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Suppression of Carnosine on Adhesion and Extravasation of Human Colorectal Cancer Cells

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Abstract. *Aim: To investigate the effect of carnosine, an active compound of dietary beef, fish and chicken, on the regulation of cell adhesion and extravasation during metastasis. Materials and Methods: Cell adhesion and extravasation abilities, and related regulating molecular mechanisms were analyzed in human colorectal cancer cells (HCT-116) and human umbilical vein cells (EA.hy926). Results: Carnosine reduced the ability of HCT-116 cells to adhere to EA.hy926 cells. The expression levels of integrin-β1 in HCT-116 cells, as well as of intercellular adhesion molecule-1 and E-selectin in EA.hy926 cells, were reduced after carnosine treatment. After EA.hy926 cells were treated with carnosine, phosphorylation of vascular endothelia-cadherin (VE-cadherin), protein levels of Ras homologous (RHO) and RHO-associated coiled-coil containing protein kinase, and levels of reactive oxygen species were reduced. After treating EA.hy926 cells with carnosine, phosphorylation of inhibitor of kappa B (IκB) and DNA binding activity of nuclear factor-κB (NF-κB) were reduced. Conclusion: Carnosine inhibits metastatic cell adhesion and extravasation by suppressing NF-κB signaling activation.*

Carnosine ((2S)-2-[(3-amino-1-oxopropyl) amino]-3-(3H-imidazol-4-yl) propanoic acid) is a natural dipeptide consisting of β-alanine and L-histidine. It is found in lean dietary beef, fish, and chicken (1). Bae *et al*. showed that carnosine exhibits no signs of toxicity in various examined organs in Sprague–Dawley (SD) rat, and that carnosine not only affected viability of normal primary cultures of cortical neurons and astrocytes from C56BL/6 mice, but also reduced reactive oxygen species (ROS) levels and the mitochondrial membrane potential (2). In sum, the safety of carnosine is not in doubt. Carnosine also has anti-aging, antioxidation, anti-inflammation, anticancer, mitochondrial bioenergetics regulatory, and physiological effects (3-7). In particular, carnosine can inhibit cancer cell proliferation by regulating the glycolysis pathway and mitochondrial bioenergetics (4, 7). Our previous study found that carnosine inhibited migration and intravasation, which are important processes in metastasis, through regulating the epithelial–mesenchymal transition and matrix metalloproteinase expression (8). However, whether carnosine plays a role in adhesion and extravasation during metastasis and the molecular regulatory mechanism(s) are not clear.

Colorectal cancer (CRC) is one of the leading causes of deaths from malignant disease worldwide (9). Notably, 40-50% of patients with CRC are susceptible to developing metastatic disease (10). Metastasis leads to a poor prognosis in CRC and is an important issue in cancer therapy and prevention. During metastasis, cancer cells leave the original tumor-bearing organ and migrate to a targeted organ through a process that involves intravasation, adhesion, and extravasation (11). Cell adhesion involves many crucial functions, including organization, structure, communication, and cohesion, within complex multicellular organisms (12). Cell–cell and cell–extracellular matrix adhesion are both involved in the above-mentioned functional regulation. There are some specific molecules that regulate these two types of adhesion and link cells to other cells or the extracellular matrix with the intracellular actin cytoskeleton (13). Among the adhesion molecules, integrin, E-selectin, and intercellular adhesion molecule-1 (ICAM1) all play various important roles in cell–cell and cell–extracellular matrix adhesion (13).

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Additionally, adhesion junctions are one of the common interfaces of cell–cell adhesion between endothelial cell monolayers. Cadherins, especially vascular endothelial cadherin (VE-cadherin), are typically the primary junction molecules involved in these cellular structures (14). ROS and Ras homologous (RHO)/RHO-associated coiled-coil containing protein kinase (ROCK) are important regulatory molecules in the phosphorylation of VE-cadherin and cell–cell adhesion and contact (15, 16). Endothelial cell–cell monolayer permeability and integrity are highly correlated with the adhesion and extravasation of tumor cells to endothelial cells (17). The use of carnosine to regulate adhesion and extravasation by altering adhesion abilities, vascular permeability, and integrity has potential as a treatment strategy.

Among these adhesion- and extravasation-related regulatory molecules, integrin-β1, ICAM1, E-selectin, ROCK and RHOA are controlled primarily at the transcriptional level by nuclear factor-κB (NF-κB) (18-20). Whether carnosine modifies CRC adhesion and extravasation by regulating the NF-κB pathway remains unclear. Therefore, it is worth investigating the anti-metastasis molecular regulatory mechanism of carnosine.

The aims of this study were to investigate the regulatory effect of carnosine on CRC cell adhesion and extravasation and the expression of their related regulatory molecule in metastasis. In this study, the adhesive ability of HCT-116 human colorectal cancer cells to EA.hy926 vascular endothelial cells was evaluated. Analysis of adhesive molecules from these cells was performed. Changes in cell shape were examined by scanning electron microscopy (SEM) using a model of induced extravasation. Phosphorylated VE-cadherin (p-VE-cadherin) expression levels and the related molecular regulation of EA.hy926 cells were evaluated. Furthermore, the effect of carnosine on NF-κB signaling activation in EA.hy926 cells was analyzed.

Materials and Methods

Materials. The HCT-116 human colorectal cancer cell line and the EA.hy926 human umbilical vein endothelial cell line were purchased from the Bioresource Collection and Research Center (Hsinchu, Taiwan, ROC). Carnosine was purchased from Sigma-Aldrich (Saint Louis, MO, USA). Antisera against E-selectin, ICAM1, p-VE-cadherin, phosphorylated IκB (p-IκB) and NF-κB were purchased from Abcam (Cambridge, MA, USA). Antisera against integrin-β1, ROCK and RHOA were obtained from GeneTex, Inc. (San Antonio, Texas, USA). 2',7'-*Bis*-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein (BCECF) was purchased from Life Technologies GmbH (Darmstadt, Germany).

Cell culture and treatment. HCT-116 and EA.hy926 cells were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin at 37˚C in a humidified 5% CO_2 atmosphere. For passaging, cells were detached with trypsin/EDTA and subsequently replated. HCT-116 cells and EA.hy926 cells at passage levels between 20~26 and 12~25, respectively, were used in the present study. Carnosine was diluted in sterilized water, and cells treated with sterile water alone served as the control.

Determination of cell viability. Cell viability was analyzed using a (3-(4,5-dimethyl-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction assay as described by Denizot and Lang (21). EA.hy926 cells were incubated in 3-cm plates (1×10^6 cells) for 24 h and then treated with 0.5, 1 or 5 mM carnosine for 12, 24 or 48 h. MTT reagent (5 mg/ml) was then added, and the optical density (OD) was measured at a wavelength of 570 nm.

Assessment of adhesive ability. The adhesive ability assay was modified from a protocol described by Braut-Boucher *et al*. (22). EA.hy926 cells (1×10^6 cells) were cultured on 3-cm plates and exposed to 0.5, 1 or 5 mM carnosine for 6 h. The EA.hy926 cells were then washed with phosphate-buffered saline (PBS) and cocultured with HCT-116 cells prelabeled with 10 μM BCECF, a probes for cell adhesion, for 1 h. After washing the cells with PBS, the cell shape and number of BCECF-stained HCT-116 cells were evaluated under an inverted fluorescence microscope (Olympus IX51 Microscope; Olympus Optical Co. Ltd., Tokyo, Japan). Furthermore, the BCECF-stained cells were collected and measured fluorometrically using an enzyme-linked immunosorbent assay reader to obtain OD values at a wavelength of 580 nm.

SEM examination. SEM was used to examine morphological changes in cell shape and to examine protruding surface structures (23). HCT-116 cells were treated with 1 μg/ml lipopolysaccharide (LPS; *Escherichia coli* O111: B4; St. Louis, MO, USA), which is an invasion inducer (24); then 0.5, 1 or 5 mM carnosine combined with 1 μg/ml LPS was added and cells were incubated for 24 h. Next, the cells were collected, washed with PBS, and fixed for 30 min in 10% glutaraldehyde solution (25%; Sigma Chemical Co.). Dehydration was achieved with a graded ethanol series (2×15 min in 50 vol.% ethanol, 2×15 min in 70 vol.% ethanol, 2×15 min in 80 vol.% ethanol, 2×15 min in 90 vol.% ethanol, and 2×20 min, 1×60 min, and overnight incubation with absolute ethanol). After dehydration, the cells were prepared on gold substrates and platinum-coated conductive substrates (JFC-1600; JEOL, Ltd., Akishima, Tokyo, Japan). SEM images were obtained using a JEOL SEM 7000F microscope at an accelerating voltage of 5 kV.

Transepithelial electrical resistance (TEER) measurements. TEER measurements were used to examine cell–cell integrity and permeability according to a modified method reported by Gopal *et al*. (25). For the TEER measurements, EA.hy926 cells (5×10^4 cells/well) were cultured for 24 h in RPMI-1640 medium containing 0.5, 1 or 5 mM carnosine. TEER values were obtained by subtracting the TEER measurement for the cell culture dish groove from the measurement obtained in the presence of a cell layer. These measurements were acquired using a Millicell-ERS voltohmmeter (Millipore Continental Water Systems, Bedford, MA, USA).

Detection of ROS. ROS levels in HCT-116 cells was examined by flow cytometry (Becton Dickinson FACSCalibur) using 2,7-dichlorodihydrofluorescein diacetate (DCFH-DA, Sigma). HCT-116 cells were seeded in a 12-well plate at a cell density of approximately 5×10^5 cells/well, treated with 0.5, 1 or 5 mM carnosine and incubated

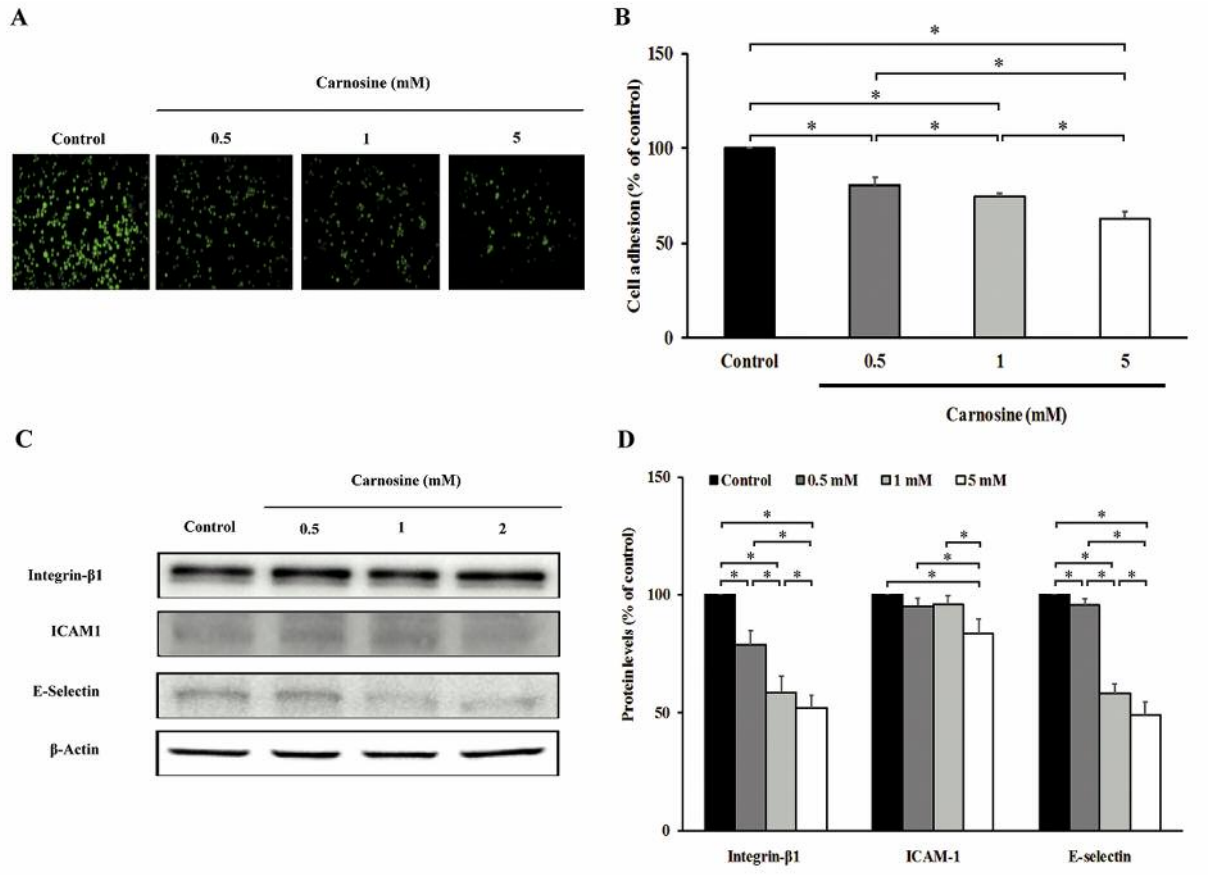

Figure 1. *Effect of carnosine on HCT-116 cell adhesion to EA.hy926 cells. EA.hy926 cells (1×10^6 cells/3-cm plate) were treated without or with 0.5, 1, or 5 mM carnosine for 24 h. HCT-116 and EA.hy926 cells were then co-treated for 1 h. (A) HCT-116 cells were pre-stained with 2',7'-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein and the adhesion of HCT-116 cells to EA.hy926 cells was observed under a fluorescence microscope. (B) HCT-116 cell staining was observed using a fluorescence EUSA reader at an excitation wavelength of 485 nm and an emission wavelength of 530 nm. (C) Immunoblotting assays were used to analyze the expression of integrin-β1 in HCT-116 cells and intercellular adhesion molecule 1 (ICAM1) and E-selectin in EA.hy926 cells. (D) Quantification of the protein expression in HCT-116 and EA.hy926 cells were quantified by densitometry; the expression levels in the control group were considered as 100%. The values are presented as the means±SD (n=3). *p<0.05 (ANOVA and Duncan's multiple comparison test).*

for 24 h to detect changes in ROS. The cells were then harvested and washed twice, resuspended in 500 μl of 10 μM DCFH-DA, incubated at 37˚C for 30 min and analyzed by flow cytometry (26).

Analysis of the expression of regulatory proteins involved in adhesion, VR-cadherin phosphorylation and the NF-κB pathway. Approximately 5×10^5 HCT-116 cells/3-cm plate were used for integrin-β1 expression analysis, and 5×10^5 EA.hy926 cells/3-cm plate were used for ICAM1, E-selectin, p-VE-cadherin, ROCK, RHOA, p-inhibitor of kB (p-IκB), IκB, NF-κB and nuclear NF-κB (p65) expression analyses. The cells were incubated in a 12-well plate with 0.5, 1 or 5 mM carnosine for 24 h, washed twice with cold PBS and harvested using 200 μl of lysis buffer containing 10 mM Tris-HCl, 5 mM EDTA, 0.2 mM phenylmethylsulfonyl fluoride, and 20 μg/ml aprotinin at pH 7.4. Quantification of cellular proteins was determined following the method described by Lowry *et al*. (27).

For each sample, 10 to 20 μg of cellular protein was added to 10% sodium dodecyl sulfate polyacrylamide gels (28). After electrophoresis, the proteins were transferred to polyvinylidene difluoride membranes (29) and incubated with anti-integrin-β1, anti-ICAM1, anti-E-selectin, anti-p-VE-cadherin, anti-VE-cadherin, anti-ROCK, anti-RHOA, anti-p-IκB, anti-IκB, anti-NF-κB and anti-p65 at 37˚C for 1 h and subsequently incubated with peroxidase-conjugated secondary antibodies. Bands were visualized using hydrogen peroxide/tetrahydrochloride diaminobenzidine or an enhanced chemiluminescence detection kit (Amersham Life Science, Buckinghamshire, UK) and quantified using a ChemiDoc™ MP Imaging System (Bio-Rad Laboratories Inc., Hercules, CA, USA).

Statistical analysis. The data were analyzed using SPSS statistical analysis software for Windows, version 20.0 (IBM, Armonk, NY, USA). One-way analysis of variance and Duncan's multiple range tests were employed to evaluate the significance of differences between two mean values. *p*-Values of less than 0.05 were considered to indicate statistically significant results.

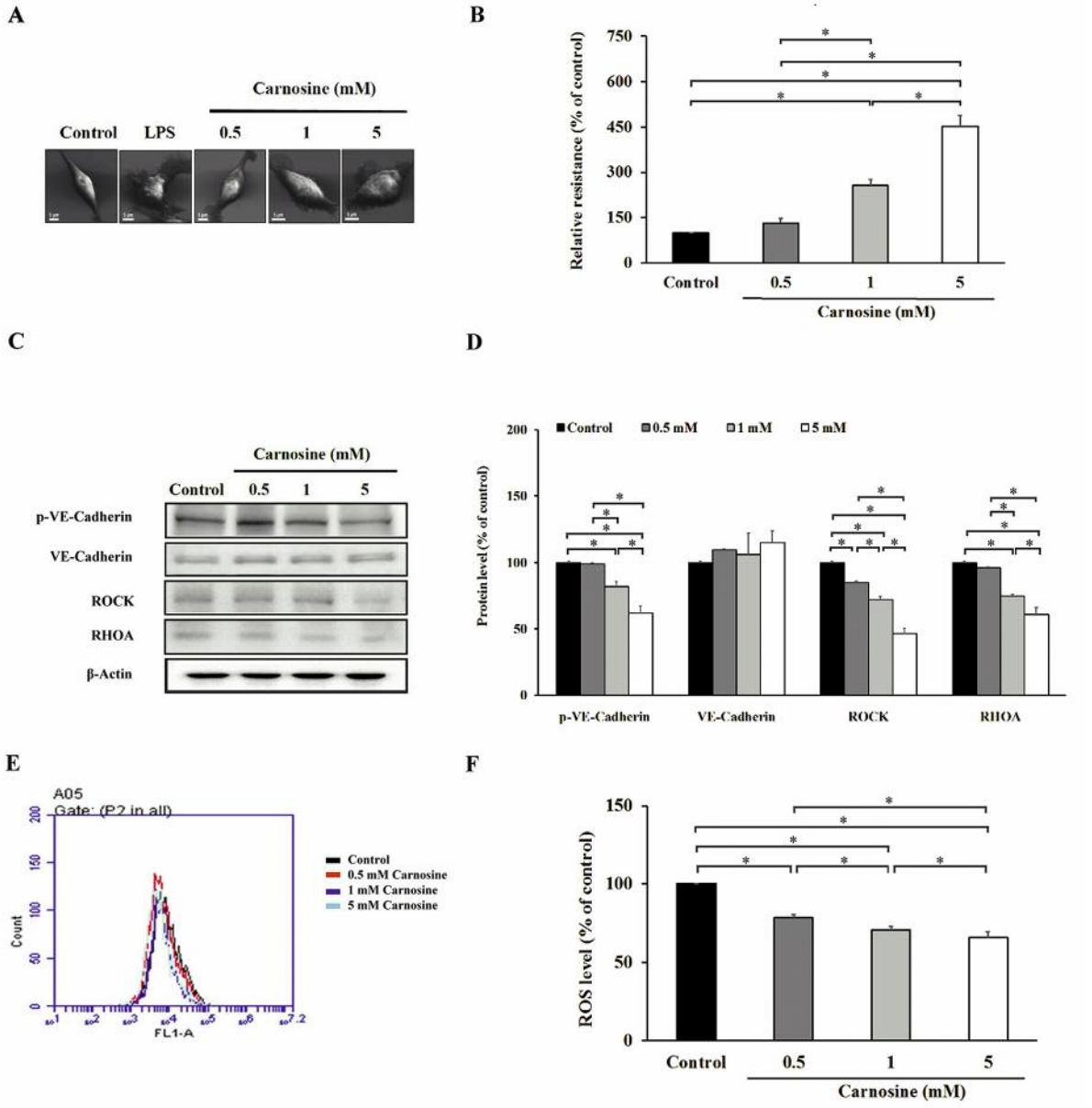

Figure 2. *Effect of carnosine on extravasation and permeability in EA.hy926 cell monolayer cell-cell junctions. HCT-116 or EA.hy926 cells (5×10^5 cells/3-cm transwell plate) were treated with 0.5, 1, or 5 mM carnosine. (A) Scanning electron microscopy images of cells exposed to lipopolysaccharide (LPS) and LPS combined with 0.5, 1 and 5 mM carnosine for 24 h. Dimethyl sulfoxide treatment served as the control. (B) The effect of carnosine on transepithelial electrical resistance in EA.hy926 cells. (C) After 48 h of incubation, immunoblotting assays were used to analyze the effects of carnosine on phosphor-vascular endothelia-cadherin (p-VE-cadherin), RHO-associated coiled-coil containing protein kinase (ROCK) and Ras homologous A (RHOA) expression in EA.hy926 cells. (D) Quantification of the protein expression in EA.hy926 cells by densitometry; the expression levels in the control group were considered as 100%. (E) Representative cytometry by reactive oxygen species (ROS) in EA.hy926 cells treated with 0.5, 1, or 5 mM carnosine. (F) Quantification of ROS in EA.hy926 cells treated with 0.5, 1, or 5 mM carnosine. The values are presented as the means±SD (n=3). *$p<0.05$ (ANOVA and Duncan's multiple comparison test).*

Results

The effect of carnosine on EA.hy926 cell viability. To find the optimum concentration of carnosine for inhibiting extravasation, an MTT assay was used to analyze cell viability in this study. Cell viability was significantly reduced by 8-14% after EA.hy926 cells were treated with carnosine for 48 h compared with that in the control group ($p<0.05$). The cell viability of carnosine-treated cells (approximately 78-80%) was significantly less than that of

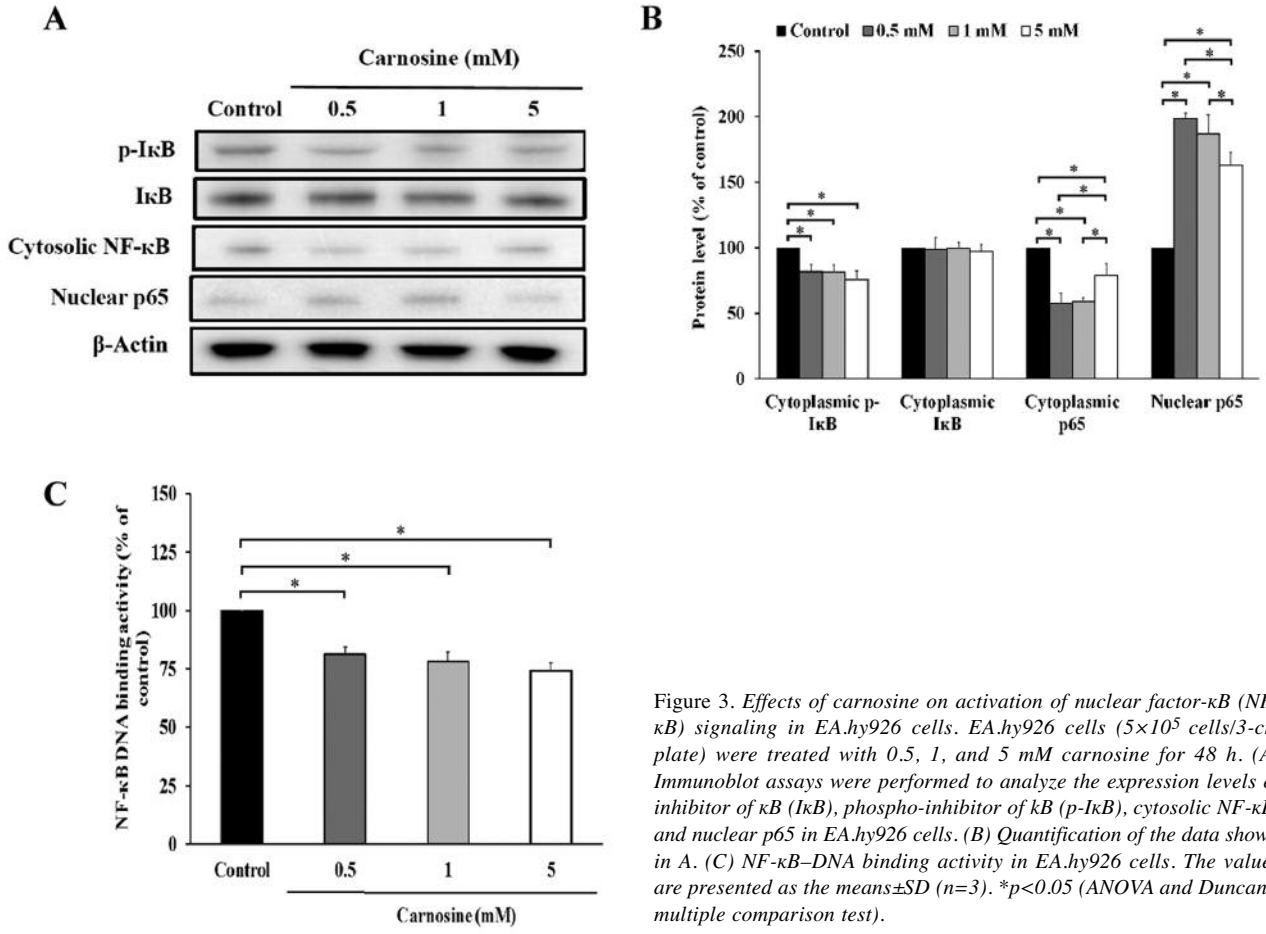

Figure 3. *Effects of carnosine on activation of nuclear factor-κB (NF-κB) signaling in EA.hy926 cells. EA.hy926 cells (5×10^5 cells/3-cm plate) were treated with 0.5, 1, and 5 mM carnosine for 48 h. (A) Immunoblot assays were performed to analyze the expression levels of inhibitor of κB (IκB), phospho-inhibitor of kB (p-IκB), cytosolic NF-κB, and nuclear p65 in EA.hy926 cells. (B) Quantification of the data shown in A. (C) NF-κB–DNA binding activity in EA.hy926 cells. The values are presented as the means±SD (n=3). *p<0.05 (ANOVA and Duncan's multiple comparison test).*

the control group (100%) ($p<0.05$) (data not shown). These results showed that 0.5-5 mM carnosine was an appropriate experimental dose range for subsequent studies.

Carnosine reduces adhesion between HCT-116 and EA.hy926 cells. The adhesion ability of HCT-116 cells, which were stained with BCECF, to EA.hy926 cells was analyzed in this study. Figure 1A shows that when EA.hy926 cells were treated with carnosine, HCT-116 cells, which were stained with a green fluorescent spot, adhered less than those cells in the control group. The quantified fluorescence spectrometric results shown in Figure 1B indicate that the cell adhesion was significantly reduced by 12-36% after carnosine treatment compared with those in the control group ($p<0.05$).

Furthermore, Figure 1 C and D shows that compared with expression in the control group, the expression of integrin-β1 in HCT-116 cells was significantly decreased after carnosine treatment for 48 h ($p<0.05$). The expression of ICAM1 in EA.hy926 cells was significantly lower than those of the controls after 5 mM carnosine treatment for 48 h ($p<0.05$). Compared with the control group, when EA.hy926 cells were treated with 1 or 5 mM carnosine for 48 h, the E-selectin expression was significantly reduced by 42-52% ($p<0.05$). These results showed that carnosine may inhibit the expression of adhesion proteins, such as integrin-β1, ICAM1, and E-selectin, reducing the ability of HCT-116 cells to adhere to EA.hy926 cells.

Carnosine reduces the permeability and extravasation of EA.hy926 cells. In this study, SEM examination was an important method for observing the cell surface and morphology under the extravasation process. Figure 2A shows that HCT-116 cells were flat and wrinkled under extravasation induced by LPS. However, when HCT-116 cells were treated with LPS combined with carnosine for 24 h, the flat and wrinkled morphology was markedly improved. Compared with cells in the control group, the TEER measurements in cells treated with carnosine were significantly increased by 131-145% ($p<0.05$) (Figure 2B). These results showed that carnosine reduces the extravasation

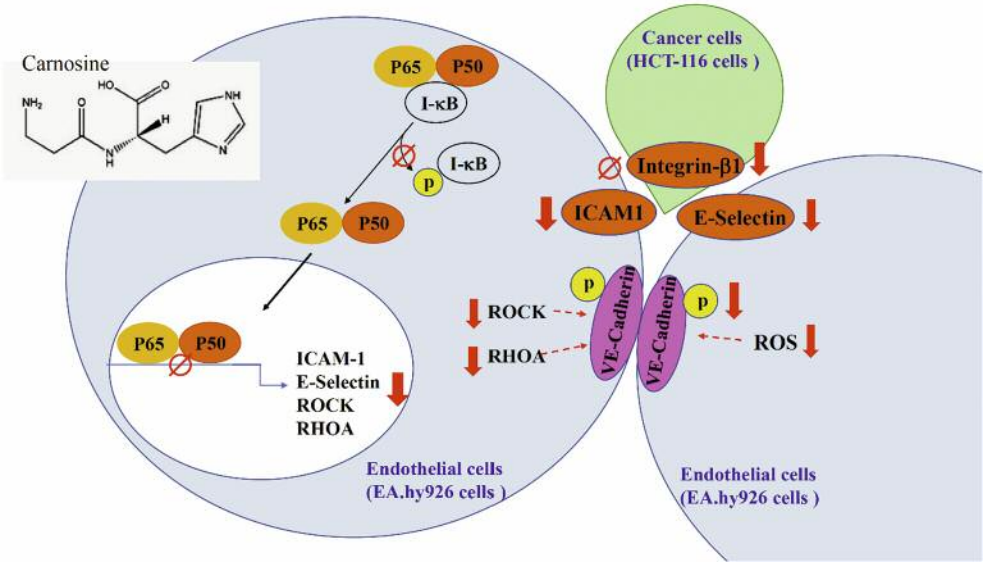

Figure 4. *Possible mechanisms by which carnosine inhibits cell adhesion and extravasation by reducing nuclear factor-kappaB (NF-κB) pathway activation. Carnosine suppresses adhesion and extravasation through the following pathway: (i) Carnosine inhibits the NF-κB activation pathway in EA.hy926 cells by reducing phosphorylation of inhibitor of kB (IκB) and reducing NF-κB activity, which then reduces levels of intercellular adhesion molecule 1 (ICAM1), E-selection, RHO-associated coiled-coil containing protein kinase (ROCK), and Ras homologous A (RHOA) proteins. (ii) Carnosine reduces the permeability of EA.hy926 cell–cell junctions by reducing levels of reactive oxygen species (ROS), ROCK, RHOA proteins and vascular endothelia-cadherin (VE-cadherin) phosphorylation. (iii) Carnosine inhibits HCT-116 cell adhesion to EA.hy926 cells by suppressing their adhesive ability and reducing molecular adhesion and levels of integrin β1, ICAM1, and E-selectin proteins.*

ability and permeability of HCT-116 cells and increases the integrity of EA.hy926 monolayer cell–cell junctions.

To determine how carnosine reduces the permeability of EA.hy926 cell–cell junctions, the phosphorylation of VE-cadherin and its regulation of molecular expression were examined in this study. The phosphorylation of VE-cadherin was significantly reduced after 1 or 5 mM carnosine treatment for 48 h in EA.hy926 cells compared with the control ($p<0.05$) (Figure 2C and D). The protein expression of VE-cadherin of carnosine-treated cells did not significantly change as compared with the control group (Figure 2C and D). The ratio of p-VE-cadherin to VE-cadherin in cells treated with 0.5, 1 and 5 mM carnosine was 92.8±15.5%, 78.1±13.8% and 51.3±4.2%, respectively, and was significantly lower than for the control cells. The protein expression of ROCK and RHOA in EA.hy926 cells significantly decreased after 1 or 5 mM carnosine treatment for 48 h compared with the controls ($p<0.05$; Figure 2C and D). Figure 2E and F shows that the levels of ROS in EA.hy926 cells significantly decreased after carnosine treatment for 48 h compared with those in the controls. These results showed that carnosine may inhibit the phosphorylation of VE-cadherin by reducing the expression levels of ROCK and RHOA and the ROS levels in EA.hy926 cells. In addition, the inhibition of VE-cadherin phosphorylation of led to reduced permeability and increased integrity of EA.hy926 monolayer cell–cell junctions.

Carnosine regulates adhesion and extravasation by suppressing NF-κB pathway activation. After EA.hy926 cells were treated with carnosine, I-κB phosphorylation was significantly reduced after 48 h of incubation ($p<0.05$). However, there were no effects on the protein level of IκB after carnosine treatment for 48 h. The ratios of p-IκB/IκB of 0.5, 1, and 5 mM carnosine-treated groups were 82.6±7.0%, 82.0±8.8% and 77.8±7.9%, respectively; they were significantly reduced as compared the control ($p<0.05$). Expression of cytosolic NF-κB significantly decreased after 0.5, 1, or 5 mM carnosine treatment for 48 h ($p<0.05$). In addition, NF-κB DNA-binding activity was significantly reduced after carnosine treatment for 48 h ($p<0.05$). These results showed that carnosine suppresses the activation of the NF-κB pathway, reducing expression of molecules related to adhesion and extravasation.

Discussion

The present study found that carnosine suppressed the adhesion and extravasation of CRC cells through regulating adhesive ability and extravasation. Carnosine inhibited adhesive ability by reducing the expression of intracellular integrin-β1 in HCT-116 cells and ICAM1 and E-selectin in EA.hy926 cells. Carnosine also inhibited extravasation by reducing cell–cell contact permeability and maintaining the

endothelial cell surface integrity of the cell monolayer. Moreover, carnosine can suppress expression of adhesion and extravasation-regulated molecules, including ICAM1, E-selectin, ROCK, and RHOA, through inhibiting the activity of NF-κB signaling in EA.hy926 cells. These findings confirmed that carnosine potentially inhibits CRC cell metastasis by suppressing adhesion and extravasation. Carnosine may be a potential compound for preventing or treating cancer metastases.

During the metastatic process, primary tumor cells migrate and adhere to the endothelium of the vasculature on the target metastatic organ. There is a multiple-step interaction between tumor cells and vascular endothelial cells (30). Several adhesion molecules, including integrin, cadherin and immunoglobulin-like molecules, have been shown to be involved in interactions between cell–cell surface adhesion properties and cell–cell communication (31). Integrins, which are cell-surface glycoproteins, are involved in cell–extracellular matrix (ECM) interactions and mediate the cellular environment through the actin cytoskeleton (32). The expression and binding of integrins and E-cadherin induce the RHO family of GTPases, such as RHO, Ras-related C3 botulinum toxin substrate (RAC) and cell division control protein 42 (CDC42), leading to changes in cell shape through adaptive actin cytoskeleton remodeling in response to adhesion (33). A previous study showed that acetin, which is a natural triterpene glycoside from the root of *Cimicifuga* species, suppresses the migration and adhesion of MDA-MB-231 and MCF-7 human breast cancer cells through inhibiting cellular integrin-1 expression (34). Recently, our previous study showed that carnosine significantly reduced the expression of E-cadherin in HCT-116 cells (8). In this study, carnosine also significantly reduced the protein expression of integrin-β1 in HCT-116 cells (Figure 1). Carnosine reduces the adhesive ability and maintains the normal morphology of HCT-116 cells by reducing the expression of integrin-β1, ICAM1 and E-cadherin. The SEM examination results strongly support these observations. Figure 2A shows that the HCT-116 cell shape became flattened, shrunken and partially collapsed after induction with the inducer of invasion LPS. The binding of metastasizing cells to the cell monolayer is an important phenomenon that leads to a flat and collapsed cell shape, disruption of endothelial cell–cell interactions and retraction of metastasizing cells (35, 36). However, carnosine treatment improved the morphological changes of HCT-116 cells after LPS induction (Figure 2). Our results demonstrated that carnosine not only reduced integrin expression but also improved cell shape by regulating the actin cytoskeleton in HCT-116 cells.

In addition to integrin-β1, the expression of ICAM1 and E-selectin were inhibited by carnosine in EA.hy926 cells. The immunoglobulin-like molecules play an important role in the signal transduction of cell membranes, cell–cell interactions, cell–endothelial cell interactions, and regulation of cell migration and adhesion (37). In addition to being involved in atherosclerosis formation, ICAM1 is also involved in the signaling response, which may enhance metastatic phenotype (38). In addition, the efficiency of E-selectin-mediated binding of colon carcinoma cells to the human endothelium is correlated with tumor progression (37). E-Selectin is expressed on activated endothelial cells for cell-surface adhesion reactions (12). Luo *et al*. showed that reduction of ICAM1 expression inhibited the invasion and metastasis of thyroid cancer cells (39). Inhibition of lung cancer cell invasion was shown to occur through the inhibition of ICAM1 expression (40). Rosmarinic acid from *Prunella vulgaris* inhibited the adhesion and metastasis of CRC cells by suppressing ICAM1, E-cadherin and integrin-β1 expression in HCT-116 cells (41). In this study, carnosine was found to inhibit the adhesion of HCT-116 cells to EA.hy926 cells by reducing ICAM1 and E-selectin expression, which is a very unique and important finding.

The endothelial lining is a semipermeable barrier. This vascular system controls the extravasation of plasma proteins, blood cells, macromolecules and foreign materials (42). The adhesion junction is one of the major connecting junctions between cell–cell connection (43). After metastatic cancer cells adhere to the vascular system of the target organ, endothelial cell–cell monolayer integrity and permeability affect the extravasation ability of migrating cancer cells. VE-cadherin plays an important role in vascular integrity and endothelial cell–cell adhesion (44). VE-cadherin binds to the cellular actin cytoskeleton and regulates cell–cell permeability. Phosphorylation of VE-cadherin leads to destabilization of the adherens junction complex and increases permeability of the cell–cell monolayer (45). A previous study showed that *honokiol*, which is a bioactive constituent of the bark from *Magnolia officinalis*, inhibits cell invasion and permeability *via* regulation of VE-cadherin phosphorylation in human glioblastoma cells (U87MG) (46). In this study, carnosine significantly reduced the extravasation ability of HCT-116 cells that invaded the cell-cell monolayer consisting of EA.hy926 endothelial cells. Additionally, the phosphorylation of VE-cadherin in EA.hy926 cells was significantly reduced after carnosine treatment. The TEER results also showed that the TEER of the EA.hy926 endothelial cell monolayer was increased after carnosine treatment. These results confirm that the permeability of the EA.hy926 endothelial cell monolayer significantly decreased after carnosine treatment.

Furthermore, VE-cadherin-based junctions are regulated by a small GTPase, which is a member of the RHO family. Small GTPase signaling is involved in remolding junction-related actin cytoskeletons and thereby affects cell–cell adhesion and permeability (18). The RHO family is a major

regulator of actin cytoskeleton dynamics, cell polarity, membrane transport and gene expression (47). RHOA promotes actin stress fiber formation and focal adhesion congregation. When RHOA binds with ROCK, this complex translocates to the cell membrane, leading to the phosphorylation of the myosin light chain of myosin II and actin-myosin contraction, ultimately enhancing permeability of the endothelial cell–cell monolayer (16). Chin *et al*. showed that suppressing RHO/ROCK signaling in cancer cells reduces angiogenesis, invasion and metastasis *in vitro* and *in vivo* (48). In this study, carnosine successfully reduced extravasation and permeability between endothelial cell–cell contacts by reducing the expression levels of RHOA and ROCK, thereby reducing the phosphorylation of VE-cadherin. Plumbagin, which is isolated from the roots of the medicinal plant *Plumbago zeylanica* L., reduced osteopontin-induced invasion of A549 non-small cell lung cancer cells by inhibiting the ROCK pathway and suppressing lung metastasis *in vivo* (49).

Notably, carnosine significantly reduced ROS levels in EA.hy926 cells. A previous study showed that carnosine has good antioxidative ability *in vivo* and *in vitro* (50, 51). The antioxidative characteristic of carnosine affects vascular permeability and cell shape by reducing levels of ROS. ROS are involved in vascular permeability by up-regulating the expression of molecules such as ICAM1 (52). ROS increase leads to cellular edge ruffling (53). ROS can regulate vascular permeability by modifying junction protein phosphate ions and can inactivate protein phosphatase by oxidizing cysteine residues in VE-cadherin (54). ROS also mediates junctional permeability by regulating the organization of the actin cytoskeleton and affecting the activity of RHO (53).

NF-κB is a crucial transcription factor that is involved in various physiological and pathological effects, including inflammation, cell proliferation, carcinogenesis and cancer cell metastasis (55). In this study, carnosine was shown to significantly suppress NF-κB signaling activation by reducing the phosphorylation of IκB and the DNA binding activity of NF-κB. These suppression effects led to significant reduction of the expression of cell adhesion- and extravasation-associated proteins, including ICAM1, E-selectin, ROCK and RHOA, in EA.hy926 cells (Figure 3). Inactivated NF-κB is sequestered in the cytoplasm by its interaction with the inhibitory protein IκB. The exposure of cells to oxidative or proinflammatory factors causes IκBα phosphorylation, which leads to the disassociation of NF-κB from IκBα (56). Activated NF-κB is then translocated to the nucleus, where it binds to the cis-acting κB enhancer element of target genes, such as ICAM1, E-selectin, ROCK and RHOA, and activates their transcription (57). These results indicated that NF-κB is an important cellular target of carnosine.

Conclusion

As shown in Figure 4, our results confirmed that carnosine can suppress adhesion of HCT-116 cells to EA.hy926 cells and extravasation. Carnosine also inhibitand the NF-κB activation pathway of EA.hy926 cells by reducing IκB phosphorylation, NF-κB–DNA binding activity and ICAM1, E-selection, ROCK and RHOA protein levels. Carnosine also reduced the permeability of EA.hy926 cell–cell junctions by reducing protein levels of ROS, ROCK, RHOA and VE-cadherin phosphorylation. Carnosine significantly inhibited HCT-116 cell adhesion to EA.hy926 cells by suppressing molecular adhesion and integrin β1, ICAM1, and E-selection protein expression.

Conflicts of Interest

The Authors declare that they have no competing interests.

Authors' Contributions

C-C Wu and S-L Hsieh conceived and designed the study and were major contributors to writing and critically revising the article. P-Y Lai and S Hsieh performed the experiments and analyzed the data. C-C Cheng provided advice on the experiments and technical assistance. S-L Hsieh supervised the study. All Authors read and approved the article and agree to be accountable for all aspects of the research and ensure that the accuracy or integrity of any part of the work is appropriately investigated and resolved.

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