Carnosol inhibits cell adhesion molecules and chemokine expression by tumor necrosis factor-α in human umbilical vein endothelial cells through the nuclear factor-κB and mitogen-activated protein kinase pathways

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Received May 12, 2013; Accepted November 13, 2013

DOI: 10.3892/mmr.2013.1839

Abstract. Inflammatory bowel diseases (IBD) are gastrointestinal disorders associated with chronic inflammatory processes. Carnosol has been demonstrated to possess anti-inflammatory properties. This study examined the suppressive effect of carnosol on the expression of cell adhesion molecules (CAMs) and chemokines in human umbilical vein endothelial cells (HUVECs) and the possible underlying mechanism. The effect of carnosol on CAM and chemokine expression in HUVECs was identified by western blotting and ELISA, respectively. Nuclear factor (NF)-κB activation of HUVECs was analyzed using the TransAM NF-κB Family kit. The effect of carnosol on the tumor necrosis factor (TNF)-α-induced activation of the NF-κB and mitogen-activated protein kinase (MAPK) pathways, and was subsequently analyzed using western blotting. Carnosol not only inhibited TNF-α-induced protein expression of intercellular adhesion molecule (ICAM)-1, vascular cell adhesion molecule (VCAM)-1 and E-selectin in HUVECs, but also suppressed interleukin (IL)-8 and monocyte chemoattractant protein (MCP)-1 expression. In addition, carnosol inhibited the TNF-α-induced phosphorylation of p-65 and IkB-α, as well as the activation of NF-κB. The same result was observed in TNF-α-stimulated phosphorylation of ERK1/2 and p-38. It was demonstrated that carnosol inhibited TNF-α-induced CAM and chemokine expression in HUVECs. The underlying mechanism may be associated with the blocking of the NF-κB and MAPK pathways. These results indicate that carnosol may be a novel therapeutic agent for targeting endothelial cells in IBDs.

Introduction

Inflammatory bowel disease (IBD), comprising Crohn's disease (CD) and ulcerative colitis (UC), is considered to be a chronic relapsing disorder involving inflammation of the gastrointestinal tract (1). Numerous IBD patients suffer from a relapse of the disease; therefore ideal therapeutic strategies are imperative.

Although the precise etiology of IBD is not well understood, certain substantial advances have been made. Among the complex pathogenesis of IBD, leukocyte recruitment is a common event, occurring in the initiation and progression of the disease (2). This recruitment is regulated by cell adhesion molecules (CAMs) (3) and chemokines (4), expressed by endothelial cells, which are activated during the development of IBD. The overexpression of CAMs [intercellular adhesion molecule (ICAM)-1, vascular cell adhesion molecule (VCAM)-1 and E-selectin in HUVECs, but also suppressed interleukin (IL)-8 and monocyte chemoattractant protein (MCP)-1 expression. In addition, carnosol inhibited the TNF-α-induced phosphorylation of p-65 and IkB-α, as well as the activation of NF-κB. The same result was observed in TNF-α-stimulated phosphorylation of ERK1/2 and p-38. It was demonstrated that carnosol inhibited TNF-α-induced CAM and chemokine expression in HUVECs. The underlying mechanism may be associated with the blocking of the NF-κB and MAPK pathways. These results indicate that carnosol may be a novel therapeutic agent for targeting endothelial cells in IBDs.

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Key words: carnosol, cell adhesion molecules, chemokine, nuclear factor-κB, mitogen-activated protein kinase
cells (HUVECs) (12). Further research has demonstrated that
carnosol may suppress tumor necrosis factor (TNF-α)-induced
ICAM-1 expression by inhibiting IκB kinase beta (IKK-β)
activity or upregulating heme-oxygenase-1 expression (13).
However, whether carnosol is capable of inhibiting the
TNF-α-induced expression of other CAMs, the production of
proinflammatory cytokines in endothelial cells and the inhibi-
tion of other potential signaling pathways, remains unknown.
This study examined the ability of carnosol to inhibit the
expression of CAMs (ICAM-1, VCAM-1 and E-selectin) and
the production of chemokines (IL-8 and MCP-1) in HUVECs,
and also investigated the possible mechanisms underlying the
anti-inflammatory activity of carnosol.

Materials and methods

Cell culture. HUVECs were isolated from human umbilical
veins by treatment with collagenase (0.1%). Subsequently,
the cells were collected and cultured in EGM-2 medium
(Lonza Inc., Walkersville, MD, USA) with 10% foetal bovine
serum (FBS, Life Technology, Carlsbad, CA, USA), 100 IU/ml
penicillin and 100 µg/ml streptomycin (Life Technology). All
cells were incubated at 37°C in an atmosphere of 95% air:5%
CO₂. The purity of the HUVECs was 98% and was evaluated
by morphology and immunofluorescent staining for cluster of
differentiation (CD)31 and von Willebrand factor (Santa Cruz
Biotechnology, Santa Cruz, CA, USA). HUVECs achieved
confluence in the flasks and cells from between three to five
passages were used for the experiment.

Cell viability assay. Cell viability was determined by using a
Cell Counting kit-8 (CCK-8) (Dojindo Molecular,
Kumamoto, Japan) as described previously (14). The HUVECs
were seeded onto a 96-well plate at a density of 1x10⁶ cells/ml
followed by preincubation with various concentrations (1,
5 and 10 µmol/l) of carnosol (Cayman Chemical Company,
Ann Arbor, MI, USA) and TNF-α (Life Technology) for
24 h. Subsequently 10 µl CCK-8 solution was added to each
well for an additional 2 h, and the absorbance at 450 nm was
assessed with a microplate reader (Multiskan MK3, Thermo
Labsystems, Vantaa, Finland).

Western blotting. HUVECs were washed with pre-chilled
phosphate-buffered saline and lysed in radioimmunopre-
cipitation assay buffer. Following sonication (Sonicator Q700;
QSonica LLC, Newtown, CT, USA), the lysate was cen-
trifuged (14,000 x g for 15 min at 4°C) and the supernatant was
transferred to a tube. The protein content was quantified with a
bicinchoninic acid protein assay kit (Keygen Biotech, Nanjing,
China). Total proteins were separated by electrophoresis on
SDS-polyacrylamide gels and were subsequently electrob-
lotted onto polyvinylidene fluoride membranes. The indicated
primary antibodies were incubated, washed and visualized by
incubation with a horseradish peroxidase (HRP)-conjugated
secondary antibody (goat anti-rabbit IgG and goat anti-mouse
IgG; 1:2,500, Santa Cruz Biotechnology) and the enhanced
chemiluminescence plus detection system (Amersham,
Arlington Heights, IL, USA). CAM expression was detected with anti-ICAM-1, anti-VCAM-1 and anti-E-selectin mouse
monoclonal antibodies, respectively (1:1,000, Santa Cruz
Biotechnology). The activation of NF-κB and mitogen-acti-
vated protein kinase (MAPK) was detected with anti-p-65,
anti-phospho-p-65, anti-phospho-IκB-α (mouse monoclonal),
anti-extracellular signal regulated kinase (ERK) 1/2,
anti-p-38, anti-phospho-ERK1/2 and anti-phospho-p-38 rabbit
monoclonal antibodies (1:1,000, Cell Signaling Technology,
Danvers, MA, USA).

ELISA. HUVECs were plated into the wells of a 24-well
cluster plate at a density of 5x10⁶ cells/ml/well. Subsequently,
the cell culture supernatants were harvested by centrifuga-
tion at 800 x g for 5 min at 4°C to remove cell debris and
were frozen at -80°C. Supernatant samples were thawed once
and assayed for IL-8 and MCP-1 content in duplicate using a
commercially available ELISA kit (R&D systems, Abingdon,
UK), as previously reported (15).

Assay of transcription factor NF-κB. NF-κB activity was
measured using the TransAM NF-κB Family kit, according to
the manufacturer's instructions (Active Motif, Carlsbad, CA,
USA). The samples were analyzed in a 96-well plate containing
the immobilized NF-κB consensus site (5'-GGGACTTTCC-3')
oligonucleotide. Nuclear extracts were prepared for analysis
of NF-κB activity; the activated form of NF-κB p-65 and p-50
subunits in the nuclear extract bind to this oligonucleotide,
respectively. Using antibodies against the p-65 and p-50
subunits and a HRP-conjugated secondary antibody, the devel-
opling solution of the TransAM NF-κB Family kit was added
to produce a blue color and subsequently quantified with the
microplate reader (Multiskan MK3, Thermo Labsystems)
at 450 nm. Data from triplicate wells were expressed as the
mean ± standard deviation (SD).

Statistical analysis. All experiments were repeated a minimum
of three times. The results were expressed as the mean ± SD
using SPSS 13.0 statistical software (SPSS Inc., Chicago, IL,
USA). Statistical analyses were performed using one-way
analysis of variance, followed by Duncan's multiple range test.
P<0.05 was considered to indicate a statistically significant
difference.

Results

Effect of carnosol on cell viability. The effect of carnosol
on cell viability was investigated using CCK-8. The results
revealed that the viability of the HUVECs was not signifi-
cantly influenced by carnosol (Fig. 1).

Carnosol inhibits TNF-α-induced CAM expression levels.
Western blotting was performed in order to investigate whether
carnosol affects TNF-α-induced CAM expression in HUVECs.
Compared with the control group, the TNF-α only group revealed
that TNF-α significantly increased the expression of ICAM-1,
VCAM-1 and E-selectin in HUVECs (Fig. 2). However, when
the cells were pretreated with 5 and 10 µmol of carnosol, the
expression was markedly inhibited in a dose-dependent manner.

Carnosol inhibits IL-8 and MCP-1 expression in HUVECs
induced by TNF-α. The ELISA results revealed that TNF-α
enhanced IL-8 expression in HUVECs (Fig. 3). However,
following treatment with carnosol only, IL-8 expression was not identified to have a significant difference. Pretreatment with higher doses of carnosol may inhibit IL-8 expression more markedly.

**Carnosol inhibits TNF-α-induced NF-κB activation.** Western blotting was used to investigate whether carnosol affected TNF-α-induced NF-κB activation in HUVECs. Compared with the control group, carnosol had no impact on the phosphorylation of p-65 and IκB-α in HUVECs, while TNF-α significantly increased their phosphorylation. When pretreated with 5 and 10 µmol carnosol, the phosphorylation levels of p-65 and IκB-α, but not the total p-65 expression, were reduced in a dose-dependent manner (Fig. 4).

**Carnosol reduces TNF-α-induced activation of NF-κB p-65 and p-50.** The nuclear translocation of p-50 and p-65 proteins of the NF-κB family of transcription factors were measured. Compared with the control group, a greater number of p-50 and p-65 proteins were located when the cells had been treated with TNF-α only (Fig. 5). Following pretreatment with carnosol, the total quantities of the two proteins were significantly reduced. These results supported the view that NF-κB-DNA binding activity was inhibited, in a dose-dependent manner,
by the pretreatment of HUVECs with carnosol prior to TNF-α stimulation.

**Effects of carnosol on MAPK signaling.** Western blotting was performed in order to investigate the effect of carnosol on TNF-α-induced MAPK activation in HUVECs. The results demonstrated that TNF-α significantly enhanced the phosphorylated expression of ERK1/2 and p38, but not that of total ERK1/2 and p38, in HUVECs (Fig. 6). Furthermore, pretreatment with carnosol was shown to decrease their expression in a dose-dependent manner.

**Discussion**

Endothelial activation induced by proinflammatory cytokines is crucial in the inflammation process, as it is directly responsible for the proinflammatory cytokines recruiting the leukocytes into the inflamed focus from blood vessels during the inflammation process. During the inflammatory process, the levels of several proinflammatory cytokines were elevated, which induced the expression of adhesion molecules, monocyte adhesion and chemokine release (16). CAMs, including VCAM-1, ICAM-1 and E-selectin, were increasingly expressed by these endothelial cells (16), in addition to the production of IL-8 and MCP-1 (4).

The stimulatory effect of TNF-α on CAM expression and chemokine production in HUVECs observed in our study concurs with that observed in previous reports (13,17,18). The present study revealed that carnosol reduced the expression of VCAM-1, ICAM-1 and E-selectin and the production of IL-8 and MCP-1 in TNF-α-stimulated HUVECs in a dose-dependent manner. These adhesion molecules have been verified as critical for the recruitment of inflammatory cells to the endothelium (19). Furthermore, previous studies have demonstrated that the functional blocking of these adhesion molecules was capable of suppressing T cell adhesion to endothelial cells (20) and that reducing the production of chemokines (21) may be a treatment for IBD. The results of the present study suggested that carnosol may retard the endothelial inflammatory process by suppressing the secretion of chemoattractant molecules and the production of chemokines.

Furthermore, this secretion of chemoattractant molecules and the production of chemokines is always dependent on the activities of NF-κB (13) and MAPK (4). The analysis in the present study indicated that carnosol suppressed TNF-α-induced NF-κB activation. Numerous genes involved in the development of IBDs are regulated by NF-κB. In addition, activated NF-κB has been identified in the inflamed gut of IBD patients (22). NF-κB is composed of p-65 and p-50 subunits, and inactive NF-κB dimers are sequestered in the cytosol in association with various inhibitory molecules of the IκB-α family. In response to TNF-α, IκB-α is phosphorylated, which in turn is targeted for ubiquitination- and proteasome-dependent degradation (23). Previous studies have demonstrated that the active forms of MAPK are upregulated in patients with IBD (24). The present study demonstrated that carnosol inhibited the phosphorylation of ERK1/2 and p38 in TNF-α-stimulated HUVECs. Due to the fact that the phosphorylation of ERK1/2 and p38 is associated with the CAM expression of endothelial cells (25), it was hypothesized that the molecular mechanism underlying the anti-inflammatory activity of carnosol is associated with its inhibitory effect on the MAPK pathway. As demonstrated in a previous study, the inhibition of MAPK- and NF-κB-signaling pathways may suppress IBD (26). Therefore, it may be assumed that carnosol may have promising anti-inflammatory ability by its inhibition of the MAPK and NF-κB pathways, leading to a reduction in CAMs and chemokines.
There are emerging therapeutic agents for IBD treatment which specifically target the endothelium. Natalizumab, the first α4-integrin inhibitor, demonstrated significant therapeutic benefit for the treatment of CD, by blocking the interaction of α4 expressing leukocytes with their ligands, including E-selectin and VCAM-1 (27). Another drug, the soluble epoxide hydrolase inhibitor, also demonstrated high potential for the treatment of IBD by decreasing the levels of IFN-γ, TNF-α, MCP-1, VCAM-1 and NF-κB signals (28). In the present study, it was demonstrated that carnosol may also inhibit the expression of CAMs and chemokines associated with leukocyte recruitment.

In conclusion, the current study demonstrated that carnosol may provide an effective approach for the treatment of chronic inflammation, including IBDS. This anti-inflammatory effect may be due to its ability to inhibit the production of CAMs (ICAM-1, VCAM-1 and E-selectin) and chemokines (IL-8 and MCP-1), which increase leukocyte infiltration to the inflammatory tissues. Furthermore, the molecular mechanism underlying this effect may be associated with the inhibitory effect of carnosol on the NF-κB and MAPK pathways.

Acknowledgements
This study was partially sponsored by the National Natural Science Foundation of China (grant nos. 91029702 and 81072046).

References