Carnosic acid prevents the migration of human aortic smooth muscle cells by inhibiting the activation and expression of matrix metalloproteinase-9

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The migration and matrix metalloproteinase (MMP) activation of vascular smooth muscle cells may play key roles in the development of atherosclerosis. Carnosic acid (CA) is a phenolic compound found in herbs, including rosemary and sage. Previous studies indicated that CA possesses antioxidant activity in vitro. In this study, we investigated the effects of CA on TNF-α-induced cell migration, the formation of intracellular reactive oxygen species, the translocation of NF-κB and the activation and expression of MMP-9 in human aortic smooth muscle cells (HASMC). The Matrigel migration assay showed that CA (10 and 20 μmol/l) effectively inhibited TNF-α-induced migration of HASMC as compared with the control group. To explain this inhibitory effect, MMP-9 was assayed by gelatin zymography and Western blot. The results indicated that CA inhibited MMP-9 activity and expression. Furthermore, the production of reactive oxygen species and the nuclear translocation of NF-κB p50 and p65 induced by TNF-α were dose-dependently suppressed by CA pretreatment. These results indicate that CA has anti-inflammatory properties and may prevent the migration of HASMC by suppressing MMP-9 expression through down-regulation of NF-κB.

**Carnosic acid: Human aortic smooth muscle cells migration: Matrix metalloproteinase-9: NF-κB: Atherosclerosis**

Atherosclerosis is a progressive pathological disorder that often leads to CVD and cerebrovascular diseases. Despite improved pharmacological agents and changes in lifestyle, atherosclerosis is still a leading cause of mortality and morbidity in industrialized countries(6–11). This chronic inflammatory disease is driven by risk factors that cause oxidative and inflammatory mechanisms(12–20). Oxidative stress may trigger the p50/p65 heterodimer. The dimer is retained in the cytoplasm in an inactive state through interaction with inhibitory proteins(21–23). Upon activation of NF-κB, a large number of genes are induced, including various inflammatory cytokines, adhesion molecules and MMP(12–14)

NF-κB activation, which is followed in vitro by elevation in free radical levels, was demonstrated to be inhibited by antioxidants such as gallates, caffeic acid, curcumin and others(15–17). Carnosic acid (CA), a major phenolic constituent in rosemary *Rosmarinus officinalis* and sage *Salvia officinalis*(18,19), has a typical O-diphenol structure (Fig. 1). Most diphenol compounds have been shown to have potent chain-breaking antioxidant activity(20–22). Previous studies have indicated that the extracts of rosemary and sage possess antioxidant, anti-inflammatory effects(23,24), and inhibit lipid absorption in man(25).

Abbreviations: CA, carnosic acid; DPPH, 2,2-diphenyl-1-picrylhydrazyl; HASMC, human aortic smooth muscle cells; MMP, matrix metalloproteinase; MTT, 3-(4,5-dimethyl-2-hydroxymethyl-1,3-propanediol hydrochloride.

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Materials and methods

2,2-Diphenyl-1-picrylhydrazyl scavenging assay

The free radical scavenging effect was determined using the free radical generator 2,2-diphenyl-1-picrylhydrazyl (DPPH) proposed by Yamaguchi et al. 

Trolox equivalent antioxidant capacity assay

The Trolox equivalent antioxidant capacity (TEAC) was determined according to the method of Miller et al. 

Inhibition of LDL oxidation

LDL was isolated after ultracentrifugation as described previously. The LDL fraction was dialysed against a PBS buffer of pH 7.4 without EDTA in the dark, filtered through a 0.45 μm filter, stored at 4°C under nitrogen, and used within 24–72 h. The LDL was oxidized using classical copper-induced LDL auto-oxidation. Increasing concentrations of CA (approximately 0–10 μmol/l) dissolved in dimethyl sulphoxide were added to the incubation media. Incubations were carried out at 37°C to measure the formation of conjugated dienes. Briefly, LDL total cholesterol (0.9 mg/ml) was incubated in PBS in the presence of CuSO₄ (50 μmol/l). After incubation, 150 μl EDTA (2 mmol/l) was added. A 100 μl portion of the mixture was then transferred to vials containing 0.9 ml 2-propanol. The precipitates were removed via centrifugation. The concentration of conjugated diene in the supernatant was determined by measuring the absorbance at 234 nm.

Cell culture

HASMC were purchased from the Food Industry Research and Development Institute, Chin-Tsu, Taiwan (CCRC 60 293). They were maintained in Ham’s F12K medium containing 10% fetal bovine serum, 2 mmol/l l-glutamine, 1.5 g/l sodium bicarbonate, 10 mmol/l HEPES, 10 mmol/l (N-tris) hydroxymethyl-2-aminoethanesulfonic acid, 0.05 mg/ml ascorbic acid, 0.01 mg/ml transferrin, 0.01 mg/ml insulin, 10 ng/ml sodium selenite and 0.03 mg/ml epidermal growth factor. All experiments were performed with HASMC in passages 21–31, which had been grown to 80–90% confluence and made quiescent by serum starvation (0.1% fetal bovine serum) for at least 24 h.

Cell viability assay using 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide

The cytotoxic effect of CA on HASMC was investigated using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay. The principle of this assay is that mitochondria dehydrogenase in viable cells reduces MTT to a blue formazan product. Briefly, the cells were grown in ninety-six-well culture plates at a density of 1 x 10³ cells per well in F-12K culture medium and incubated with various concentrations of CA for 24 h. Then, 10 μl MTT (5 mg/ml) was added to each well and incubation was allowed to continue at 37°C for an additional 4 h. The medium was then carefully removed, so that the formazan crystals that had formed were not disturbed. Dimethyl sulphoxide (100 μl), which solubilizes formazan crystals, was added to each well, and absorbance of the solubilized blue formazan was measured at an optical density of 590 nm using the μQuant Microplate Spectrophotometer (Bio-Tek, VT, USA). All determinations were performed according to three individual experiments. Each individual experiment included two duplicated experiments. The data are shown as means and standard deviations, as a percentage of the control.

Examination of matrix metalloproteinase-9 by gelatin zymography

MMP-9 activity in conditioned medium of cultured HASMC was analysed by substrate-gel electrophoresis (zymography) using SDS-PAGE (10%) containing 0.1% gelatin. Substrate gel zymographic quantification of the activity of MMP-9 was performed with a Mini-Protein II apparatus from Bio-Rad, according to a method described previously. Cells were grown to sub-confluence, rinsed with PBS, and then incubated in serum-free medium for 24 h. Equal volumes of samples of conditioned cell culture medium were mixed with sample buffer containing 62.5 mmol/l 2-amino-2-hydroxyethyl-1,3-propanediol hydrochloride (Tris–HCl) (pH 6.8), 10% glycerol, 2% SDS and 0.00625% (w/v) bromophenol blue, loaded on to the gel and separated by electrophoresis.
Thereafter, gels were washed three times for 30 min at room temperature in buffer (50 mmol/l Tris–HCl, pH 8.0, 5 mmol/l CaCl2, 0.02 % Na3VO4, and 2.5 % Triton X-100) and incubated for 18 h at 37°C with the same buffer, minus Triton X-100. Gels were stained with Coomassie Brilliant Blue R-2500 (0.1 %) and destained in 5 % methanol and 7 % acetic acid. Gelatinolytic activity was represented as a clear band on a blue background.

**Western blot analysis for the expression of matrix metalloproteinase-9**

HASMC were treated with various concentrations of CA in the presence of 100 ng/ml TNF-α. Cellular lysates were prepared in a lysis buffer containing 10 mmol/l Tris–HCl (pH 8), 0.32 mol/l sucrose, 5 mmol/l EDTA, 1 % Triton X-100, 2 mmol/l 1,4-dithiothreitol and 1 mmol/l phenylmethylsulphonyl fluoride. The cells were disrupted and extracted at 4°C for 30 min. After centrifugation at 13 000 rpm for 15 min, the cell lysate was obtained as the supernatant. Protein concentrations were measured using the Bradford assay. Total protein (20 µg) was subjected to SDS–PAGE (10 %) and blotted on polyvinylidene difluoride membranes. Soaking the membrane in PBS–Tween 20 buffer containing 50 g/l non-fat milk blocked non-specific binding. The membrane was incubated with monoclonal mouse anti-human β-actin (1:1000) and polyclonal rabbit anti-human MMP-9 (1:1000). Subsequently, the membrane was incubated with sheep anti-mouse IgG antibody (1:5000) and goat anti-rabbit IgG antibody (1:5000) (Abcam, Cambridge, UK). The protein levels were determined using enhanced chemiluminescence film (Amersham, IL, USA). Incubation with mouse anti-human β-actin antibody was also performed as an internal control. Results were quantified with a scanning densitometer using an image analysis system with software.

**ELISA-based NF-κB assay**

In addition to gel-shift assays, an ELISA-based kit was used for quantitative detection of NF-κB activity. Nuclear protein extracts of HASMC were prepared using a Trans AM Nuclear Extract Kit (Trans AM, CA, USA). For each sample, 20 µl of nuclear extracts (5 µg protein) were used according to the manufacturer’s instructions. Nuclear extracts were incubated in the oligonucleotide-coated wells for 60 min. Where indicated, a competitor for NF-κB binding (NF-κB wild-type consensus oligonucleotide) was added in molar excess prior to the probe. The wells were then washed and incubated with the primary antibodies for p50 and p65 for 60 min. After incubation with a horseradish peroxidase-conjugated secondary antibody, a substrate was added to produce a colour reaction; the reaction was quantitated by a µQuant Microplate Spectrophotometer (Bio-Tek). The absorbance was read at 590 nm and the blank was subtracted from all measurements.

**Cell migration assay**

The invasion of vascular SMC through the extracellular matrix was determined by a commercial cell invasion assay kit (Chemicon, CA, USA) (31). HASMC (1.5 × 10^5 cells/300 µl) were resuspended in conditioned medium collected after pretreatment with CA and TNF-α-treated cells for 23 h, and added to the upper compartments of the migration chamber. Then, 500 µl of the same conditioned medium was added to the lower compartment of the migration chamber. Cells without TNF-α-treated conditioned medium served as the control. The migration chambers were incubated at 37°C for 24 h in 5 % CO2. After incubation, the inserts were removed from the wells, and the cells on the upper side of the filter were removed using cotton swabs. The filters were fixed, and stained according to the manufacturer’s instructions. Then, 100 µl of the dye mixture was transferred to a ninety-six-well plate, and the optical density was measured at 560 nm.

**Measurement of intracellular reactive oxygen species**

HASMC were pretreated with 10 and 20 µmol/l CA for 1 h and induced by TNF-α (100 ng/ml) for 23 h. The cells were then incubated for 30 min with 10 µmol/l 2,7-dichlorofluorescein diacetate, which is converted to dichlorofluorescein by intracellular esterase. The latter was then oxidized by ROS to the highly fluorescent dichlorofluorescein. The fluorescence of each dish was immediately analysed at an excitation wavelength of 485 nm and an emission wavelength of 528 nm by an FL× 800 microplate fluorescence reader (Bio-Tek) (32). All measurements were at least triplicated.

**Statistical analyses**

Results are shown as means and standard deviations. Statistical analyses of MTT were performed using one-way ANOVA followed by Dennett’s test; the other analyses were performed using one-way ANOVA followed by Duncan’s multiple range test. A value of P<0.05 was considered statistically significant.

**Results**

**The antioxidative capacity of carnosic acid in vitro**

The free radical scavenging effect of CA was determined using the free radical generator DPPH; the IC50 of the DPPH assay was 35.9 (SD 1.7) µmol/l (Table 1). The LDL oxidation assay was inhibited by CA and the IC50 of the inhibited LDL oxidation was 5.63 (SD 0.19) µmol/l (Table 1). The TEAC assay is based on the reduction of the 2, 2′-azino-bis

**Table 1. Antioxidative capacity of carnosic acid in vitro**

<table>
<thead>
<tr>
<th>Inhibition of LDL oxidation</th>
<th>DPPH radicals scavenging ability</th>
<th>TEAC assay</th>
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<tr>
<td>(IC50, µmol/l)</td>
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<td>Mean</td>
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<tr>
<td>TEAC</td>
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DPPH, 2,2-diphenyl-1-picrylhydrazyl; TEAC, Trolox equivalent antioxidant capacity.

*For details of procedures, see Materials and methods IC50 values were obtained from the concentration response curves.*
Carnosic acid (CA) inhibits the protein expression of matrix metalloproteinase-9 (MMP-9) in human aortic smooth muscle cells

The effect of CA on TNF-α-induced expression of MMP-9 was assessed by Western blot analysis. (A), Representative Western blot showing MMP-9 protein levels in cell lysates (top) and suppressed by CA pretreatment. The 20 μmol/l CA treatment was more effective at decreasing nuclear translocation of NF-κB p65 than the 10 μmol/l CA treatment.

Carnosic acid suppresses nuclear translocation of NF-κB p50 and p65 in TNF-α-induced human aortic smooth muscle cells

To determine whether the inhibitory effect of CA on the TNF-α-induced expression of MMP-9 is mediated via NF-κB, we measured the nuclear translocation of p50 and p65 of the NF-κB family. Treatment of TNF-α (100 ng/ml) for 23 h enhanced the nuclear translocation of p50 (Fig. 4) and p65 (Fig. 5). Pretreatment of HASMC with 10 and 20 μmol/l CA prior to TNF-α stimulation significantly prevented the nuclear translocation of p50 and p65. In Fig. 4, the 20 μmol/l CA treatment was more effective at decreasing nuclear translocation of NF-κB p65 than the 10 μmol/l CA treatment.

Carnosic acid suppresses TNF-α-induced human aortic smooth muscle cell migration

As shown in Fig. 6, the migration of HASMC increased after treatment with TNF-α relative to that of TNF-α-untreated control cells. The stimulatory effect of TNF-α significantly decreased after CA pretreatment. The 20 μmol/l CA treatment was more effective on decreasing the level of HASMC migration than the 10 μmol/l CA treatment.

Fig. 2. Effect of carnosic acid (CA) on the matrix metalloproteinase-9 (MMP-9) activity of TNF-α-induced human aortic smooth muscle cells. HASMC were pretreated with 10 and 20 μmol/l CA for 1 h, and induced by TNF-α (100 ng/ml) for an additional 23 h. (A), The activation of MMP-9 was assessed by Western blot analysis. (B), Densitometric analysis was conducted with image analysis system software to quantify MMP-9 protein levels in cell lysates (top) and β-actin (bottom). (A), Representative Western blot showing MMP-9 protein levels in cell lysates (top) and β-actin (bottom). (B), Densitometric analysis was conducted with image analysis system software to quantify Western blot data. Values are means with their standard deviations depicted by vertical bars (n = 3). Each individual experiment included two duplicated experiments. a,b,c Mean values with unlike letters were significantly different (P<0.05). C, control without TNF-α or CA; CA10, 10 μmol/l CA; CA20, 20 μmol/l CA; TNF, TNF-α.

Fig. 3. Carnosic acid (CA) inhibits the protein expression of matrix metalloproteinase-9 (MMP-9) in human aortic smooth muscle cells (HASMC). HASMC were pretreated with 10 and 20 μmol/l CA for 1 h, and induced by TNF-α (100 ng/ml) for 23 h. The expression of MMP-9 was assessed by Western blot analysis. (A), Representative Western blot showing MMP-9 protein levels in cell lysates (top) and β-actin (bottom). (B), Densitometric analysis was conducted with image analysis system software to quantify Western blot data. Values are means with their standard deviations depicted by vertical bars (n = 3). Each individual experiment included two duplicated experiments. a,b,c Mean values with unlike letters were significantly different (P<0.05). C, control without TNF-α or CA; CA10, 10 μmol/l CA; CA20, 20 μmol/l CA; TNF, TNF-α.
Carnosic acid suppresses TNF-α-induced reactive oxygen species generation

The production of ROS was induced by TNF-α and decreased by CA. The 20 μmol/l CA treatment was more effective at reducing ROS generation than the 10 μmol/l CA treatment (Fig. 7).

Discussion

In the present study, we investigated the effect of CA on HASMC migration and TNF-α-induced MMP-9 activation. Gelatin zymography and Western blot assays revealed that CA lowered the level of secretion and protein expression of MMP-9, as well as suppressed the nuclear translocation of the NF-κB p50 and p65. In addition, CA effectively inhibited the TNF-α-induced migration of HASMC.

Sage (*S. officinalis*) and rosemary (*R. officinalis*) are Labiate herbs commonly used in cooking and folk medicines around the world (21). CA is a phenolic diterpene compound found in sage (2–5 mg CA/g sage) and rosemary (12–15 mg CA/g rosemary) (18, 19). It is a lipophilic antioxidant that scavenges singlet oxygen, hydroxyl radicals and lipid peroxyl radicals, thus preventing lipid peroxidation (20–22). In the present *in vitro* study, we found that CA could scavenge DPPH radicals, alkoxyl radicals (RO) and lipid peroxyl radicals (ROO) (Table 1). Furthermore, we found that CA has the ability to suppress TNF-α-induced intracellular ROS production (Fig. 7), and that its antioxidative ability is approximately 5–6-fold more potent than Trolox (Table 1).

The migration of SMC from the tunica media to the subendothelial region is a key event in the development and progression of atherosclerosis (7). MMP (MMP-2 and MMP-9) activity may contribute to the pathogenesis of atherosclerosis by facilitating the migration of SMC (33, 34). Although MMP-2 and MMP-9 have similar substrate specificities, the regulation of their expression is different. MMP-2 is constitutively expressed in several cell types, including SMC, and its expression is not induced by cytokines or growth factors. In contrast, MMP-9 can be induced by TNF-α in SMC (33, 34). Therefore, we have investigated the effect of CA on TNF-α-induced SMC migration and activation of MMP-9. The present results indicate that the migration of HASMC is significantly induced by TNF-α and suppressed by 10 and 20 μmol/l CA pretreatment (Fig. 6). This inhibition of TNF-α-induced migration of HASMC is consistent with the inhibition of activation and expression of MMP-9 (Figs. 2 and 3). Similar results were seen when SMC were pretreated with other polyphenolic compounds, such as tea flavonoid epigallocatechin-3-gallate (20 μmol/l), quercetin (40 μmol/l) and other flavonoids (35–37).

Further research beyond the scope of the present study is necessary to elucidate the mechanisms underlying the synergistic regulation of MMP secretion by cytokines. In the
present study, we focused instead on defining the role played by the NF-κB transcription factor in the regulation of MMP in HASMC. A functional NF-κB site occurs in the proximal stimulatory region of the MMP-9 promoter, and deletion of this site reduces up-regulation of reporter gene constructs in response to cytokines. Until now, however, it has been unclear as to the role NF-κB plays in the up-regulation of the endogenous MMP-9 gene. Bond et al. have demonstrated that transient overexpression of inhibitory protein κBα in vascular SMC only partially impaired up-regulation of MMP-9, suggesting that NF-κB might play a simple permissive role. In the present study, CA reduced cytokine-induced expression of MMP-9 and prevented the nuclear translocation of p50 (Fig. 4) and p65 (Fig. 5) in HASMC.

We suggest that inhibitory mechanisms of CA might interrupt a signalling cascade involving MMP-9 transcription-mediated activation of NF-κB.

Several studies have indicated that ROS are implicated in the activation of NF-κB. The current study showed that CA pretreatment in HASMC decreased the TNF-α-stimulated production of ROS (Fig. 7). Based on this result, we propose that the inhibitory effect of CA on MMP-9 expression and NF-κB activation may be due to its antioxidant and anti-inflammatory properties. Since atherosclerosis is a chronic inflammatory disease associated with increased oxidative stress in HASMC, it would be conceivable that the antiatherogenic effects of CA might be caused by its antioxidative and anti-inflammatory properties.

Fig. 7. Effect of carnosic acid (CA) on TNF-α-induced reactive oxygen species (ROS) production in human aortic smooth muscle cells (HASMC). (A), Microphotograph of ROS production in HASMC without TNF-α or CA (a), with TNF-α (100 ng/ml) (b), with TNF-α (100 ng/ml) and 10 μmol/l CA (c), with TNF-α (100 ng/ml) and 20 μmol/l CA (d). HASMC were pretreated with 10 and 20 μmol/l CA for 1 h and induced by TNF-α (100 ng/ml) for 23 h. Values are means with their standard deviations depicted by vertical bars (n = 3). Each individual experiment included two duplicated experiments. a,b,c Mean values with unlike letters were significantly different (P < 0.05). C, control without TNF-α or CA; CA10, 10 μmol/l CA; CA20, 20 μmol/l CA; TNF, TNF-α.
In conclusion, CA effectively inhibited the TNF-α-induced migration of HASMC. The levels of ROS production, MMP-9 activation and expression, and nuclear translocation of NF-κB p50 and p65 were also all reduced by CA pretreatment. The present results led us to conclude that CA inhibits TNF-α-induced nuclear translocation of p50 and p65, thereby suppressing the activation and protein expression of MMP-9, resulting in decreased HASMC migration. Thus, CA may play an important role in the prevention of atherosclerosis.

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