Original Article

Caffeic acid inhibits the formation of 1-hydroxyethyl radical in the reaction mixture of rat liver microsomes with ethanol partly through its metal chelating activity

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Effect of caffeic acid on the formation of 1-hydroxyethyl radicals *via* the microsomal ethanol-oxidizing system pathway was examined. The electron spin resonance spin trapping showed that 1-hydroxyethyl radicals form in the control reaction mixture which contained 0.17 M ethanol, 1 mg protein/ml rat river microsomes, 0.1 M α-(4-pyridyl-1-oxide)-*N-tert*-butylnitrone, 5 mM nicotinamide adenine dinucleotide phosphate and 30 mM phosphate buffer (pH 7.4). When the electron spin resonance spectra of the control reaction mixtures with caffeic acid were measured, caffeic acid inhibited the formation of 1-hydroxyethyl radicals in a concentration dependent manner. Gallic acid, dopamine, L-dopa, chlorogenic acid and catechin also inhibited the formation of 1-hydroxyethyl radicals. Above results indicated that the catechol moiety is essential to the inhibitory effect. Caffeic acid seems to chelate of iron ion at the catechol moiety. Indeed, the inhibitory effect by caffeic acid was greatly diminished in the presence of desferrioxamine, a potent iron chelator which removes iron ion in the Fe (III)-caffeic acid complex. Since Fe (III)-desferrioxamine complex is active for the 1-hydroxyethyl radicals formation, caffeic acid inhibits the formation of 1-hydroxyethyl radicals in the reaction mixture partly through its metal chelating activity.

***Key Words*:** **P450, microsomes, 1-hydroxyethyl radical, caffeic acid, chlorogenic acid**

1-Hydroxyethyl radical (HER) is generated from ethanol *via* the microsomal ethanol-oxidizing system (MEOS) pathway. It can promote toxicity by enzyme inactivation through the protein oxidation, oxidative damage to the DNA, and disturbing cell membranes via lipid peroxidation and production of reactive lipid aldehydes. It is also known that HER formed via MEOS damages mitochondria.[1,2]

Chronic excessive use of alcohol by human results in liver disease[3,4] characterized by fatty infiltration, which lead to fibrotic degeneration and necrosis.[3,5,6] Some investigations have indicated that lipid peroxidation occurs in the livers of animals soon after the administration of an acute dose of alcohol. Much of the evidence for the lipid peroxidation has been based on the thiobarbituric acid assay for malondialdehyde in livers of animals treated with ethanol. On the other hand, increases in conjugated dienes as well as decreases in hepatic glutathione have also been reported.[7,8] In addition, Muller and Sies[9] have reported an enhanced production of ethane and n-pentane, which are believed to be products of the peroxidative degradation of membrane lipids, during the metabolism of ethanol by perfused livers. These various findings have been interpreted as evidence that lipid peroxidation has occurred in the liver as a result of ethanol metabolism. Lipid peroxidation has also been proposed as a mechanism of ethanol-induced toxicity in the heart,[10] gastric mucosa[11] and testes.[12]

Polyphenols are compounds having two or more phenolic hydroxy groups in the molecule.[13] There are many reports showing protective effects of the polyphenols against oxidative stresses. Caffeic acid (CA) and chlorogenic acid have been known to be inhibitors of the mutagenicity of bay-region diol epoxides of polycyclic aromatic hydrocarbons,[14] of retinoic acid 5,6-epoxidation,[15] of hydroxyl radical formation[16] and of lipid peroxidation.[17,18] Chlorogenic acid and CA also act as scavengers of superoxide radical, hydroxyl radical[19] and peroxy radical.[20] On the other hand, catechins exert protective effects against oxidative damage of erythrocyte membrane,[21] ethanol-induced fatty livers,[22] cardiovascular diseases,[23,24] inflammatory[25] and cancer.[26] Catechins from *Camellia sinenesis* decreases α-(4-pyridyl-1-oxide)-*N-tert*-butylnitrone (4-POBN)/radical adducts in bile of rats after transplantation of ethanol-induced fatty livers.[27] Liu and Mori reported that monoamine metabolites, *i.e.*, norepinephrine and dopamine provide an antioxidant defense in the brain against oxidant and free radical-induced damage.[28] Dopamine and L-dopa inhibit the peroxidation of ox-brain phospholipids, with IC_{50} values of 8.5 μM for dopamine and 450 μM for L-dopa.[29] Galloyl derivatives work as highly efficient antioxidants against the chemically induced LDL oxidation.[30] Their antioxidative activities are achieved through the preventing the formation of the free radical by catechol moiety.[31]

In this study, HER formed in the reaction mixture of rat liver microsomes with ethanol is separated, detected and identified using the high performance liquid chromatography-electron spin resonance-mass spectrometry (HPLC-ESR-MS).[32] Furthermore, the inhibitory effects of CA and its related compounds on the formation of HER are examined, in order to clarify the mechanism through which CA and its related compounds inhibit the formation of HER in MEOS.

Materials and Methods

Chemicals. 4-POBN and desferrioxamine mesylate (Des) were obtained from Sigma Chemical Co. (St. Louis, MO). CA, ferulic acid, chlorogenic acid, D-(−)-quinic acid, gallic acid, D-(+)-catechin, dopamine hydrochloride and L-dopa were purchased from Tokyo Kasei Kogyo, Ltd. (Tokyo, Japan). Ethanol and

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salicylic acid were obtained from Katayama Chemical, Ltd. (Osaka, Japan). Nicotinamide adenine dinucleotide phosphate (NADPH) was obtained from Oriental Yeast Co., Ltd. (Tokyo, Japan). Ferric chloride was obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). All other chemicals used were of analytical grade.

Preparation of rat liver microsomes. Male Sprague-Dawley rats, body weight 250–300 g, were used in the experiments. The rat livers were removed immediately after decapitation. The livers were homogenized in 9 volumes of 0.25 M sucrose. The liver homogenate was centrifuged at 16,000 g for 30 min at 4°C. The supernatant fraction was then centrifuged at 120,000 g for 30 min at 4°C. The pallet was resuspended in 0.15 M KCl and then centrifuged twice again at 120,000 g. The pallet was resuspended in 0.15 M KCl. Protein concentration of the suspention was 3.1 mg/ml. It was kept at −80°C before use. All experimentals were performed under the guideline for animal experiments in Wakayama Medical University.

Control reaction mixture of microsomes with ethanol. The control reaction mixture of microsomes with ethanol contained 0.17 M ethanol, 1 mg protein/ml microsomes, 0.1 M 4-POBN, 5 mM NADPH and 30 mM phosphate buffer (pH 7.4). The reaction was performed for 10 min at 37°C. The reaction mixture was applied to ESR, the HPLC-ESR and the HPLC-ESR-MS analyses.

CA concentration-dependence of the formation of the HER. In order to examine the CA concentration-dependence of the formation of the HER, ESR spectra were measured for the control reaction mixture with various concentration of CA (0.17 mM, 0.33 mM, 0.50 mM and 1.00 mM).

Effect of CA on the formation of HER in the control reaction mixture in the presence of Des. In order to know whether CA inhibits the formation of HER through a chelate of ferric ions or not, ESR spectra were measured for the control reaction mixture, the control reaction mixture with 0.17 mM CA, the control reaction mixture with 0.50 mM Des and the control reaction mixture with both 0.17 mM CA and 0.50 mM Des.

ESR measurements. The ESR spectra were obtained using a model JES-FR30 Free Radical Monitor (JEOL Ltd., Tokyo, Japan). Aqueous samples were aspirated into a Teflon tube centered in a microwave cavity. Operating conditions of the ESR spectrometer were: power, 4 mW; modulation width, 0.1 mT; center of magnetic field, 336.300 mT; sweep time, 4 min; sweep width, ±5 mT; time constant, 0.3 s. Magnetic fields were calculated by the splitting of MnO ($\Delta H_{3\text{-}4}$ = 8.69 mT).

HPLC-ESR analysis. The HPLC used in the HPLC-ESR consisted of a model 7125 injector (Reodyne, Cotati, CA) with a 5 ml sample loop, a model L-7100 pump (Hitachi Ltd., Ibaragi, Japan). A guard column (Bondapak™ C18 10 μm 125A Guard-Pak™, Waters Co., Wexford, Ireland) was used. The semi-preparative column (300 mm long × 10 mm i.d.) packed with TSKgel ODS-120T (TOSOH Co., Tokyo, Japan) was used. The columns were kept at 20°C throughout the analyses. For the HPLC-ESR analyses, two solvents were used: solvent A, 50 mM acetic acid; solvent B, 50 mM acetic acid/acetonitrile (20:80, v/v). A combination of isocratic and linear gradient was used: 0–40 min, 100% A to 20% A (linear gradient) at a flow rate of 2.0 ml/min; 40–60 min, 20% A (isocratic) at a flow rate of 2.0 ml/min. The ESR spectrometer was connected to the HPLC by a Teflon tube, which passed through the center of the ESR cavity. The operating conditions for the ESR spectrometer were: power, 4 mW; modulation width, 0.2 mT; time constant, 1 s. The magnetic field was fixed at the third ESR peak indicated by an arrow (Fig. 1) throughout the experiments.

HPLC-ESR-MS analysis. The HPLC and ESR conditions for the HPLC-ESR-MS analyses were the same as in the HPLC-ESR analysis. The mass spectrometer (MS) used in the HPLC-ESR-MS was a model M-1200 HS electrospray ionization (ESI)-MS (Hitachi Ltd., Ibaragi, Japan). The operating conditions of the mass spectrometer were: nebulizer, 180°C; aperture 1, 120°C; N_2 controller pressure, 2.0 kg/cm^2; drift voltage, 70 V; multiplier voltage, 1,800 V; needle voltage, 3,000 V; polarity, positive; resolution, 48. The control reaction mixture of microsomes with ethanol was applied to the HPLC-ESR-MS. Mass spectra were obtained by introducing the eluent from the ESR detector into the LC-MS system just before the respective peaks were eluted. The flow rate was kept at 50 μl/min while the eluent was introducing into the LC-MS system.

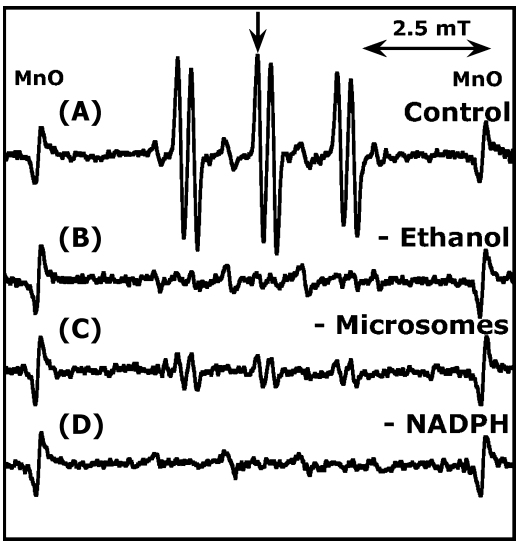

Fig. 1. ESR measurements of the reaction mixtures of microsomes with ethanol. ESR conditions were as described in Materials and Methods. (A) Control reaction mixture (B) Without ethanol (C) Without microsomes (D) Without NADPH.

Visible absorption spectra. Visible absorption spectra were measured using a model UV-160A ultraviolet-visible spectrometer (Shimadzu Co., Kyoto, Japan). The spectrometer was operated from 350 nm to 700 nm. The measurements were performed at 25°C. The measurements of the visible absorption spectra were carried out in a cuvette with a 10 mm light path. In the reference cell, 50 mM phosphate buffer (pH 7.4) was contained. The visible absorption spectra were measured for the complete mixture which consisted of 50 mM phosphonate buffer (pH 7.4), 0.17 mM CA, 0.085 mM ferric chloride and 0.5 mM Des.

Results

ESR measurements of the control reaction mixture. ESR spectra of the control reaction mixture, the control reaction mixture without ethanol, the control reaction mixture without microsomes and the control reaction mixture without NADPH were measured (Fig. 1). A prominent ESR spectrum (a^N = 1.58 mT and $a^H\beta$ = 0.26 mT) was observed for the control reaction mixture (Fig. 1(A)). The structures of these radicals are difficult to determine based on their ESR spectra alone because, in general, the spin-trapped radicals of 4-POBN show only six-line signals typical of carbon-centered radicals, with only small differences in hyperfine coupling. Weak ESR signals were observed in the absence of microsomes (Fig. 1(C)). In the absence of ethanol (or NADPH), the ESR signals were hardly observed (Fig. 1(B) and (D)).

HPLC-ESR analysis of the control reaction mixture. HPLC-ESR analysis was performed for the control reaction mixture (Fig. 2). On the HPLC-ESR elution profile of the control reaction mixture, two prominent peaks were observed at the retention times of 23.5 min (peak 1) and 27.9 min (peak 2) (Fig. 2(A)).

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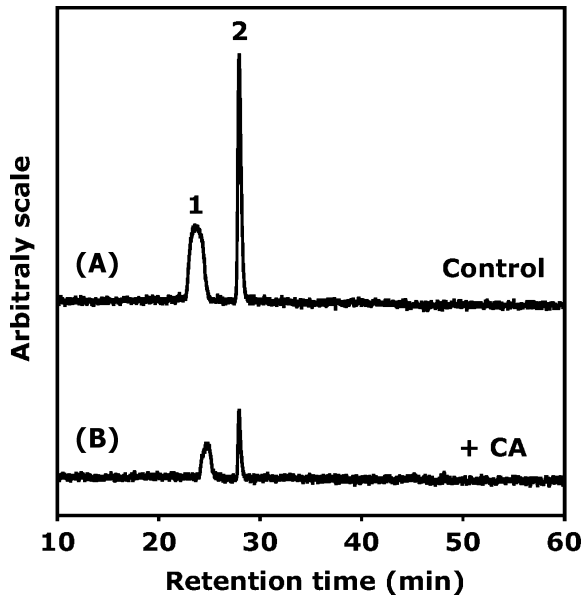

Fig. 2. HPLC-ESR analyses of the reaction mixtures. The reaction and HPLC-ESR conditions were as described in Materials and Methods. (A) Control reaction mixture (B) With 0.17 mM CA.

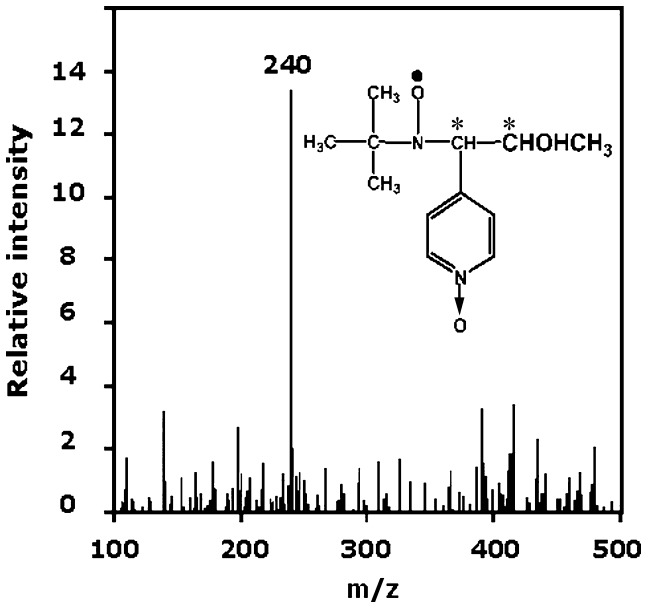

Fig. 3. An HPLC-ESR-MS analysis of the peak 2 fraction of Fig. 2. The reaction and HPLC-ESR-MS conditions were as described in Materials and Methods. Asymmetric carbon atoms are indicated by marks (*).

HPLC-ESR-MS analysis of the control reaction mixture. To identify the peak 1 and peak 2 observed on the elution profile of the HPLC-ESR (Fig. 2), the HPLC-ESR-MS analyses of the peak 1 and the peak 2 were performed. The HPLC-ESR-MS analysis of the peak 2 gave ions at m/z 240 (Fig. 3). Since the protonated molecular ions, $(M + H)^+$ have been observed for the various kinds of 4-POBN radical adducts,[33,34] we assign the ion m/z 240 to the protonated molecular ion of 4-POBN/ HER adduct, $(M + H)^+$. The MS spectrum of the peak 1 compound could not be observed since a huge 4-POBN peak was eluted at almost the same retention time as the peak 1. Since 4-POBN/HER radical adduct has two asymmetric carbon atoms (Fig. 3), two diastereoisomers could form. Indeed, two diastereoisomers of the DMPO/HER radical adducts have detected.[35,36] Thus, the peak 1 seems to be a diastereoisomer of the peak 2.

CA concentration-dependence of the formation of the HER. In order to examine the CA concentration-dependence of the formation of the HER, ESR spectra were measured for the control reaction mixture with various concentration of CA (Fig. 4). The ESR peak heights decreased to 49% (0.17 mM CA), 43% (0.33 mM CA), 40% (0.50 mM CA) and 37% (1.00 mM CA) of the control. CA inhibited the formation of HER in a concentration dependent manner.

Effects of CA and its related compounds on the formation of HER. Effects of CA and its related compounds on the overall formation of the 4-POBN/HER adducts were examined (Fig. 5). On addition of 0.17 mM CA, or gallic acid, or dopamine, or L-dopa, or chlorogenic acid, or D-(+)-catechin to the control reaction mixture, the ESR peak heights of the 4-POBN/HER adducts were decreased to 49 ± 4% (CA), 48 ± 1% (gallic acid), 44 ± 3% (dopamine), 72 ± 10% (L-dopa), 55 ± 1% (chlorogenic acid) and 65 ± 2% (D-(+)-catechin) of the control. On the other hand, addition of 0.17 mM salicylic acid (or quinic acid, or ferulic acid) showed no effects on the overall formation of the 4-POBN/HER adducts.

HPLC-ESR analysis of the control reaction mixture with CA. HPLC-ESR analysis was performed for the control reaction mixture with CA (or without CA) (Fig. 2). On addition of CA to the control reaction mixture, the peak height of the peak 1 decreased to 46% of the control, and the peak height of the peak 2 decreased to 28% of the control (Fig. 2(B)).

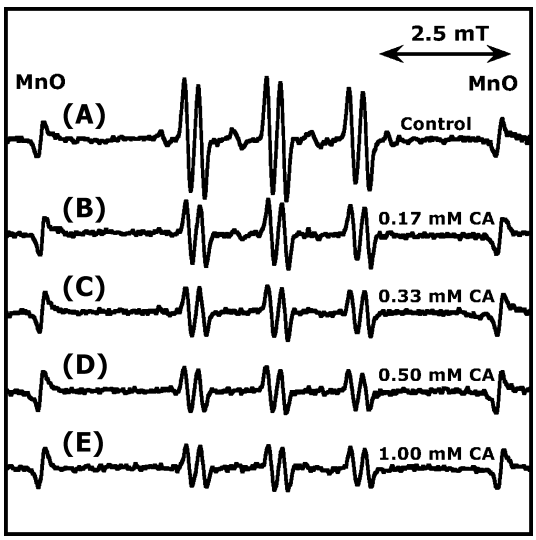

Fig. 4. CA concentration-dependence of the formation of HER. The reaction and ESR conditions were as described in Materials and Methods. (A) Control reaction mixture (B) With 0.17 mM CA (C) With 0.33 mM CA (D) With 0.50 mM CA (E) With 1.00 mM CA.

Visible absorption spectra of the mixtures containing ferric ions and CA in the presence of Des. In order to know whether CA chalets of ferric ions in the control reaction mixtures or not, visible spectra of the mixtures of ferric ions with CA were measured (Fig. 6). The mixture, which contained both ferric ions and CA, showed a visible spectrum with λ_{max} of 610 nm (Fig. 6(B)), while the mixture of ferric ions with CA did not show the visible spectrum in the presence of Des (Fig. 6(A)).

Effect of CA on the formation of HER in the control reaction mixtures in the presence of Des. In order to know whether CA inhibits the formation of HER through a chelate of

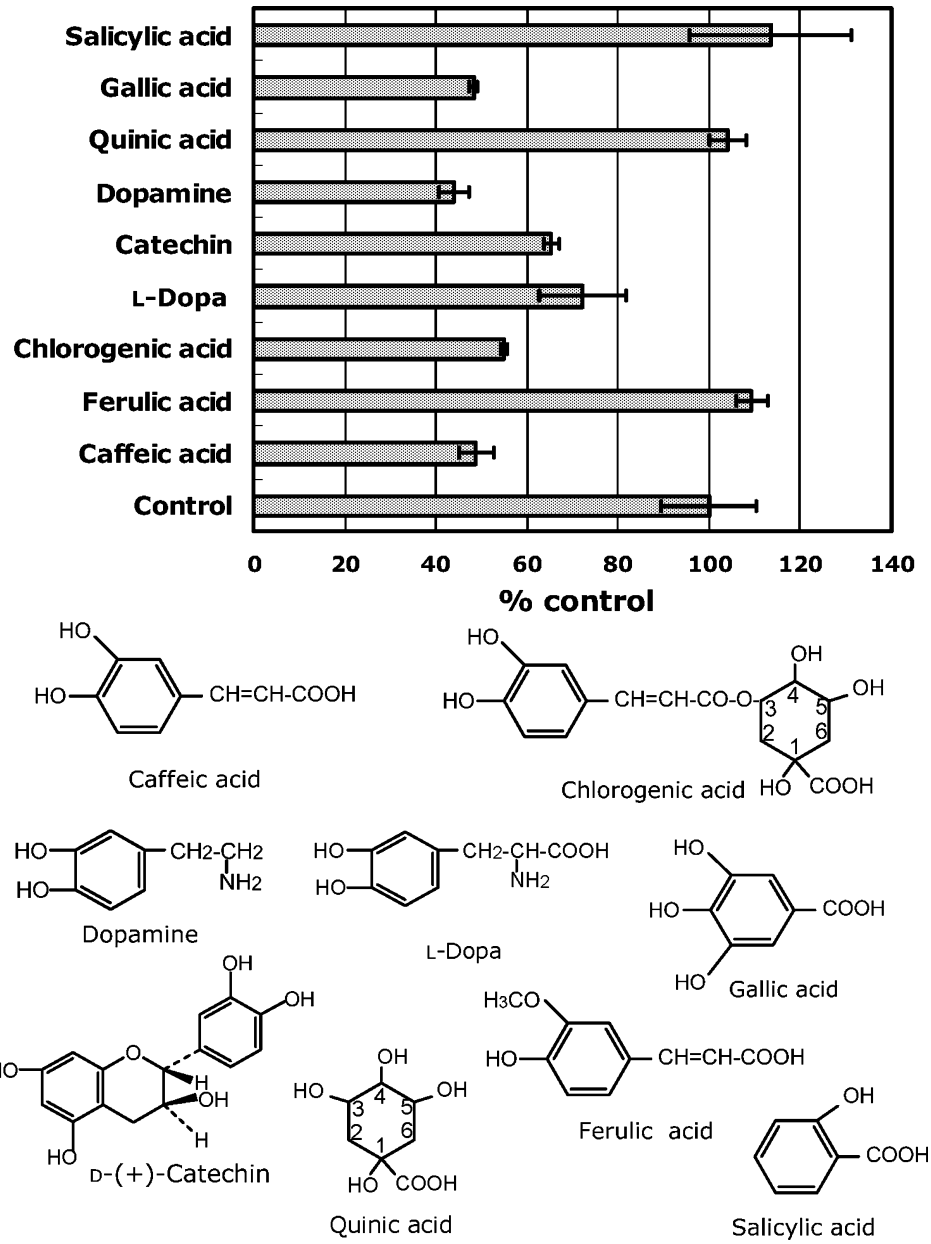

Fig. 5. Effect of CA and its related compounds on the formation of HER and chemical structures of CA and its related compounds. The reaction and ESR conditions were as described in Materials and Methods. The ESR spectra were observed for the control reaction mixture with 0.17 mM CA, or salicylic acid, or gallic acid, or quinic acid, or dopamine, or L-dopa, or chlorogenic acid, or ferulic acid, or D-(+)-catechin. Signal intensities were evaluated from the peak height of the third ESR signal of 4-POBN/HER adducts. The control value 100% represents the level of 4-POBN/ HER adducts formed in the absence of CA and its related compounds. The respective values are means ± SD of three determinations.

ferric ions or not, ESR spectra were measured for the control reaction mixture, the control reaction mixture with CA, the control reaction mixture with Des and the control reaction mixture with both CA and Des (Fig. 7). On addition of CA to the control reaction mixture, the ESR peak heights decreased to 50 ± 10% of the control. On the other hand, on addition of CA to the control reaction mixture in the presence of 0.50 mM Des, the ESR peak heights decreased to 72 ± 7% of the control with 0.50 mM Des.

Discussion

In the MEOS, the HER was generated from ethanol.[1] Knecht *et al.* has reported an overall scheme describing the production of the HER. The key feature of the mechanism is the postulation of an as yet uncharacterized oxidizing species (**Ox**), which can oxidize so-called "hydroxyl radical scavengers" but does not appear to be the ˙OH radical itself. The formation of this reactive species is dependent upon superoxide ($O_2^{\bullet -}$) and trace transition metal (M^{n+}).[37]

In this study, the effect of CA and its related compounds on the formation of 4-POBN/HER adducts was examined for the control reaction mixture of microsomes with ethanol. The formation of 4-POBN/HER adducts was inhibited by some polyphenols such as CA, gallic acid, dopamine, L-dopa, chlorogenic acid and catechin (Fig. 5). Ferulic acid, quinic acid and salicylic acid showed no inhibitory effect. Since catechol moieties do not occur in ferulic acid, quinic acid and salicylic acid, the catechol moiety seems to be essential to the inhibitory effect.

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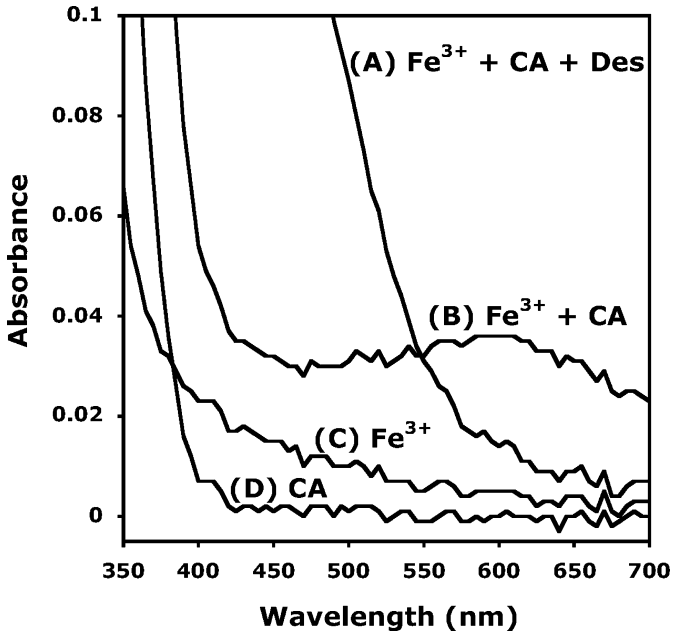

Fig. 6. Visible absorption spectra of the mixture containing both ferric ions and CA in the presence of Des. The conditions were as described in Materials and Methods. (A) 0.085 mM ferric chloride, 0.17 mM CA and 0.5 mM Des (B) 0.085 mM ferric chloride and 0.17 mM CA (C) 0.085 mM ferric chloride (D) 0.17 mM CA.

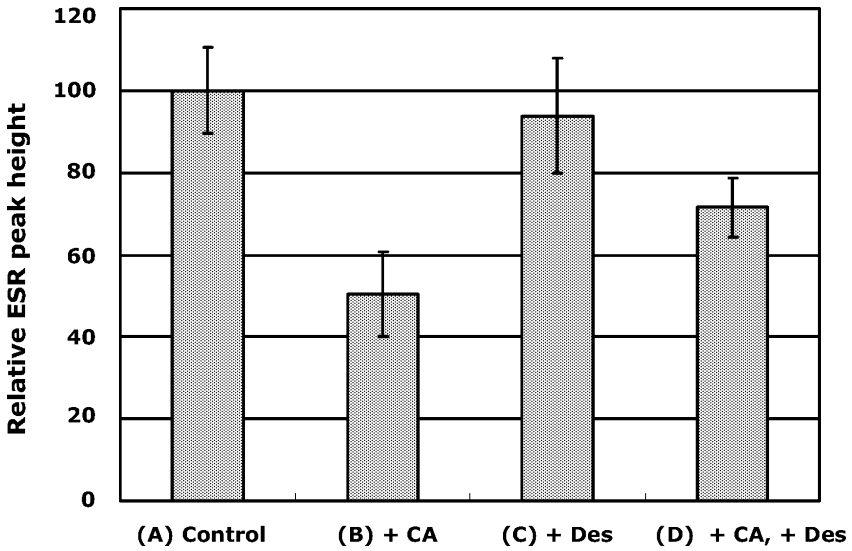

Fig. 7. Effect of CA on the formation of HER in the control reaction mixtures in the presence of Des. The reaction and ESR conditions were as described in Materials and Methods. (A) Control reaction mixture (B) With 0.17 mM CA (C) In the presence of 0.5 mM Des (D) With 0.17 mM CA in the presence of 0.5 mM Des.

Visible absorption analyses showed that the mixture, which contained both ferric ions and CA, showed a characteristic visible spectrum with λ_{max} of 610 nm (Fig. 6(B)) in the absence of Des, while the mixture of ferric ions with CA did not show the visible spectrum in the presence of Des (Fig. 6(A)). Some papers have reported the formation of the Fe (III)-CA complex[16,38] which showed a characteristic visible spectrum. The Fe (III)-CA complex does not form in presence of Des because the Des, a potent iron ion chelator removes iron ion in the Fe (III)-CA complex. Thus, the characteristic visible spectrum with λ_{max} of 610 nm is due to the Fe (III)-CA complex.

The HER formation from ethanol in microsomes is mediated by transition metals and P450 derived superoxide-dependent oxidizing species even in the presence of Des.[37] On addition of CA to the control reaction mixture, the ESR peak heights decreased to $50 \pm 10\%$ of the control reaction mixture without Des (Fig. 7(B)), while on addition of Des to the control reaction mixture, the ESR peak heights kept unchanged (Fig. 7(C)), suggesting that Des-transition metal complex is active for the HER formation. On the other hand, on addition of CA to the control reaction mixture with Des, the ESR peak heights decreased to $72 \pm 7\%$ of the control reaction mixture with Des (Fig. 7(D)).

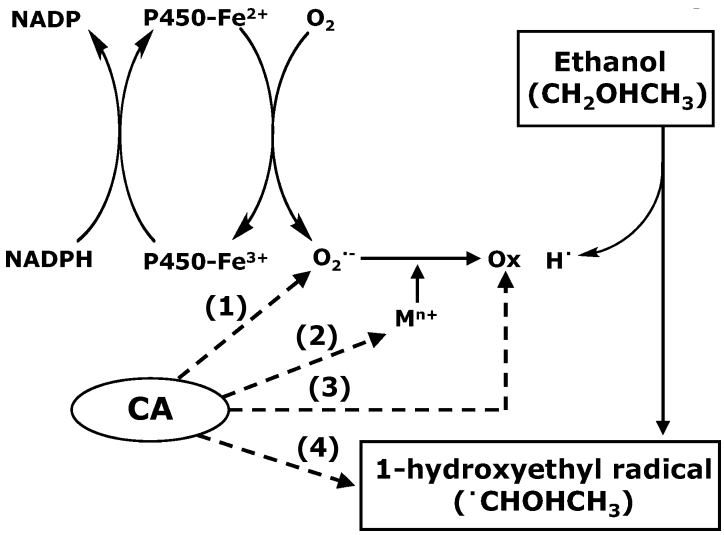

Fig. 8. A possible mechanism for the inhibitory effect on the formation of HER by CA. The **Ox** represents an as yet uncharacterized oxidizing species. The arrows (1), (2), (3) and (4) indicate scavenging $O_2^{\bullet-}$, chelating of iron ions, scavenging the uncharacterized oxidizing species (**Ox**) and scavenging HER by CA, respectively.

Since CA-iron ion complex does not form in the presence of Des, the decrease of the ESR peak heights (72 ± 7% of the control reaction mixture with Des) in the presence of Des appears to be due to the other antioxidative activities rather than chelating. Scavenging $O_2^{\bullet-}$, or scavenging HER, or scavenging the uncharacterized **Ox** may be involved in the other antioxidative activities (Fig. 8). Indeed, CA, gallic acid, dopamine, L-dopa, chlorogenic acid and catechin were reported to be $O_2^{\bullet-}$ scavenger,[21,22,29,30] although quinic acid and ferulic acid cannot scavenge $O_2^{\bullet-}$.[32,39] It has also been known that some flavonoids scavenge the HER generated by radiolysis.[40] Transition metal chelating activity by polyphenols has been reported.[13]

CA inhibits the HER formation through both chelating and the other antioxidative activities in the absence of Des, while CA inhibits the HER formation through the other antioxidative activities rather than chelating in the presence of Des. Therefore, the difference in the decreases of the relative ESR peak height, 50% (in the absence of Des) and 72% (in the presence of Des), seems to be due to the CA chelating of ferric ions. CA seems to chelate of free iron ions since CA can not remove iron ions in hemoproteins. Thus, both chelating and the other antioxidative activities of CA appear to be involved in those inhibitory effects.

Abbreviations

CA	caffeic acid
Des	desferrioxamine mesylate
ESI	electrospray ionization
ESR	electron spin resonance
HER	1-hydroxyethyl radical
HPLC-ESR-MS	high performance liquid chromatography-electron spin resonance-mass spectrometry
MEOS	microsomal ethanol-oxidizing system
MS	mass spectrometry
NADPH	nicotinamide adenine dinucleotide phosphate
Ox	an as yet uncharacterized oxidizing species
4-POBN	α-(4-pyridyl-1-oxide)-*N*-*tert*-butylnitrone

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