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ORIGINAL CONTRIBUTION

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Carnosic acid reduces cytokine-induced adhesion molecules expression and monocyte adhesion to endothelial cells

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Abstract *Background* Expression of cell adhesion molecules on the endothelium and the attachment of monocytes to endothelium may play a major role in the early atherogenic process. *Aim of the study* We investigated the effects of carnosic acid on the adhesion of U937 cells to IL-1β-treated human umbilical vein endothelial cells (HUVECs), as well as on the expression of adhesion molecules. *Results* Our data showed that pretreatment with 10 and 20 μmol/l carnosic acid significantly reduced the number of U937 cells adhering to IL-1β-treated HUVECs. In addition, we found that 20 μmol/l carnosic was more effective than 10 μmol/l carnosic acid at inhibiting expression of cell adhesion molecules (ICAM-1, VCAM-1, and E-selectin), the nuclear translocation of NF-κB subunits p65 and p50, and the production of ROS in IL-1β-stimulated HUVECs. *Conclusions* We conclude that carnosic acid inhibits IL-1β-induced ICAM-1, VCAM-1 and E-selectin expression in HUVECs through a mechanism that involves NFκB. We propose that the reduction in binding of human monocytic cell line U937 to IL-1β-treated HUVECs is due to the anti-inflammatory properties of carnosic acid.

Key words carnosic acid – adhesion molecules – NFκB – IL-1β – reactive oxygen species

Introduction

Atherosclerosis is a progressive pathological disorder that often leads to cardiovascular and cerebrovascular diseases [17]. Adhesion of circulating leukocytes to the vascular endothelium is a critical early event in the early development of atherosclerosis [4]. This process is dependent on the interaction between cell adhesion molecules expressed on the surface of endothelial cells and their cognate ligands on leukocytes [16].

Previous studies have indicated that NF-κB/Rel transcription factors may play an important role in the development of atherosclerosis [22]. The activation of NF-κB in endothelial cells is associated with the activation of the genes responsible for increased transcription of adhesion molecules, cytokines, and chemokines [23, 25].

Plant polyphenols are a large group of naturally occurring antioxidants, and epidemiological studies have suggested that higher polyphenol intake from fruits and vegetables are associated with decreased risk for cardiovascular disease [8]. Previous studies showed that polyphenolic compounds, such as dietary flavonoids and red wine polyphenols, could prevent atherosclerosis by inhibiting the expression of adhesion molecules in endothelial cells [15, 20]. Carnosic

acid, a polyphenol derived from Sage (*Salvia oddicinalis*) and Rosemary (*Rosmarinus officinalis*), is a lipophilic antioxidant that scavenges singlet oxygen, hydroxyl radicals, and lipid peroxyl radicals, thus preventing lipid peroxidation and disruption of biological membranes [2, 6]. It has also been demonstrated that carnosic acid can inhibit plasma triglyceride elevation in olive oil-loaded mice and reduce body weight gain and the accumulation of epididymal fat in high fat diet-fed mice [10]. Therefore, we examined the effect of carnosic acid on monocyte adhesion to cultured human endothelial cells and on the expression of adhesion molecules (VCAM-1, ICAM-1 and E-selectin). We also attempt to elucidate the possible mechanisms associated with such an effect.

Materials and methods

Cell viability assay (MTT test)

The viability of the cells was assessed by the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide) assay [14].

Adhesion of U937 cells to endothelial cells

Adhesion was evaluated using human leukemia promonocytic U937 cells labeled with calcein AM (10 nmol/l; Molecular Probe; Invitrogen) [27]. HUVECs (2×10^5) were distributed into 6-well plates and allowed to reach confluence. They were then incubated for 18 h with medium supplemented with CA at concentrations of 10 and 20 μmol/l followed by incubation for 6 h with 10 ng/ml of IL-1β in the continued presence of CA. U-937 was grown in RPMI-1640 medium (Gibco, NY, USA) containing 10% FBS and subcultured at a 1:5 ratio three times per week, were labeled for 30 min at 37°C with calcein AM (10 nmol/l, Molecular Probe) in RPMI-1640 medium, washed with PBS to remove free dye and then resuspended in 10% M-199 medium. Labeled U937 cells (1×10^6) were added to each HUVEC-containing well and incubated for 1 h. Non-adherent cells were removed by two gentle washes with PBS. Then, adherent U937 cells were determined by a fluorescence plate reader at an excitation wavelength of 485 nmol/l and emission at 530 nmol/l; HUVEC cell monolayers served as the blank.

Western blotting assay for the expression of adhesion molecules

Cells were lysed with a lysis buffer and centrifuged at 12,000×*g* for 30 min at 4°C. Cell extracts were resolved by 10% SDS-PAGE and transferred at room temperature by blotting to a polyvinylidene difloride (PVDF) membrane [18]. Then incubated with mouse anti-human-VCAM-1, ICAM-1, and E-selectin antibodies. The membrane was also incubated with mouse anti-human β-actin antibody as an internal control.

Measurement of NF-κB activation

Nuclear protein extracts from HUVECs were prepared using a nuclear extract kit (TransAM nuclear extract kit, CA, USA) to assay NF-κB activity. Nuclear proteins were prepared as described previously [5]. NF-κB activation was analyzed with the TransAM NF-κB Family kit (TransAM, Active Motif, CA, USA) [27]. In this assay, 96-well plates were coated with an oligonucleotide containing the consensus binding sequence for NF-κB 5′-GGGACTTTCC-3′. Specific primary antibodies included in the kit detected the binding of NF-κB family transcription factors to their consensus sequence Experiments were analyzed by an ELISA-based assay. A total of 10 μg of nuclear extract was used in each experiment and processed according to the manufacturer's protocol. Briefly, nuclear extracts were incubated with 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 p65, p50, c-Rel, p52, and RelB for 60 min. After incubation with a horseradish peroxidase-conjugated secondary antibody, a substrate was added to produce blue color and then for quantitation by a standard ELISA reader. The absorbance was read at 450 nmol/l and the blanks were subtracted from all measurements. The data presented is the result of three independent duplicated experiments.

Measurement of reactive oxygen species

ROS generation in cells was assessed using the probe 2,7-dichlorofluorescein (DCF) (Molecular Probes Europe BV, Leiden, Netherlands) [26]. The fluorescence intensity (relative fluorescence units) was measured at 485-nm excitation and 530-nm emission wavelengths using a fluorescence microplate reader.

Statistical analysis

Statistical analyses of MTT assay results were performed using One-way ANOVA, followed by Dunnett's test. Other statistical 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

Viability of HUVECs exposed to carnosic acid

Cell viability was determined by the MTT assay after 24 h of incubation with 5, 10 and 20, 40 and 60 μmol/l carnosic acid, cell viability was 105.1 ± 2.5, 106.3 ± 5.4, 99.1 ± 5.2, 88.2 ± 3.3, and 71.5 ± 3.8%.

Carnosic acid inhibited the adhesion of U937 cells to IL-1β-stimulated HUVECs

The control group showed minimal binding of U937 cells to HUVECs, however, adhesion significantly increased when the HUVECs were treated with IL-1β. Pretreatment with 10 and 20 μmol/l carnosic acid significantly reduced the number of U937 cells adhering to IL-1β-stimulated HUVECs. Furthermore, the concentration of 20 μmol/l carnosic acid was more effective on reducing the level of monocyte adhesion than 10 μmol/l carnosic acid (Fig. 1a, b).

Carnosic acid inhibits IL-1β-induced expression of VCAM-1, ICAM-1and E-selectin in HUVECs

The expression of ICAM-1, VCAM-1, and E-selectin increased after IL-1β stimulation. Pretreatment of HUVECs with 10 μmol/l carnosic acid significantly inhibited the expression of VCAM-1 and E-selectin, but not ICAM-1. However, pretreatment of HUVECs with 20 μmol/l carnosic acid significantly inhibited the expression of ICAM-1, VCAM-1, and E-selectin. (Fig. 2a–c).

Carnosic acid decreases activation of NF-κB and nuclear translocation of NF-κB subunits p65 and p50 in IL-1β-stimulated HUVECs

Incubation of IL-1β (10 ng/ml) for 6 h induced the nuclear translocation of p65 and p50. Pretreatment of HUVECs with 10 and 20 μmol/l carnosic acid significantly attenuated the nuclear translocation of p65 and p50 (Fig. 3a, b). In addition, pretreatment of HUVECs with 20 μmol/l carnosic acid was more effective in decreasing the nuclear translocation of p65 and p50 than 10 μmol/l carnosic acid.

Carnosic acid inhibits IL-1β-induced ROS in HUVECs

The production of ROS was induced by IL-1β and significantly decreased after pretreatment with 10 and 20 μmol/l carnosic acid (Fig. 4a, b). In addition, pretreatment of HUVECs with 20 μmol/l carnosic acid was more effective in decreasing the production of ROS than 10 μmol/l carnosic acid.

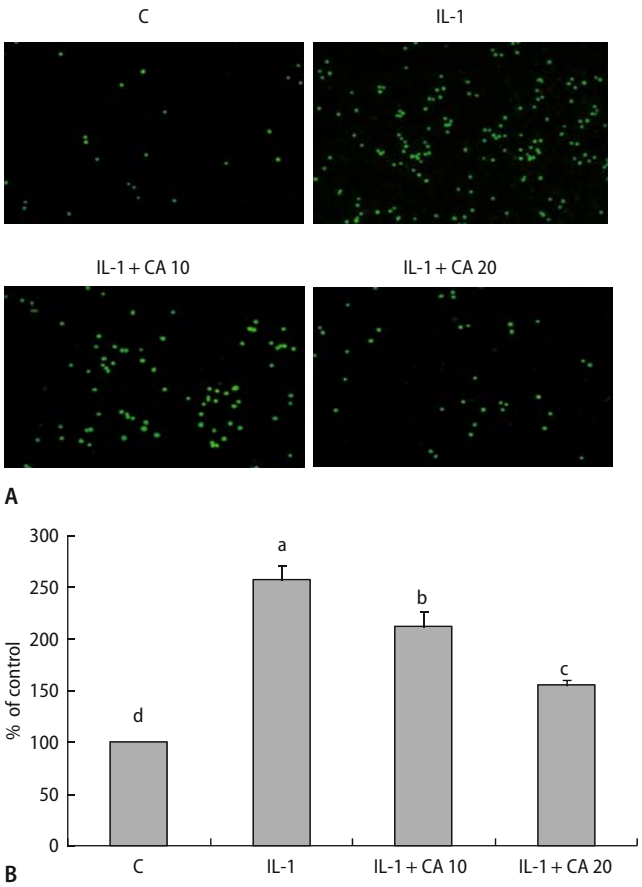

Fig. 1 Effect of carnosic acid on IL-1β-induced adhesion of U-937 cells to HUVECs. **a** Representative images of the reduction of IL-1β-induced adhesion of U-937 cells to HUVECs monolayers after pretreatment with 10 and 20 μmol/l carnosic acid for 18 h. HUVECs (2×10^5) were incubated for 18 h with medium supplemented with CA at concentrations of 10 and 20 μmol/l followed by incubation for 6 h with 10 ng/ml of IL-1βin the continued presence of CA. **b** Mean ROS production was expressed as % of control. Values are means with their standard deviations depicted by vertical bars ($n = 3$). Each individual experiment included two duplicated experiments. $^{a-d}$Mean values with unlike letters were significantly different ($P < 0.05$)

Discussion

Expression of cell adhesion molecules (CAM) by the endothelium, and the attachment of monocytes to endothelium may play a major role in the early atherogenic process [4]. Carnosic acid is an antioxidant polyphenol derived from Sage (*Salvia oddicinalis*) (2–5 mg CA/g sage) and Rosemary (*Rosmarinus officinalis*) (10–15 mg CA/g rosemary) [2, 6]. In the present study, we found that carnosic acid significantly suppressed IL-1β-induced intracellular ROS

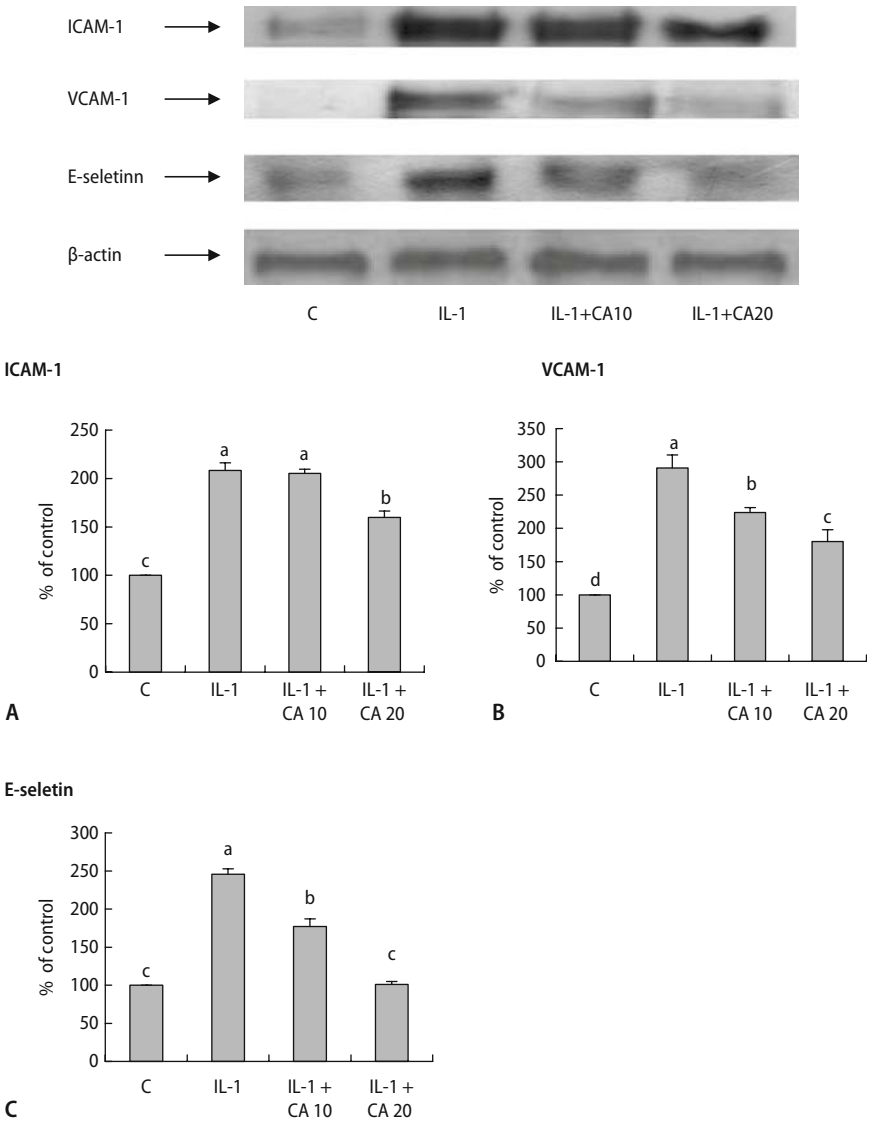

Fig. 2 Effect of carnosic acid on the protein levels of ICAM-1, VCAM-1 and E-seletin in cultured HUVECs. Representative images of the reduction of IL-1β-induced expression of ICAM-1 (**a**), VCAM-1 (**b**) and E-seletin (**c**) by carnosic acid in HUVECs. Densitometric analysis was conducted with image 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. $^{a-c}$Mean values with unlike letters were significantly different ($P < 0.05$)

production, attenuated the activation of redox-sensitive transcription factors NF-κB p50 and p65, reduced the expression of ICAM-1, VCAM-1 and E-selectin, and suppressed monocyte (U937) adhesiveness to endothelial cells. These results demonstrate the inhibitory effect that carnosic acid has on the proatherosclerotic mechanism in vitro.

Adhesion and transendothelial migration of monocytes into the surrounding tissues are crucial steps in inflammation, immunity, and atherogenesis [4, 16]. Vascular endothelial cells play an active role in this process by expressing cell adhesion molecules that enhance the adhesion of monocytes to the endothelium [1]. In the present study, we found minimal binding of U937 cells to endothelial cells in the control group, but that the level of adhesion increased when HUVECs were treated with IL-1β. Pretreatment with 10 and 20 μmol/l carnosic acid reduced the number of U937 cells adhering to IL-1β-stimulated HUVECs. Previous studies have also found that monocyte adhesion was reduced when HUVECs were pretreated with other polyphenolic compounds, such as vitamin E (40 μmol/l), ellagic acid (50 μmol/l), or tea flavonoid (60 μmol/l) [9, 12, 27].

The translocation of the transcription factor NF-κB is involved in the signal transduction pathways for IL-1β-induced adhesion molecule expression [11]. The activated form of NF-κB is a heterodimer, which usually consists of two proteins, a p65 (also called relA) subunit and a p50 subunit [3]. Udalova et al. [24] described the involvement of the p50–p65 heterodimer in enhancing the transcription of adhesion molecules, cytokines, and chemokines. In the present study, we found that the IL-1β-induced nuclear

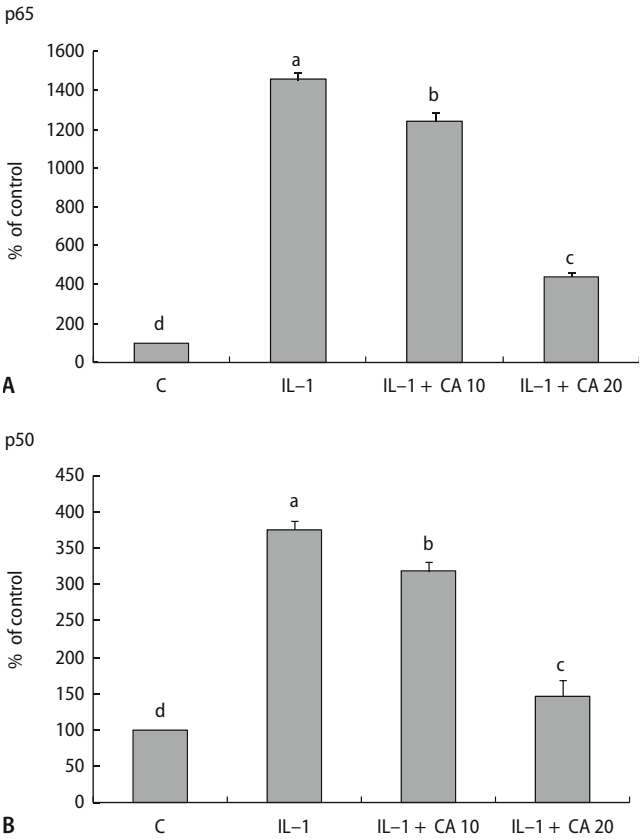

Fig. 3 Effect of carnosic acid on IL-1β-induced activation of NF-κB p65 and p50. Nuclear extracts were prepared and analyzed for activation of NF-κB p65 (**a**) and p50 (**b**). Ten micrograms of nuclear protein was used in each experiment. Values are means with their standard deviations depicted by *vertical bars* ($n = 3$). Each individual experiment included two duplicated experiments. [a–d]Mean values with unlike letters were significantly different ($P < 0.05$)

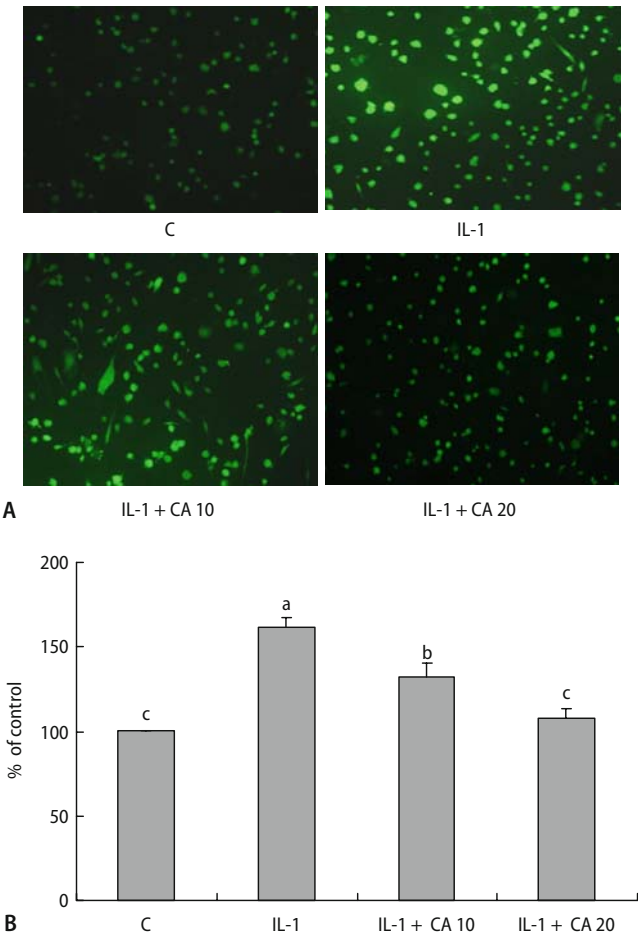

Fig. 4 Effect of carnosic acid on IL-1β-induced ROS production in HUVECs. **a** Fluorescent images show the reduction of IL-1β-induced ROS production in HUVECs after pretreatment with 10 and 20 μmol/l carnosic acids for 18 h. **b** Mean ROS production was expressed as % of control. Values are means with their standard deviations depicted by *vertical bars* ($n = 3$). Each individual experiment included two duplicated experiments. [a–c]Mean values with unlike letters were significantly different ($P < 0.05$)

translocation of the p65 and p50 subunits decreased in HUVECs that had been pretreated with carnosic acid. This finding confirms that carnosic acid has an anti-inflammatory effect, based on its ability to partially interfere with NF-κB activation.

Harrison et al. [7] reported that ROS play a central role in the pathogenesis of endothelial dysfunction and atherosclerosis. Previous studies have also shown ROS are involved in the activation mechanism of NF-κB nuclear translocation [19, 21]. In the present study, we found the IL-1β-induced production of ROS decreased in HUVECs pretreated with carnosic acid. Previous studies showed that the activation of NF-κB could be inhibited by different antioxidants, suggesting that endogenous ROS may play an important role in these redox-sensitive transcription pathways in atherogenesis [13, 21]. Furthermore, in our in vitro study, we found that CA could scavenge DPPH (2, 2-diphenyl-1-picrylhydrazyl) radicals, alkoxyl radicals ($RO^{\bullet}$), and lipid peroxyl radicals ($ROO^{\bullet}$) [28]. Based on the present results, we propose that the inhibitory effect of carnosic acid on the expression of adhesion molecules and activation of NF-κB is due to its anti-oxidant and anti-inflammatory properties.

In conclusion, we found that carnosic acid can inhibit monocyte adhesion to endothelial cells, reduce the expression of adhesion molecules, attenuate the translocation of NF-κB, and suppress the production of ROS in HUVECs. These findings may provide a rationale for the potential use of carnosic acid in the prevention of atherosclerosis.

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