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Prooxidant action of rosmarinic acid: Transition metal-dependent generation of reactive oxygen species

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Abstract

Rosmarinic acid and its constituent caffeic acid produced reactive oxygen species in the presence of transition metals. Complex of rosmarinic acid or caffeic acid with iron inactivated aconitase the most sensitive enzyme to oxidative stress. The inactivation of aconitase was iron-dependent, and prevented by TEMPOL, a scavenger of reactive oxygen species, suggesting that the rosmarinic acid/iron-mediated generation of superoxide anion is responsible for the inactivation of aconitase. Direct spectrophotometric determination of hydrogen peroxide and superoxide anion confirmed the rosmarinic acid/iron-dependent production of reactive oxygen species. Treatment of DNA from plasmid pBR322 and calf thymus with rosmarinic acid plus copper caused strand scission and formed 8-hydroxy-2′-deoxyguanosine in DNA. Rosmarinic acid and caffeic acid showed a potent activity that reduces transition metals. These results suggest that transition metals reduced by rosmarinic acid can form superoxide radical by one electron reduction of oxygen molecule: superoxide radical in turn converts to hydrogen peroxide and hydroxyl radical causing the formation of DNA base adduct. Cytotoxicity of rosmarinic acid may be related to the prooxidant action resulting from metal-reducing activity.

Keywords: Rosmarinic acid; Transition metals; Reactive oxygen species; DNA damage; 8-Hydroxy-2′-deoxyguanosine

1. Introduction

Rosmarinic acid is found as a secondary metabolite in medicinal plants such as rosemary and salvia, which is widely used as a culinary herb, especially in Mediterranean dishes, and is also used as a fragrant additive in soaps and other cosmetics (Martinez-Tome et al., 2001). Rosmarinic acid and its constituent caffeic acid have a polyphenolic structure, which can act as antioxidants (Liu et al., 1992; Ho et al., 2000); on the contrary, rosemary extract and rosmarinic acid show antimicrobial, antiviral and anti-inflammatory activities (Aruoma et al., 1996; al-Sereiti et al., 1999; Elgayyar et al., 2001), and induce apoptosis (Hur et al., 2004), suggesting that these compounds can generate reactive oxygen species. In this study we describe the rosmarinic acid-mediated generation of reactive oxygen species. Production of reactive oxygen species was demonstrated by the inactivation of aconitase (EC 4.2.1.3) the sensitive enzyme to active oxygen, and further by the copper-dependent formation of 8-hydroxy-2′-deoxyguanosine in DNA, which is an indicator of hydroxyl radical. Antimicrobial and antiviral effects of rosmarinic acid may be explained by its prooxidant activity, which is closely related to the metal-reducing activity.

2. Materials and methods

2.1. Materials

The sources of materials used in this work were as follows: rosmarinic acid, caffeic acid, *threo*-Ds-isocitrate, TEMPOL (4-hydroxy-2,2,6,6-tetramethylpiperidine-1-oxyl), 8-hydroxy-2′-deoxgyguanosine, neocuproine, and enzymes for DNA hydrolysis from Sigma–Aldrich Japan (Tokyo,

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Japan); NADP-isocitrate dehydrogenase from Oriental Yeast Co. (Tokyo, Japan); double stranded supercoiled plasmid pBR322 from Takara Biomedicals (Shiga, Japan), and 4-aminoantipyrine and sodium *N*-ethyl-*N*-(3-sulfopropyl) *m*-anisidine from Toyobo Co (Osaka, Japan). Other chemicals were obtained from commercial sources. Baker's yeast was purchased locally.

2.2. Determination of the aconitase activity

Yeast cells (10 mg/ml) permeabilized with toluene (Murakami et al., 1980) were treated with rosmarinic acid or caffeic acid in the presence of 0.05 mM $FeSO_4$ and 0.5 mM NaN_3 with or without 1 mM TEMPOL in 40 mM Tris–HCl (pH 7.1). After incubation at 37 °C for 10 min, cells were collected by centrifugation at 800*g* for 5 min and suspended with 50 mM Tris–HCl (pH 7.1) containing 0.5 M sorbitol at the concentration of 200 mg/ml. Aconitase activity was determined by increase in absorbance at 340 nm in the presence of 5 mM citrate, 0.25 mM NADP, 4 mM $MgCl_2$, 10 unit/ml of NADP-isocitrate dehydrogenase (Murakami et al., 2006).

2.3. Determination of hydrogen peroxide and superoxide anion

Determination of rosmarinic acid/metal-mediated formation of reactive oxygen species was carried out on the basis of the measurement of hydrogen peroxide, which depends on the peroxidase-dependent formation of quinoneimine dye according to the method used for determining glucose, glycerol with glucose oxidase and glycerol kinase (Barham and Trinder, 1972). Peroxidase (POD) catalyzes the coupling of H_2O_2 with 4-aminoantipyrine and sodium *N*-ethyl-*N*-(3-sulfopropyl) *m*-anisidine to produce a quinoneimine dye that shows an absorbance maximum at 540 nm (Barham and Trinder, 1972). The increase in absorbance at 540 nm is directly proportional to the free glycerol concentration of the sample. The reaction mixture of 1 ml contained 0.2 mg *N*-ethyl-*N*-(3-sulfopropyl) *m*-anisidine, 0.15 mg 4-aminoantipyrine, 0.5 unit peroxidase, 10 mM Mops-KOH buffer (pH 7.1), and 0.2 mM rosmarinic acid in the absence and presence of 50 μg superoxide dismutase. Reaction was started by addition of 5 μl of $FeSO_4$ (at final concentration of 0.05 mM), and the absorbance at 540 nm was recorded. Formation of superoxide anion was calculated by the difference in the absorbance between the values with or without superoxide dismutase.

2.4. Quantitation of 8-hydroxy-2′-deoxgyguanosine in calf thymus DNA treated with rosmarinic acid and caffeic acid in the presence of copper

Calf thymus DNA was treated with rosmarinic acid or caffeic acid. The reaction mixture of 0.2 ml contained 30 μg of calf thymus DNA, 0.1 mM $CuCl_2$ and various concentrations of rosmarinic acid or caffeic acid in 10 mM Tris–HCl buffer (pH 7.4). The mixture was incubated at 37°C for 60 min. Aliquots were enzymatically hydrolyzed and used for the determination of 8-hydroxy-2′-deoxyguanosine (8-OHdG) with HPLC-ECD method (Yoshino et al., 1999). Statistical analysis was performed by Student's *t*-test.

2.5. Reduction of copper ion

Copper reduction was followed by determining the cuprous ion concentration with neocuproine (Ito et al., 2005). The samples of 0.3 ml contained 10 mM Tris–HCl (pH 7.1), 0.05 mM $CuSO_4$, various concentrations of rosmarinic acid or caffeic acid. The mixture was incubated at room temperature, and the absorbance at 450 nm was recorded.

3. Results

We examined the effect of rosmarinic acid and caffeic acid on the activity of aconitase the most sensitive enzyme to reactive oxygen species (Gardner and Fridovich, 1992; Murakami and Yoshino, 1997; Gardner, 2002) with the permeabilized yeast cells. Addition of rosmarinic acid and caffeic acid with iron caused an effective inactivation of aconitase, whereas, rosmarinic acid alone did not affect the aconitase activity (Fig. 1). The activities of aldolase and glyceraldehyde 3-phosphate dehydrogenase were not at all

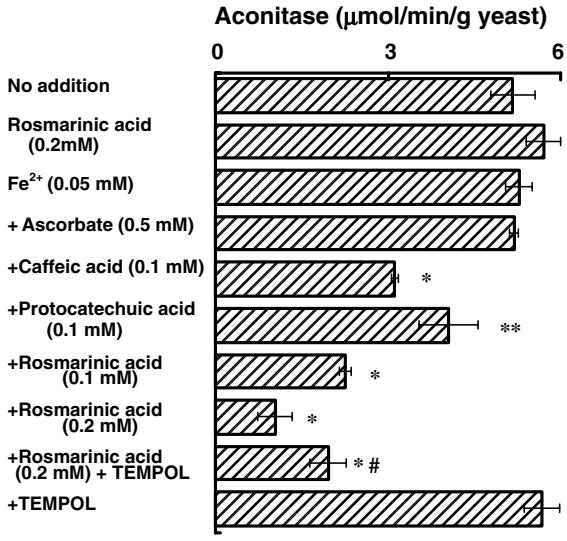

Fig. 1. Effect of rosmarinic acid and its related compounds on the aconitase activity in permeabilized yeast cells. Baker's yeast cells (10 mg/ml) permeabilzied with toluene (Murakami et al., 1980) were incubated with the indicated concentrations of rosmarinic acid or related compounds, 0.05 mM $FeSO_4$ in the presence of 0.5 mM NaN_3 with or without 1 mM TEMPOL in 40 mM Tris–HCl (pH 7.1) at 37 °C for 10 min. Cells were collected and used for determination of enzyme activity. Aconitase activity was determined by increase in absorbance at 340 nm in the presence of 5 mM citrate, 0.25 mM NADP, 4 mM $MgCl_2$, 10 unit/ml of NADP-isocitrate dehydrogenase and 1 mg/ml of yeast. *, $p < 0.001$ (vs control group); **, $p < 0.01$ (vs control group); #, $p < 0.05$ (vs rosmarinic acid-added group).

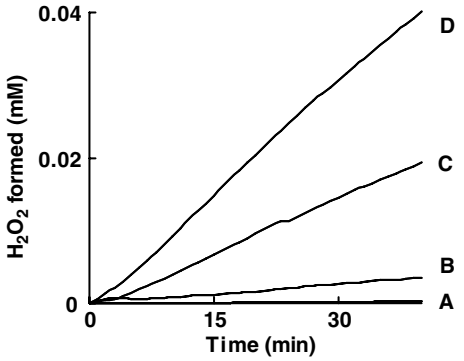

Fig. 2. Rosmarinic acid/iron-mediated formation of hydrogen peroxide and superoxide. The reaction mixture of 1 ml contained 0.2 mg *N*-ethyl-*N*-(3-sulfopropyl) *m*-anisidine, 0.15 mg 4-aminoantipyrine, 0.5 unit peroxidase, 10 mM Mops-KOH buffer (pH 7.1), and 0.2 mM rosmarinic acid in the absence and presence of 50 μg superoxide dismutase. Reaction was started by addition of 5 μl of $FeSO_4$ (at final concentration of 0.05 mM), and the absorbance at 540 nm was recorded. Formation of superoxide anion was calculated by the difference in the absorbance between the values with or without superoxide dismutase. Curve A, No addition; curve B, 0.05 mM $FeSO_4$ added; curve C, 0.2 mM rosmarinic acid with 0.05 mM $FeSO_4$; curve D, 0.2 mM rosmarinic acid with 0.05 mM $FeSO_4$ in the presence of 50 μg/ml superoxide dismutase.

inactivated by rosmarinic acid (data not shown). Pretreatment of cells with TEMPOL (4-hydroxy-2,2,6,6-tetramethylpiperidine-1-oxyl), a scavenger of reactive oxygen species, appreciably protected aconitase from the rosmarinic acid-mediated inactivation (Fig. 1). These results suggest that rosmarinic acid/iron complex produced reactive oxygen species causing the inactivation of aconitase.

Rosmarinic acid/iron-mediated formation of reactive oxygen species was confirmed by the direct determination of hydrogen peroxide and superoxide anion. Fig. 2 shows that addition of iron to the rosmarinic acid solution effectively generated hydrogen peroxide, although iron alone caused only a little production of hydrogen peroxide (Fig. 2, curves A–C). Addition of superoxide dismutase enhanced the formation of hydrogen peroxide (curve D), indicating that the difference between curve C and D corresponds to the production of superoxide anion.

We further evaluated the rosmarinic acid/metal-mediated formation of reactive oxygen species by the strand breaks of plasmid DNA. Double stranded supercoiled structure of plasmid pBR322 DNA with a relatively high electrophoretic mobility is disrupted upon formation of strand breaks, resulting in an open-circle conformation with a reduced electrophoretic mobility in agarose. Linear DNA, formed either by double strand breaks or closely opposed single strand breaks, has a mobility intermediate between that of the supercoiled and open-circular conformation of plasmid DNA (Rahman et al., 1992). Treatment of plasmid with rosmarinic acid plus copper produced a major band of open circular DNA, and the increase in the concentration of rosmarinic acid raised the ratio of a linear form of DNA (Fig. 3). Replacing copper by iron also caused a degradation of plasmid DNA to a similar extent (data not shown).

When calf thymus DNA was treated with ascorbic acid in the presence of $CuCl_2$, 8-OHdG was effectively formed (Yoshino et al., 1999). We examined the effect of rosmarinic acid and caffeic acid on the copper-dependent 8-OHdG formation. Rosmarinic acid effectively produced 8-OHdG in the presence of $CuCl_2$, and addition of caffeic acid with copper also produced 8-OHdG to a lesser extent (Fig. 4). Replacing copper by iron strikingly decreased the production of 8-OHdG as described in the flavonoid-mediated formation of 8-OHdG (Yoshino et al., 1999).

Transition metal-dependent generation of reactive oxygen species requires the reduction of metals for activation of oxygen molecules, and we, thus, analyzed the copper-reducing activity of rosmarinic acid and caffeic acid. Rosmarinic

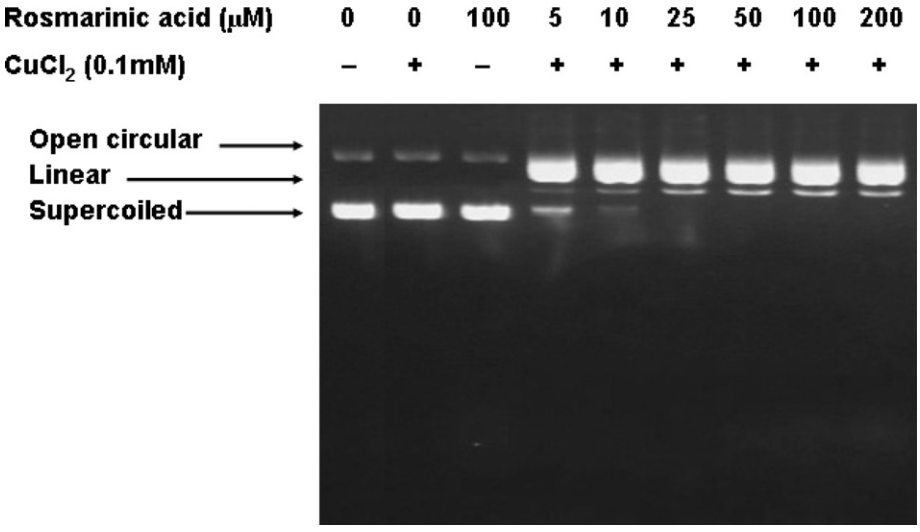

Fig. 3. Agarose gel electrophoretic patterns of plasmid DNA by rosmarinic acid in the presence of Cu(II) ion. pBR322 plasmid DNA (0.5 μg) was incubated for 1 h at 37 °C in the presence of 0.2 mM $CuSO_4$ with the indicated concentrations of rosmarinic acid.

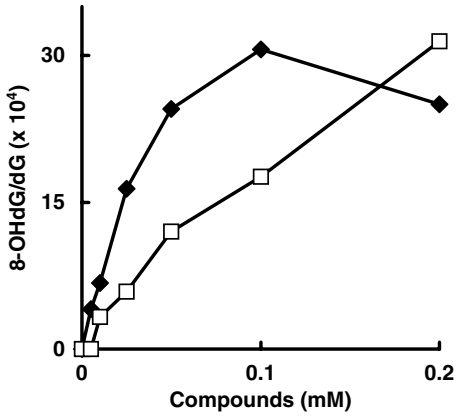

Fig. 4. Effect of rosmarinic acid and caffeic acid on the copper-dependent formation of 8-hydroxy-2′-deoxyguanosine in DNA. Calf thymus DNA was treated with rosmarinic acid or caffeic acid at the indicated concentrations and 0.1 mM $CuCl_2$ in 0.2 ml of 10 mM Tris–HCl buffer (pH 7.1) for 1 h, and 8-hydroxy-2′-deoxyguanosine wad determined by HPLC-ECD method as described previously (Yoshino et al., 1999). ◆, rosmarinic acid; □, caffeic acid.

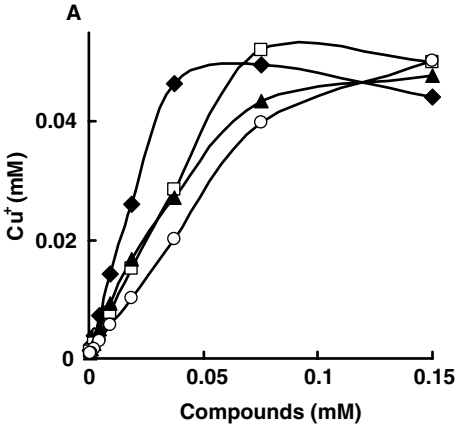

Fig. 5. Effect of rosmarinic acid and its related compounds on the reduction of copper ion. Reaction mixture of 0.3 ml contained 0.05 mM $CuSO_4$, 10 mM Tris–HCl (pH 7.1), various concentrations of additives, and 0.5 mM neocuproine. Mixture was incubated at room temperature. The concentrations of Cu^+ were determined by measuring the absorbance at 450 nm by microplate reader. ◆, rosmarinic acid; ○, ascorbic acid; ▲, protocatechuic acid; □, caffeic acid.

acid reduced cupric ion to cuprous ion potently, and caffeic acid also showed a copper-reducing activity. Reducing activity of rosmarinic acid and caffeic acid was more potent than ascorbic acid (Fig. 5).

4. Discussion

Rosmarinic acid consists of the dimeric structure of caffeic acid, and these compounds are principal ingredients of rosemary extracts, which has been used as food additives, especially in Mediterranean dishes, and food preservative effects of rosmarinic acid were explained by antioxidant action (Martinez-Tome et al., 2001). Recently, we showed that rosmarinic acid can act as a potent inhibitor of superoxide and NO syntheses, and an effective protector against peroxynitrite-mediated damage: their inhibition mechanisms are demonstrated to be partly based on the ability to inhibit the serine phosphorylation of Iκ–Bα (Qiao et al., 2005).

On the other hand, antimicrobial, antiviral and apoptosis-inducing effects of rosmarinic acid may be related to the production of reactive oxygen species (Elgayyar et al., 2001; Aruoma et al., 1996; Hur et al., 2004). Generally, polyphenolic compounds show a potent metal-reducing activity, which is often responsible for the generation of reactive oxygen species by reducing dioxygen in the presence of transition metals. For example, flavonoid compounds can act as a potent prooxidant, causing an oxidative inactivation of aconitase and the formation of 8-hydroxy-2′-deoxyguanosine in DNA (Yoshino et al., 1999). In the present study we examined the prooxidant action of rosmarinic acid with polyphenolic structure in relation to the metal-reducing activity. Rosmarinic acid and caffeic acid could act as prooxidants by generating reactive oxygen species, which was demonstrated by the inactivation of aconitase, the most sensitive to reactive oxygen species (Gardner and Fridovich, 1992; Murakami and Yoshino, 1997; Gardner, 2002). Production of superoxide radical by rosmarinic acid in the presence of iron was confirmed by the inhibitory effect of TEMPOL the scavenger on the inactivation of aconitase. We further ascertained the rosmarinic acid/iron-dependent production of reactive oxygen species by direct measurement of hydrogen peroxide in the presence of superoxide dismutase. Generation of superoxide radical by rosmarinic acid/iron complex can be accounted for by its metal-reducing activity. Rosmarinic acid reduces transition metals including iron and copper. Oxygen molecules accept one electron from ferrous ion to form superoxide radical, which inactivates aconitase by oxidizing the prosthetic iron–sulfur cluster $[4Fe–4S]^{2+}$ at the active site, resulting in the formation of the inactive $[3Fe–4S]^{1+}$ enzymes and then in the release of iron(II) from the enzyme active sites. Superoxide radical in turn generates hydrogen peroxide through the dismutation reaction. Finally, hydrogen peroxide, by interacting with the reduced transition metal such as Fe^{2+}, produces hydroxyl radical the most potent oxidant by means of Fenton reaction. Aconitase is a sensitive indicator of reactive oxygen species, and rosmarinic acid-mediated inactivation of the enzyme may further participate in the enhanced production of hydroxyl radical.

Prooxidant property of rosmarinic acid was further demonstrated by the copper-mediated oxidative formation of DNA base adduct. Rosmarinic acid or caffeic acid/copper-dependent formation of 8-hydroxy-2′-deoxyguanosine is also related to the metal-reducing properties of these compounds. Rosmarinic acid or caffeic acid reduces copper ion located at poly G and GC sequences in DNA, and cuprous ion formed further activates oxygen molecule to form superoxide radical and hydrogen peroxide as discussed above. Hydroxyl radical generated from hydrogen peroxide can react with guanine base to form 8-hydroxy-2′-deoxyguanosine (Yoshino et al., 1999). Rosmarinic acid with

iron could also induce DNA fragmentation to the same extent, but produced 8-OHdG in DNA to a lesser extent, suggesting that iron cannot bind DNA at the specific sites, unlike copper ion that binds DNA preferentially at polyG and GC sequences.

Antimicrobial, antiviral and anti-inflammatory action (Aruoma et al., 1996; al-Sereiti et al., 1999; Elgayyar et al., 2001), and apoptosis-inducing properties of rosmarinic acid (Hur et al., 2004) can be explained by the production of reactive oxygen species due to its metal-reducing activity, and may have a potential for chemotherapeutic use.

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