Antiangiogenic Effect of Carnosic Acid and Carnosol, Neuroprotective Compounds in Rosemary Leaves

Tomoko KAYASHIMA¹ and Kiminori MATSUBARA²,†

¹Human Life and Environment Course, Faculty of Culture and Education, Saga University, 1 Honjou, Saga 840-8502, Japan
²Department of Human Life Science Education, Graduate School of Education, Hiroshima University, 111 Higashi-Hiroshima, Hiroshima 739-8524, Japan

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Carnosic acid, a diterpene in rosemary, is considered to be beneficial in the prevention of chronic neurodegenerative diseases. Recently, it has been found that drugs with antiangiogenic activity lower the risk of neurodegenerative diseases. Thus it is of interest whether carnosic acid has antiangiogenic activity. In this study, carnosic acid suppressed microvessel outgrowth on ex vivo angiogenesis assay using a rat aortic ring at higher than 10 μM. The antiangiogenic effect of carnosic acid was found in angiogenesis models using human umbilical vein endothelial cells with regard to tube formation on reconstituted basement membrane, chemotaxis and proliferation. Although the carnosol in rosemary also suppressed angiogenesis, its effect was not more potent than that of carnosic acid in the ex vivo model. These results suggest that carnosic acid and rosemary extract can be useful in the prevention of disorders due to angiogenesis, and that their antiangiogenic effect can contribute to a neuroprotective effect.

Key words: angiogenesis; aortic ring; endothelial cells; carnosic acid; carnosol

Carnosic acid, a diterpene, is abundant in the herb rosemary (Rosmarinus officinalis)¹–³ and is also found in the herb sage (Salvia officinalis).¹,² It is well known that carnosic acid has potent antioxidant activity in vitro. An extract of rosemary leaf has strong antioxidant activity due to carnosic acid and carnosol.¹,² Since antioxidants in food protect cells against free-radical species and oxidative stress, they have been studied extensively.⁴ Carnosic acid has a neuroprotective effect in vitro and in vivo⁵ due not only to its antioxidant activity but also to the induction of antioxidant enzyme expression via activation of the Kelch-like ECH-associated protein 1 (Keap1)/nuclear factor erythroid 2-related factor 2 (Nrf2) transcriptional pathway.⁵,⁶ suggesting that it is a novel neuroprotective compound. It is expected to be beneficial in the prevention of chronic neurodegenerative diseases such as Alzheimer’s disease and dementia, that will probably become more serious concerns around the world.

Dietary factors have been studied as to whether they reduce the risk of neurodegenerative diseases. Although there are no definitive conclusions on dietary factors reducing at the risk, much research suggests that antioxidants including vitamins and phenolic compounds can lower it,⁶ since reactive oxygen species and oxidative stress are associated with neuronal damage.⁷ In fact, a recent epidemiological study found that consumption of green tea, which contains antioxidant phenolic compounds such as catechins, lowers the risk of dementia.⁸ Thus, antioxidant compounds in food should protect against neuronal damage due to oxidative stress. On the other hand, a new concept of the neuroprotective effects of biologically active substances has emerged: an inhibitory effect on angiogenesis should contribute to a neuroprotective effect.⁹ We have reported the antiangiogenic effects of several antioxidants including vitamins and phenolic compounds.⁴,⁵,¹⁰ The antioxidant activity of polyphenols should be involved in the antiangiogenic effects because reactive oxygen species stimulate angiogenesis.¹¹⁻¹³ In sum, antioxidant compounds with antiangiogenic activity should be more potent neuroprotective compounds.

Angiogenesis is the formation of new blood vessels from a pre-existing blood vessel. It is involved in tumor growth and various other diseases.¹⁵ Thus an angiogenesis inhibitor should be useful in the prevention and treatment of the angiogenesis-dependent disorders. In addition, as described above, accumulating evidence suggests that an angiogenesis inhibitor would contribute to suppression of neurodegenerative diseases.⁹ Thus, it is of interest to clarify whether carnosic acid inhibits angiogenesis and has a protective effect against neurodegenerative diseases by suppressing angiogenesis. In this study, we explored the effect of carnosic acid and carnosol, which co-exists in rosemary, on angiogenesis.

Materials and Methods

Materials. Carnosic acid and carnosol were purchased from Sigma-Aldrich (St. Louis, MO) and were prepared as stock solution in dimethyl sulfoxide (DMSO). Human recombinant vascular endothelial growth factor (VEGF) was from R&D Systems (Minneapolis, MN). WST-1 reagent was from Dojindo Laboratories (Kumamoto, Japan). The other reagents used were of special grade and were commercially available.

Ex vivo angiogenesis assay. Male Wistar rats (6 weeks old, Charles River Laboratories, Kanagawa, Japan) were housed in metal cages in a room under controlled temperature (24 ± 1°C) and a 12 h light-dark cycle (lights on 08:00–20:00 h). The rats had free access to food and

¹ To whom correspondence should be addressed. Fax: +81-82-422-7133; E-mail: kmatsuba@hiroshima-u.ac.jp
deionized water, and were maintained according to the guide lines of the Hiroshima University Animal Research Committee. We obtained Committee approval for this animal experiment.

The ex vivo angiogenesis assay was performed by slightly modified methods, as described previously.16,17 A male Wistar rat (body weight about 200 g) was sacrificed by bleeding from the right femoral artery under anesthesia with diethyl ether. To avoid contamination with blood, the thoracic aorta was removed and washed with RPMI 1640 Medium (Gibco, Grand Island, NY). The artery was then turned inside out and cut into segments of about 1.0–1.5 mm. A collagen gel (gel matrix solution) was then prepared with 8 volumes of porcine tendon collagen solution (3 mg/mL) (Cellmatrix IA, Nitta Gelatin, Osaka, Japan), 1 volume of 10 × Eagle’s MEM (Gibco), and 1 volume of reconstitution buffer (80 mM NaOH and 200 mM HEPES). These solutions were mixed gently at 4°C. The aortic segments were placed in the center of a well on a 6-well culture plate and covered with 0.5 mL of gel matrix solution, which was reconstituted as described above. The solutions were allowed to gel at 37°C for 20 min. The collagen gel was overlaid with 2 mL of RPMI 1640 Medium containing 1% ITS+ (BD Biosciences, Bedford, MA) and carnosic acid (5–100 μM). This level of carnosic acid is nontoxic to neural cells, as a neuroprotective compound.14 Incubation was carried out for 7 d in a humidified atmosphere containing 5% CO2 in air at 37°C. Capillary length was estimated by phase-contrast microscopy by measuring the distance from the cut end of the aortic segment to the approximate mid-point of the capillary. Microscopic fields were photographed using a digital camera (DS-5330A System, Olympus, Tokyo). Capillary length was measured using Adobe Photoshop software CS3. Reported values represent averages of 3–6 culture samples.

Endothelial cell functions.

Cell culture. HUVECs were purchased from Kurabo Industries (Osaka, Japan). The cells were grown in HuMedia EG2 Medium (Kurabo), which is modified MCDB 131 Medium containing 2% fetal bovine serum, 10 ng/mL of recombinant human epidermal growth factor, and 10 μg/mL of amphotericin B, 5 ng/mL of recombinant human basic fibroblast growth factor, and 10 μg/mL of heparin, in a humidified incubator with 5% CO2 at 37°C. Subcultures were obtained by treating the HUVEC culture with Hanks’-based enzyme-free cell dissociation buffer solution (Gibco). We used HUVECs in passages 3–7 in this experiment.

HUVEC tube formation assay. Tube formation assay was performed using BD Matrigel™ (BD Biosciences). Briefly, solid gels were prepared following the manufacturer’s manual on a 96-well tissue culture plate. HUVECs (1 × 104 cells/mL) in HuMedia EG2 Medium containing carnosic acid (5–100 μM), carnosol (10, 50 μM), or vehicle (DMSO). These solutions were mixed gently at 4°C, and 5 μg/mL of recombinant human basic fibroblast growth factor, and 10 μg/mL of heparin in a humidified incubator with 5% CO2 at 37°C. Tube cultures were obtained by treating the HUVEC culture with Hanks’-based enzyme-free cell dissociation buffer solution (Gibco). We used HUVECs in passages 3–7 in this experiment.

HUVEC chemotaxis assay. Chemotaxis assay was carried out in a modified Boyden chamber.18 A microporous membrane (8 μm) of 24-well tissue culture inserts (Becton, Dickinson and Company, Tokyo) was coated with 0.1% gelatin. HUVECs were suspended in Medium 199 (Gibco) with 0.1% bovine serum albumin (BSA), and then seeded in the chamber (2.5 × 104 cells/mL). The well was filled with 400 μL of Medium 199 containing 0.1% BSA and 10 ng/mL of human recombinant VEGF (R&D Systems) with carnosic acid (5–100 μM), carnosol (10, 50 μM), or vehicle (DMSO). The assembled chamber was incubated for 6 h in humidified 5% CO2 at 37°C. Non-migrated cells on the upper surface of the membrane were removed by scrubbing with a cotton swab. The cells on the lower surface of the membrane were fixed with methanol and stained with Diff-Quick Stain (Sysmex, Kobe, Japan). Migrated cells were counted in three fields of each membrane in control and treated groups. The average number of cells in each field was calculated. The experiment was performed in triplicate or quadruplicate.

HUVEC proliferation assay. A HUVEC suspension (1.5 × 104 cells/mL) was plated onto 96-well plates (100 μL/well), cultured in HuMedia EG2 Medium, and incubated for 24 h in a humidified incubator under 5% CO2 at 37°C. The medium was replaced with fresh HuMedia EG2 Medium containing carnosic acid (5–100 μM), carnosol (5–100 μM), or vehicle (DMSO). After 72 h, 10 μL of WST-1 reagent was added to each well of a 96-well plate and incubated for 4 h. Absorbance was measured at 450 nm using a microplate spectrophotometer (Model 680, Bio-Rad, Hercules, CA). Reported values represent averages for 5 or 6 wells.

Statistical analysis. Values are presented as means ± SEM. Data were analyzed by one-way analysis of variance followed by the Dunnet test. Differences with p < 0.05 were considered significant.

Results and Discussion

Effects of Carnosic Acid and Carnosol on ex vivo angiogenesis

To determine whether carnosic acid has antiangiogenic activity, we examined its effect in an ex vivo angiogenesis model using a rat aortic ring. As shown in Fig. 1A, in the absence of carnosic acid, microvessels appeared from the ends of aortic rings and elongated. On the other hand, in the presence of carnosic acid (100 μM), microvessel growth was completely inhibited (Fig. 1A). The inhibitory effect of carnosic acid occurred in a dose-dependent manner and statistically significant at values higher than 10 μM (p < 0.05) (Fig. 1B). The present study suggests that carnosic acid, at the lower concentrations found in rosemary leaves (about 2–7% of dry material),3,19 functions as an angiogenesis inhibitor. Although the carnosic acid content of rosemary leaves is variable due to seasonal and environmental factors, genetics, and leaf age,3 carnosic acid is the major component of rosemary leaf extract. In an animal model, an anticancer effect of rosemary leaf extract has been reported.20 Furthermore, administration of rosemary extract (2 g/kg of body weight) had no serious side
Carnosic acid significantly inhibited HUVEC migration at more than 10 µM (p < 0.05) (Fig. 3). To determine whether carnosic acid inhibits endothelial cell growth, we examined the effect of carnosic acid on HUVEC proliferation. HUVECs were treated for 72 h with various concentrations of carnosic acid. As shown in Fig. 4, there were significant differences (p < 0.01) between control and carnosic acid-treated HUVECs (10–100 µM). This suggests that carnosic acid exerts an antiangiogenic effect by suppressing endothelial cell functions, especially chemotaxis and proliferation.

Carnosol also inhibited HUVEC tube formation on reconstituted basement membrane, chemotaxis and proliferation (Figs. 2–4). Both carnosic acid and carnosol strongly inhibited HUVEC proliferation (Fig. 4), suggesting that the main antiangiogenic mechanism of these compounds in rosemary is suppressing proliferation, but effects of carnosol on tube formation and chemotaxis were different from those of carnosic acid. Hence the antiangiogenic mechanisms of carnosic acid and carnosol on endothelial cells might be different, at least in part, and they were involved in the weak inhibitory effect of carnosol in the ex vivo model.

**Effects of carnosic acid and carnosol on HUVEC functions**

Because angiogenic processes involve endothelial cell migration, proliferation, and tube formation, we examined the effects of carnosic acid and carnosol on endothelial cell functions to determine how carnosic acid and carnosol exert antiangiogenic effect. First, HUVEC tube formation assay was performed. HUVECs inoculated on reconstituted basement membrane (Matrigel™) migrated, attached to each other, and finally formed tube structures (Fig. 2A). Carnosic acid suppressed HUVEC tube formation at 50 µM (p < 0.001), and completely inhibited it at 100 µM (Fig. 2A and B). Next, the effect of carnosic acid on HUVEC migration stimulated with VEGF was examined in a gelatin-coated Boyden chamber. As shown in Fig. 3, VEGF strongly stimulated HUVEC migration. Carnosic acid significantly inhibited HUVEC migration stimulated with VEGF at more than 10 µM (p < 0.05) (Fig. 3). To determine whether carnosic acid inhibits
Recently, the neuroprotective effect of carnosic acid has attracted considerable attention, since neurodegenerative diseases such as Alzheimer’s disease and dementia are public health problems in modern society.25–27) Although carnosol also protects neuronal cells against oxidative stress, the neuroprotective effect of carnosic acid is much more potent than that of carnosol.28) Various risk factors for neurodegenerative diseases are associated with vascular disease, smoking, and diabetes.29) On the other hand, it has been suggested that antioxidants in food can reduce the risk of Alzheimer’s disease.30) This is consistent with the conjecture that oxidative stress might be related to Alzheimer’s disease.30) It is notable that carnosic acid induces the expression of antioxidant enzymes through activation of the Keap1/Nrf2 transcriptional pathway and protects neurons from oxidative stress.5) Thus it can be a beneficial food compound in the prevention of neurodegenerative diseases as a potent antioxidant and inducer of antioxidant enzymes.

Our finding as to the antiangiogenic effect of carnosic acid suggests a new function of carnosic acid as a neuroprotective compound. There is an emerging concept that angiogenesis plays an important role in Alzheimer’s disease and that antiangiogenic agents can lower the risk.29) It has been suggested that angiogenesis induces the deposition of β-amyloid plaques and the secretion of a neurotoxic peptide, leading to the progression of Alzheimer’s disease.31) More recently, angiogenesis has been observed in the brain of an Alzheimer’s disease model mouse.32) Hence antiangiogenic compounds in food can contribute to brain protection, and it is of interest whether carnosic acid has antiangiogenic activity. Although it has been reported that a carnosic acid synthesis derivative, peracetylated carnosic acid, strongly inhibited endothelial functions in using human microvascular endothelial cells on in vitro assay,32) the effect of carnosic acid, a natural compound in rosemary, on angiogenesis was not clear. Here, we report the first evidence that carnosic acid has antiangiogenic activity, suggesting that it is a potent neuroprotective compound, in part through inhibiting angiogenesis. The exact mechanism by which carnosic acid inhibits angiogenesis is not clear at this moment. However, like several antioxidant compounds, the antioxidant activity of carnosic acid probably plays an important role in suppressing angiogenesis.

Carnosic acid and carnosol have catechol structure that is oxidized to a quinone type, and antioxidant property of carnosic acid at least is influenced by its chemical structure.33) The various effects of carnosic acid and carnosol on angiogenesis are also of interest. Although the two compounds have similar biological activities in PC12 cells and 3T3-L1 cell differentiation into adipocytes, the details of their molecular mechanisms are different.33–35) Both compounds phosphorylate Akt and ERK (extracellular signal-regulated kinase) in PC12 cells, but the peaks of phosphorylation of ERK are different, 12 h and 0.5 h after stimulation by carnosic acid and carnosol respectively.33,34) In addition, carnosic acid suppresses 3T3-L1 cell differentiation more effectively than carnosol.35) Thus it is possible that various antiangiogenic effects of carnosic acid and carnosol are due to their effects on endothelial cell signal pathways. Research on the molecular mechanisms and structure-activity relationships is currently underway.

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