow, electron-dense and auto fluorescent material that accumulates progressively over time in lysosomes of post-mitotic cells, such as in neurons and in cardiac myocytes (Peyroux and Sternberg, 2006). Lipofuscin is primarily composed of carbonyl-amino cross-linked protein residues and mainly comes from the "carbonyl stress" of advanced lipid peroxidation end products (ALEs) and advanced glycation end products (AGEs). The carbonyl-amino compounds are difficult to scavenge due to their low-energy potential structures, such as cyclization and/or conjugation (Yin, 1995). As a result, steady and irreversible cross-linking of carbonyl-amino compounds have been found to be a common and essential toxiferous process *in vivo*, which might therefore be at the root of disease and aging-related functional losses (Berlett and Stadtman, 1997; Suc et al., 1998).

The publications above show that the best way to inhibit the formation of age pigment is to prevent the carbonyl-amino cross linked reaction. Generally, carbonyl stress involves the use of free-radical scavengers and antioxidants that prevent the generation of lipid peroxidation products. However, this is inefficient toward pre-formed reactive carbonyl compounds. Conversely, carbonyl scavengers prevent carbonyl stress by inhibiting the formation of protein cross-links. Various AGE and ALE inhibitors have been developed, but most of the clinical trials have proved to be unsatisfactory, in part because of side effects. The long-term therapeutic effect of the most recently developed AGE and ALE inhibitors or breakers remains to be demonstrated (Terman and Brunk, 1998). In this study, we will focus on the application of the plant functional components to treat the carbonyl stress-associated diseases with low toxicity.

Tea catechins, as the main functional components of tea, play large roles in human health, display pharmacologic activity and are not known to be toxic. Evidence has accumulated that tea catechins prevent carbonyl stress-associated diseases *in vitro* and *in vivo* (Banach et al., 2009; Lo et al., 2006; Nakae et al., 2008;

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Noda and Peterson, 2007; Stangl et al., 2007) and recent studies have also shown that tea catechins manifest remarkable quenching activity against unsaturated aldehydes, especially with regard to formation of covalent adducts and they are even more reactive than the pharmaceutical agent aminoguanidine (Beretta et al., 2008; Cheng et al., 2009; Sang et al., 2007; Zhu et al., 2009). On the other hand, the structure-activity relationship studies revealed that the gallate groups, especially the galloyl groups (at C-3 position), appeared to be essential for physiologic acitivity, most of which is related to the specific binding with protein (Isaacs et al., 2008; Kusuda et al., 2006; Stangl et al., 2007). The molecule-docking studies showed that these gallate-group tea catechins occupy the hydrophobic crevice and reliably bind with positively charged residues, such as Lys, Arg and His (Cao et al., 2009; Leone et al., 2003; Maiti et al., 2006; Pellecchia and Reed, 2004), which are the binding sites of many reactive carbonyl compounds (Burcham and Kuhan, 1996; Chowdhury et al., 2004; Uchida et al., 1998). According to aforementioned results, we hypothesize that besides their powerful anti-oxidative function, preventing carbonyl-amino cross-linking reactions (anticarbonyl stress) maybe an underlying mechanism by which the ester-type catechins prevent carbonyl stress-associated diseases.

In the present study, we first examined the anti-carbonyl stress activities of EGCG *in vitro*, and the structure-activity relationship was investigated by comparing EGCG with other several phenolic compounds isolated from either tea or plants and second, we tested the inhibiting effect of EGCG on the formation of lipofuscin *in vivo*.

MATERIALS AND METHODS

Materials

Highly purified tea epicatechins (-)-Epigallocatechin gallate (EGCG) (≥98% pure), (-)-epicatechin (EC) (≥ 98%), (-)-Epicatechin gallate (ECG) (≥ 98%) and (-)-Epigallocatechin (EGC) (≥ 95%) were isolated from *Camellia sinensis* leaves and both propyl gallate (PG) (≥ 99%) and gallic acid (GA) (≥ 99%) were isolated from plants. Human serum albumin (HSA) was purchased from Sigma. 1, 1, 3, 3-tetramethoxypropane (TMP) (≥ 98%) was obtained from Fluka Chemie AG (Buchs, Switzerland). Other chemicals used were all of analytic grades.

Preparation of malondialdehyde (MDA)

A fresh MDA stock solution (10 mmol/L) was prepared by hydrolyzing TMP according to a method described by Kikugawa et al. (Kikugawa et al., 1980).

Briefly, 0.085 mL (0.5 mmol) of TMP was mixed with 4.5 ml of 1.0 mol/L HCl and shaken at 40°C for about 2.5 min. After the TMP was fully hydrolyzed, the pH was adjusted to 7.0 with 6.0 mol/L NaOH and the stock solution was finally made up to 50 mL with PBS. The stock solution was checked by measuring absorbance at 267 nm using ε_{MDA} = 31500.

Treatment of HSA with MDA at different pHs

A solution containing HSA (1 mg/mL) and MDA (2.0 mmol/L) in PBS was incubated at 37°C in a sirocco-blasting drying trunk for 24 h. Double-distilled water was added to make the corresponding blank control.

Treatment of tea catechins in MDA-modified HSA reaction systems

Different concentrations from 150 to 750 µmol/L of the six phenolic compounds (EGCG, EGC, ECG, EC,GA and PG) were added to the HSA or MDA/HSA reaction system at 37°C in a sirocco-blasting drying trunk for 24 h. Double-distilled water was added to make the corresponding blank control.

Preparation of protein products

Modified protein was precipitated using a one-fifth volume of 20% TCA followed by centrifuging (11,000 rpm) for 5 min and the pellet were washed repeatedly twice in order to remove fluorescent MDA oligomers formed during modification reactions (Hipkiss et al., 1998). Finally, the precipitated protein was re-dissolved in PBS and the concentration was adjusted to 2 mg/mL.

Fluorescence spectroscopy analysis of protein products

Fluorescence measurements on control or modified proteins were made by using FL Solutions 2.0 spectrofluorimeter (λ_{ex} = 395 nm, λ_{em} = 460 nm), so as to evaluate the effects of inhibiting the formation of lipofuscin-like fluorescence. The sample was diluted 5-fold or 50-fold before the fluorescence data were analyzed. The IC50 of the test sample was obtained from the least-squares regression line of the plots of the logarithm of the sample concentration (log) versus the fluorescence intensity of lipofusin in HSA/MDA system.

HPLC-PDA analysis of the reaction products of tea catechins and MDA

To understand the reactivity between tea catechins and MDA, tea catechins (0.7 mmol/L) were lixiviated with MDA (2 mmol/L) in PBS at pH 7.0 and 37°C for 24 h and passed through a 0.45 µm filter. Reaction products were analyzed by HPLC on a Shim-pack ODS C18 column (4.6×150 nm, 5 µm). The mobile-phase composition (v/v) was: 40.0 n, n-dimethylformamide, 2.0 methanol, and 1.5 acetic acid. The following mobile-phase was used to separate components in the reaction system: 14% mobile phase at 0 min, then increased linearly to 24% at 13 min and 34% at 22 min, and finally reduced to 14% at 30 min. The flow rate was 1 ml/min, and the reaction products of tea catechins were detected at 278 nm and the content of MDA was at 266 nm with photodiode array detector (PDA, Shimadzu LC-10 ATVP) at the same time.

Scanning electron microscope (SEM) analysis of the microstructure of protein products

The centrifugal protein products fixed with glutaraldehyde, stored at 4°C for 12 h or more. The SEM samples were coated with gold using coating machine (JEOL JFC-1600) before analysis of surface morphology and the composition of the protein products using

scanning electron microscope (SEM. JSM-6360LV).

FTIR analysis of protein products

A WQF-310-type FTIR spectrometer equipped with a KBr-splitter was employed for infrared measurements. The FTIR spectra of the samples have been recorded at room temperature between 4400 and 400 cm^{-1} at a 4 cm^{-1} nominal resolution, accumulating 64 scans per spectrum. A blank spectrum was registered to avoid any cross contamination. Each sample was vacuum dried at -42°C for 24 h to remove the absorbed water in the sample.

IR spectra processing procedure

The protein secondary structure was determined from the amide Iband located between 1700 and 1600 cm^{-1}. Fourier self-deconvolution and secondary derivative calculations were applied to estimate the number and position of the component bands. Based on these parameters, a multiple Gaussion curve-fitting process was performed in the region 1700 to 1600 cm^{-1} of the amide Iband to quantify the area of each component. The relative percentage of the secondary structural elements was obtained from the area under the Gaussion curve (Maiti et al., 2006).

Animals and experimental design

Healthy female Kunming mice, aged 7 weeks and weighing about 20 to 25 g, were obtained from the Hunan Slack King Animal Co. (Changsha, P. R. China). They were housed in plastic cages under standard conditions and allowed free access to standard laboratory food and water in a 12-h light/dark cycle. All animals were used only once. The principles of laboratory animal care were followed in accordance with the Chinese Experimental Animal Adiministration Legislation. After acclimatization to the laboratory for one week, the mice were randomly divided into three groups (n = 8 in each group): Control group, D-gal model group and EGCG-treatment group. Mice in both the D-gal model group and EGCG-treatment group were subcutaneously injected with 3% D-gal at the dose of 150 mg/kg body weight once daily for 6 weeks, while those in the control group were treated with the same volume of normal saline. From the third week, mice in the EGCG-treatment group were given EGCG at a dose of 6 mg/kg/d by the intragastric gavage (i.g.) route and mice in both the control and model groups were administered the same volume of vehicle (distilled water) for 4 weeks. After finishing all treatments, the animals were then immediately killed to dissect the brains for various experiments.

Histology and ultrastructural transmission electron microscopy (TEM) observation

The brains of mice were enucleated at week 8 and fixed immediately with a mixture of 2.5% glutaraldehyde and 2% paraformaldehyde in 0.1 mol/L PBS (pH 7.4) for histology and transmission electron microscopy (TEM). After fixation for 30 min, the brains were post-fixed in the same fixative for another 24 h at 4°C. The brains were dehydrated in a series of graded alcohols, embedded in paraffin, and 5-µm sections cut. These sections were then stained with hematoxylin and eosin for light microscopy.

Following aldehyde fixation, the cerebral cortex in the identical place in each mouse was obtained and fixed in 1% osmium tetroxide for 1 h, dehydrated, embedded in epoxy resin, trimmed and cut into ultrathin sections. Sections ranging from 40 to 60 nm thick were mounted on uncoated mesh copper grids, constructed with uranyl acetate for 30 min and lead citrate for 2 min and examined with a Zeiss 900 electron microscope.

Statistical analysis

The software package SPSS 18.0 was applied for statistical analysis. Measurement data were compared with one-way analysis of variance as well as repetitive-measurements analysis of variance. The results were depicted as mean ± standard error of the mean (S.E.M). Results were considered significantly different at a level of $p < 0.05$.

RESULTS

Fluorescence spectroscopy analysis of lipofuscin-like fluorescence intensity of proteins

Tea catechins have A-, B-, C-rings and galloyl-D-ring (Figure 1) and the number and position of the gallate groups are different, such that EGC and EGCG possess a 3,4,5-trihydroxyl structure on the pyrogallol (B ring) while, EGCG and ECG have a galloyl moiety at the C-3 position (D-ring) and EC has no gallate group. Both phenolic compounds gallic acid (GA) and propyl gallate (PG) were used as the adjuvant ingredients to analyze the structure-activity relationships.

Our study revealed that the above six phenolic compounds, especially EGCG, could remarkably inhibit the lipofuscin-like fluorescence intensity of the MDA-modified HSA and the half-inhibition concentration (IC50) of the six phenolic compounds (EGCG, EGC, ECG, EC, PG and GA) was 144.76, 196.70, 177.57, 430.39, 257.60 and 324.40 µmol/L, respectively (Figure 2).

HPLC studies of the reaction products in MDA-modified HSA reaction system

The results of HPLC studies of the reaction products in the MDA/tea catechin reaction systems showed that the phenolic compounds could greatly reduce the content of MDA, especially the gallate B-ring tea catechins such as EGCG, EGC and GA (Figures 3 and 4). The results suggested that the gallate B-ring could promote scavenging effects of MDA.

SEM study surface morphology and composition of protein products

Reaction between tea epicatechins and MDA-modified HSA had been investigated for the first time by using SEM. The results showed that in the control group, the protein crystal structure of HSA was still relatively regular arrangement and resent a complete granular crystal structure (Figure 5a). After HSA incubated with MDA, the crystal structure of protein had been damaged due to

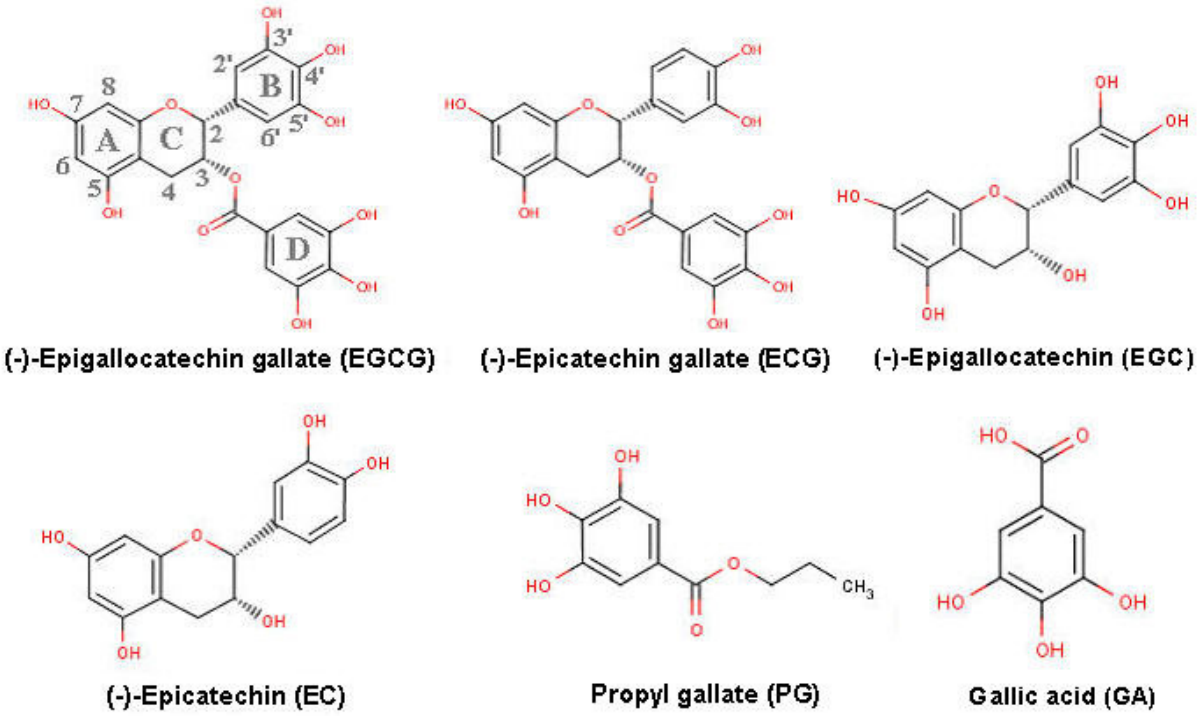

Figure 1. Chemical structures of tea catechins, gallic acid (GA) and propyl gallate (PG).

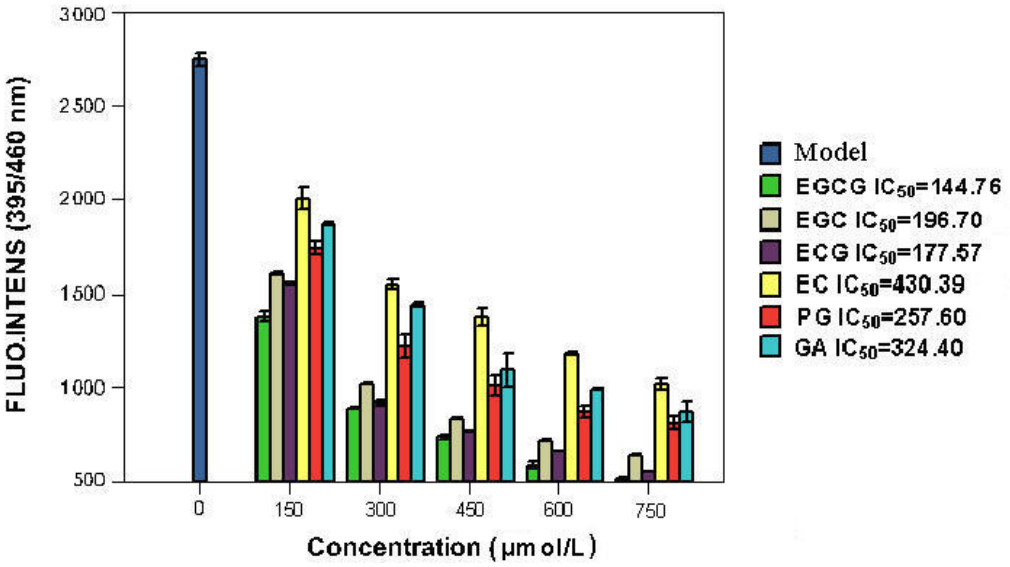

Figure 2. A comparison activities and the IC50 of tea catechins blocking fluorescence intensity of lipofusin in HSA / MDA system.

increased abnormal cross linking (Figure 5b). While ester-type catechins, especially EGCG, remarkably inhibited the formation of cross-linked structure of protein and recovered to the more completed crystal structure (Figure 5c).

FTIR spectrometer study of protein structure in the MDA-modified HSA reaction system

Infrared spectrometry techniques are extremely valuable in studying structural modifications of a protein. Figure 6A

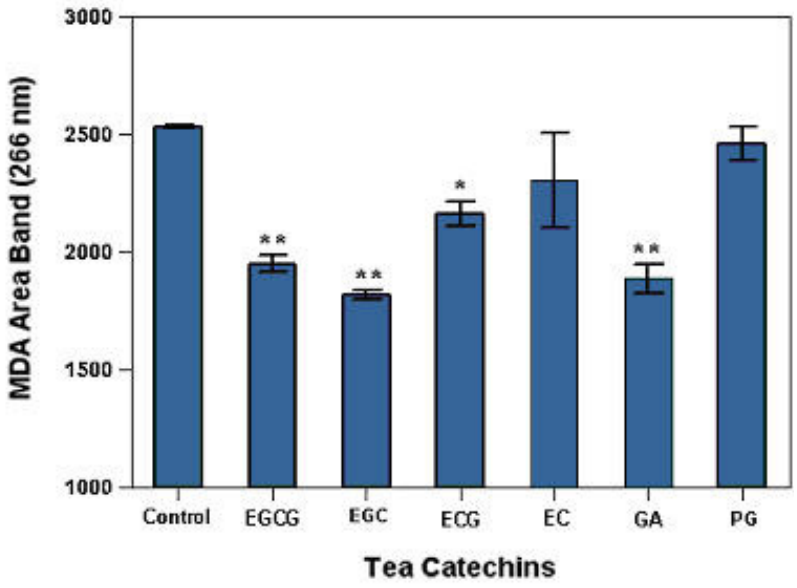

Figure 3. HPLC quantification of MDA in MDA/tea catechins reaction system. Each value is expressed as mean ±S.E. of three replications. *Represents statistical significance vs. control ($p<0.05$), and **Represents statistical significance vs. control ($P < 0.01$).

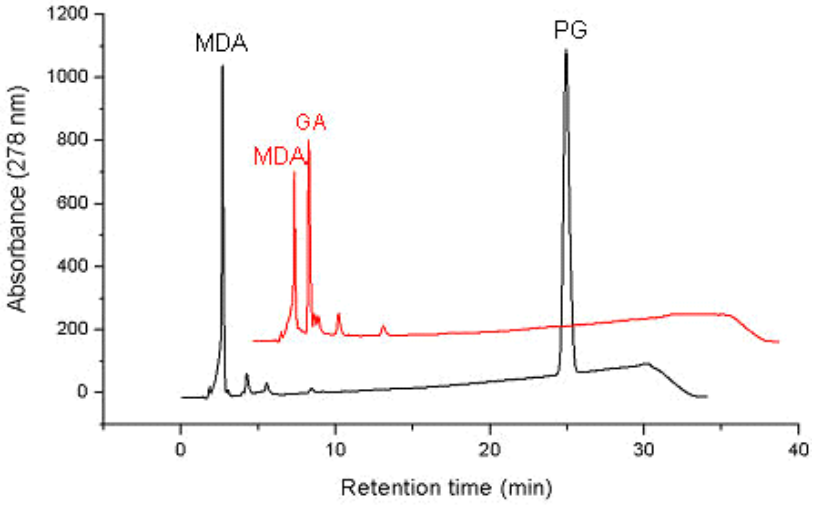

Figure 4. Products analysis of the reaction between GA, PG and MDA by HPLC.

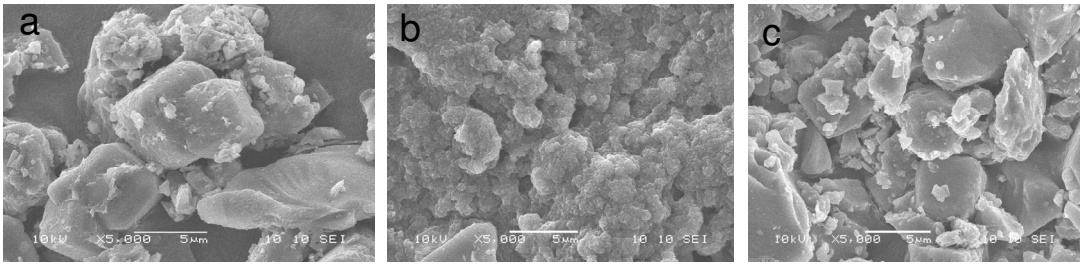

Figure 5. SEM micrographs of protein products. (a) Protein crystal structure of control group; (b) Protein crystal structure under carbonyl stress; (c) Protein crystal structure after treating with EGCG.

(1, 2) shows the infrared spectra of protein products of HSA in different treatments; and the absorption region between 1400 and 1800 cm^{-1} revealed two peaks, corresponding to the region of amidel and amidell(NH-CO).

After HSA was incubated with MDA, the amidel band height of the MDA-modified HSA was obviously decreased (Figure 6A, 2 and 2´, HSA-MDA), revealing structural modifications in the protein (Chesne et al., 2006). On the other hand, the derivative spectrum showed that tea catechins--especially EGCG and ECG--remarkably changed the spectrum structure and improved the peak values of HSA in the MDA/HSA reaction system (Figures 6A, 1 and 2).

To further understand the changes in protein structure, a quantitative analysis of the secondary structure of protein products in different treatments was made. As shown in Figure 6B, the secondary structures of the α-helix for HSA, HSA-MDA, and HSA-MDA-EGCG were 31.51, 18.14 and 35.95%, respectively. Because MDA modified the HSA, the α-helix content was reduced from 31.51 to 18.14% and the α-helix content was increased after tea catechin treatment.

Histology and TEM analysis of the effects of EGCG on brain tissues *in vivo*

The analysis of the pathologic sections of mouse cortex showed that there was no significant difference between negative and positive controls. The number of neurons in the EGCG-treatment group appeared, increased but not to a statistically significant extent. TEM examination showed that the sizes of lipofuscin particles in lysosomes were increased and mitochondra were sparsely distributed and showed vacuolar degeneration with disrupted cristae in the D-gal treated group. The degenerative changes were circumvented after EGCG treatment: the lipofuscin was obviously decreased and the structure of mitochondra showed improved recovery compared with the aging model group.

DISCUSSION

Pyrogallol and gallate rings contribute to the inhibition of lipofuscin-like fluorescence intensity

Previous studies have shown that the gallate B-ring of tea catechins exhibits a stronger tendency to become oxidized, which appears to be due to favored removal of lone electrons from free radicals (Mochizuki et al., 2002; Muzolf-Panek et al., 2008). Our present study showed that the redox centers of the gallate B-ring of tea catechins might also contribute to facilitating the addition of tea catechins to the electron-deficient regions in electrophiles, such as MDA (Figure 3) (Hernaez et al., 1998).

More importantly, the potent inhibition of lipofuscin-like fluorescence intensity was not corrected by the MDA-scavenging ability of the six phenolic compounds in the MDA-modified reaction system, especially for PG and the gallate D-ring tea catechins (Figures 2 and 3), the reason for this might be that the gallate rings specifically bind proteins sites in the MDA reaction.

Gallate D-rings may be the key moieties in EGCG's neutralization of the formation of carbonyl-amino cross-linking adducts

In the FTIR spectra study, the amidel absorption contains contributions from the C=O stretching vibration of the amide group (about 80%). The stronger the hydrogen bond involving the amide C=O, the lower the electron density in the C=O group and the lower the amidel absorption appears. Using this approach, one would predict the lowest amidel frequency to occur for extended polypeptide chains, such as those found in denatured proteins (Jackson and Mantsch, 1995). Accordingly, the decrease in the protein amidel absorption in the MDA/HSA reaction system contributed to the amino-carbonyl cross-linking reaction. It is reported that a well-documented change in MDA-modified proteins involves formation of fluorescent 1-amino-3- imino-propene cross-links produced when enamines condense with amine groups on adjacent proteins (Burcham and Kuhan, 1996).

After incubation with tea catechins, especially the EGCG and ECG, the changing trends were diminished and the peak values were increased. This revealed to us that tea catechins can protect proteins from amino-carbonyl reactions.

Our study showed that tea catechins could increase the protein aminde absorption in the MDA/HSA reaction system and increase the α-helix content of the protein products in the MDA/HSA reaction system (Figures 6A). A previous study has shown that because of the interaction of EGCG with HSA, the α-helix content was decreased (Maiti et al., 2006), possibly due to the pro-oxidation of EGCG and the forming of a Schiff base (Azam et al., 2004).

Based on the present results we can conclude that in the MDA/HSA reaction system, the binding of tea catechins--especially the galloyl-D rings--with protein and formation of macromolecular complexes by weak bonds, was not harmful to the secondary structure of protein. In contrast, it appeared to neutralize the formation of carbonyl-amino cross-linking adducts. However, more detailed information about the binding between the galloyl-D ring tea catechins and MDA binding sites of proteins is beyond the scope of the present study.

EGCG can decrease the deposition of lipofuscin in lysosomes of brain *in vivo*

The mechanism of D-galactose in inducing aging is based on the free radical theory of aging. Histology and TEM analysis showed that EGCG has neuroprotective effects

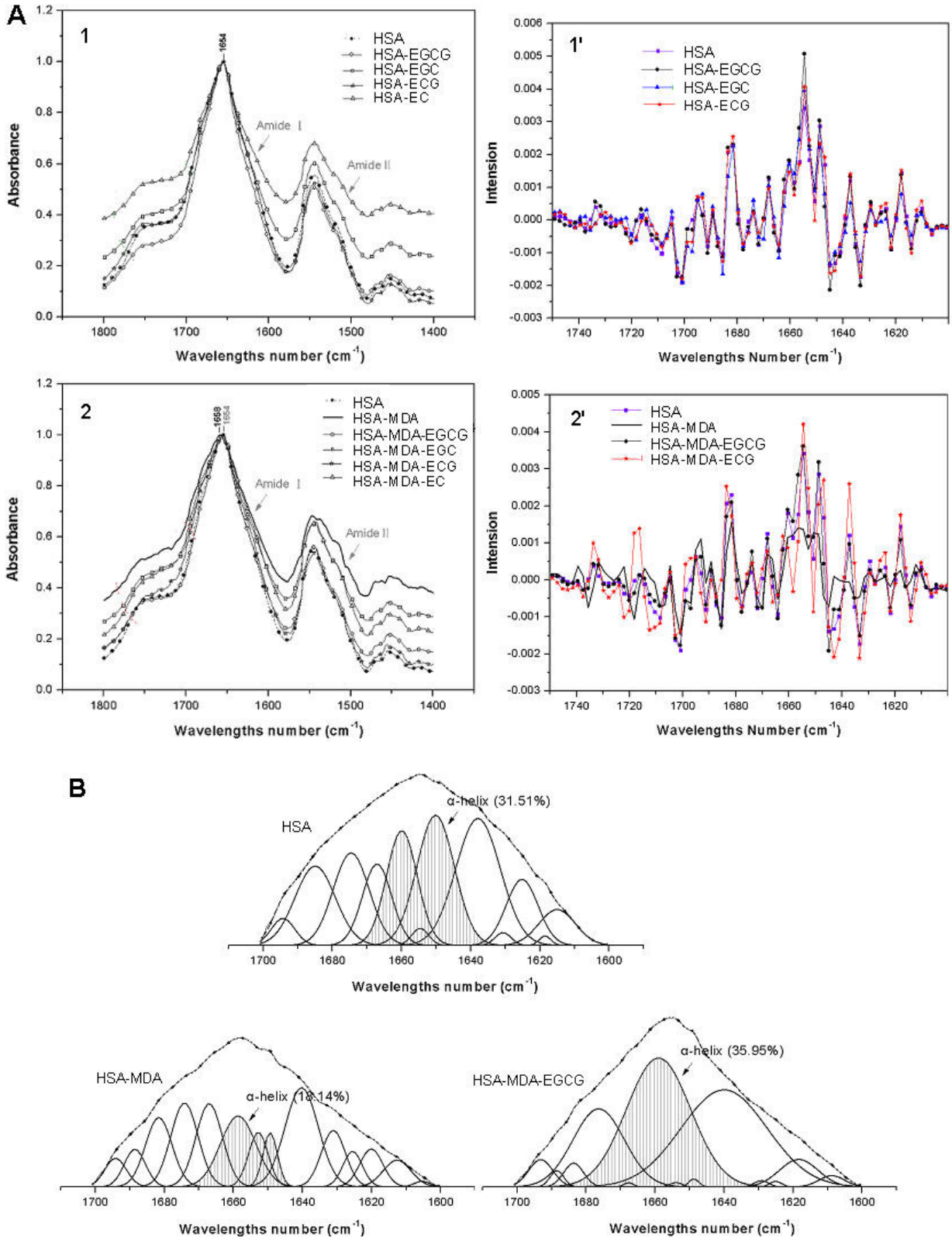

Figure 6. FTIR spectra of amide Iand II band (normalization processing) and the derivative spectrum of the protein products of HSA in different treatment groups (A), and deconvolution into different secondary structure bands by using Gaussian curve fitting program Origin 8.0 (B). HSA represent the control group, HSA-MDA represent MDA-modified HSA in reaction system, and HSA-MDA-EGCG, HSA-MDA-ECG and HSA-MDA-EGC represent EGCG, ECG and EGC incubated in MDA-modified HSA reaction system, respectively.

on mouse aging induced by D-galactose, especially in the alleviation of lipofuscin deposition.

Lipofuscin is difficult to scavenge due to its low energy potential. This indicates that one of the challenges to developing carbonyl scavengers is the inhibition of the formation of low-energy potential by-products.

A previous study showed that tea catechin radicals are stabilized by the interaction between the rings and that the B-ring and D-ring in EGCG interact especially strongly (Takeuchi et al., 2007). This indicates that the modification of the B-ring (di- vs trihydroxybenzene) or C-ring (gallate ester or hydroxyl group), can alter the phenolic reactions. We believe that when the gallate B-ring is oxidized, EGCG becomes a newly conjugated system during the transition from the ground-state to the excited-state, generating an aromatic by de-localizing oxygen electrons from phenyl rings and that this new species is more likely to react with α, β-unsaturated carbonyl compounds, which will interrupt the carbonyl-amine reactions.

EGCG (molecular weight, 458.4) is the largest of the simple isoflavanoids found in tea but is still a small molecule and is on the FDA's list of compounds generally recognized as safe and approved for human consumption (Isaacs et al., 2008). Our study suggests that beyond its ability to scavenge free radicals, the prohibitive effect on blocking carbonyl-amino cross-linking reactions may be one of the molecular mechanisms by which EGCG plays a role in human health and demonstrates pharmacologic activity.

Our immediate future studies will focus on achieving more detailed information on the galloyl-D-ring and its specific binding to unsaturated aldehydes sites in pathologic microenvironments, especially in the acidic environment within lysosomes; this will provide essential information for us in the screening for carbonyl scavenger drugs from plant functional components.

In summary, our results suggest that the synergistic effects between the gallate B-ring and the galloyl D-ring is important in allowing tea catechins to inhibit the carbonyl stress in the MDA-modified HSA reaction system. Similarly, the binding of the galloyl D-ring tea catechins with protein is a key mechanism in neutralizing the carbonyl stress process, where the anti-oxidative gallate B-ring contributes to trapping the reactive unsaturated aldehydes effectively in a local concentrated drug environment.

ACKNOWLEDGMENTS

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