The Antioxidant Effect of Carnosol in Bovine Aortic Endothelial Cells Is Mainly Mediated via Estrogen Receptor $\alpha$ Pathway

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Antioxidant action is critical for maintaining the normal cardiovascular function and vascular endothelial cell is an important target of estrogen action through estrogen receptor (ER) pathway. This study is carried out to explore the antioxidant effect of carnosol in bovine aortic endothelial cells (BAECs) via ER pathway. The ER subtype specific estrogenic effect of carnosol was further demonstrated by luciferase reporter gene assay in human embryonic kidney (HEK) 293 cells. Carnosol was extracted from Chinese medicine Rosmarinus officinalis. ER positive BAECs were employed in cell proliferation assay and cell apoptosis tests. Oxidative stress by intracellular reactive oxygen species (ROS) were measured via 2’7’-dichlorofluorescein (DCF) production. ER$\alpha$ and ER$\beta$ specific antagonists 1,3-bis(4-hydroxyphenyl)-4-methyl-5-[4-[2-piperidinylethoxy]pheneno]-1H-pyrazole (MPP) and 4-[2-phenyl-5,7-bis(trifluoromethyl)pyrazole][1,5-$\alpha$]pyrimidine-3-yl]phenol (PHTPP) were employed as tools in the experiment. ER negative HEK 293 cells were employed in luciferase reporter gene assay. The results indicate that carnosol can effectively attenuate H$_2$O$_2$ induced slowing down of cell growth and increasing of cell apoptosis. At the meantime, carnosol pretreating can also effectively reduce the H$_2$O$_2$ induced intracellular ROS elevation in BAECs. ER$\alpha$ and ER$\beta$ antagonist, especially ER$\alpha$ antagonist, can effectively decrease the above antioxidant effects of carnosol. The reporter gene analysis further demonstrates that the action of carnosol on inducing ERE dependent luciferase expression is realized via ER pathway. The conclusion is that carnosol can exert antioxidant effects towards oxidative stress induced by H$_2$O$_2$ in BAECs. And such effects are realized via ER, especially ER$\alpha$ pathway. The results contribute to explain the mechanism of cardiovascular protective function of carnosol in postmenopausal women.

Key words carnosol; antioxidant effect; estrogen receptor; phytoestrogen; bovine aortic endothelial cell; reporter gene

Endogenous estrogens play essential roles not only in the hypothalamic-pituitary-gonadal axis, but also in cardiovascular and central nervous systems as well as bone and lipid metabolism. It has been reported that the cardiovascular risk rises significantly in recent years. The statistical results indicated that cardiovascular disease (CVD) remains one of the main causes of death among women, especially aged women experiencing postmenopausal periods.$^{1,2}$ Besides the side effect of estrogen replacement therapy (ERT) on leading to higher breast cancer incidence, Women’s Health Initiative (WHI) and the heart and estrogen/progestin replacement study (HERS) found that estrogen did not reduce adverse cardiovascular events and, in fact, increased the number of cardiovascular events.$^{3}$ Thus people are paying much more attention to phytoestrogens in recent years.

Phytoestrogens are a group of compounds with chemical structures resembling natural and synthetic estrogens and thus enable them the binding affinity to estrogen receptors (ERs). Through activation or up-regulation and inactivation or down-regulation of certain genes, they can mimic some actions of human estrogens.$^{5}$ Various kinds of phytoestrogen perform different biological activity due to their structure and deviations in the structure. Phytoestrogen genistein, found in soy products, has been reported to possess antioxidant and estrogenic activity.$^{5}$ Although estrogen always shows similar high affinity with both ER$\alpha$ and ER$\beta$, phytoestrogens often have relatively different and lower affinity upon ER$\alpha$ and ER$\beta$. Thus they may display some effects different from those of estrogen. It has been reported that some active components in Chinese medicine such as resveratrol (RVT) possessed antioxidant properties.$^{7}$ As a phytoestrogen, RVT can regulate the transcription of estrogen-responsive target genes via estrogen receptors.$^{8,9}$ In bovine aortic endothelial cells (BAECs), RVT exerts endothelial improving function through both ER$\alpha$ and ER$\beta$.$^{10}$

Carnosol is a dietary diterpene isolated from culinary herbs such as Rosemary, Sage and Oregano.$^{11}$ The antioxidant properties of rosemary, Rosmarinus officinalis L. (Labiatae), have been known for centuries.$^{12}$ Carnosol has been proved to be an important antioxidant compound and contributes much to the corresponding function of Rosemary.$^{13}$ It has also been reported to exert brand functions involving in human health such as anti-angiogenic activity, antiproliferative properties towards cancer cells of intestinal, mammary and prostate tumor, anti-inflammatory, anti-infective and anti-human immunodeficiency virus (HIV) function.$^{9,14-18}$ Carnosol has also been known to be an active anti-metastatic agent against malignant melanocytes.$^{19}$

As an essential part in treating postmenopausal syndromes, antioxidant action is critical for maintaining the normal cardiovascular function in women experiencing postmenopausal period. As mentioned above, estrogen receptors are main target points, pathway and of great significance in dealing with menopausal syndromes. But till now, there has been no report

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concerning about the correlation between antioxidant effects of carnosol and function of estrogen receptor subtypes. This experiment is designed and conducted to study the mechanism through which carnosol realizes its antioxidant effect via ER pathway. In order to analyse the ER subtype dependent estrogenic effects of carnosol much directly, its action on inducing ERE linked to the luciferase reporter gene was transfected in the assay system as an internal standard.

**Materials and Methods**

**Drugs and Chemicals** The dried leaves of *Rosmarinus officinalis* (100 g) were powdered and extracted with acetone for three times at room temperature, and the solution was evaporated under reduced pressure to give a residue. The acetone extract (5 g) was chromatographed over silica gel (200 g) using CH2Cl2 (Sigma Chemical Co., St. Louis, MO, U.S.A.) with increasing amounts of MeOH (Sigma Chemical Co.) to give carnosol (12 mg). The chemical structure of carnosol (Fig. 1) was further proved by HPLC and identified by comparison with the published NMR data.

**Plasmids** Professor Satoshi Inoue at the University of Tokyo, Japan, graciously provided plasmids containing either ERα or ERβ. Plasmid containing 5× estrogen–response element (ERE) linked to the luciferase reporter gene was recombined and stored by our laboratory. An additional β-galactosidase plasmid (Clontech Laboratories Inc., Mountain View, CA, U.S.A.) was co-transfected in the assay system as an internal standard.

**Culture of ER-Positive BAECs and ER-Negative Human Embryonic Kidney (HEK) 293 Cells** BAECs and HEK 293 cells (American Type Culture Collection, Manassas, VA, U.S.A.) were maintained and subcultured in Dulbecco’s modified Eagle’s medium (DMEM) (Gibco, Grand Island, NY, U.S.A.) with 10% heat-inactivated fetal bovine serum (HyClone, Logan, UT, U.S.A.), 100 U/mL penicillin, 100 µg/mL streptomycin (HyClone) under humidified conditions at 5% CO2.

*Fig. 1. Chemical Structure of Carnosol*

**Morphological Assessment of Apoptosis by Hoechst 33342 Staining** BAECs were cultured on 8-well Chamber slides. After pre-treating with 0.1–1 µM carnosol in the presence or absence of 10⁻⁸ M estrogen receptor specific inhibitor MPP or PHTPP for 24 h, the BAECs were stimulated with oxidative stress by 1 mM H2O2 for 30 min. Then the cells were trypsinized, washed with PBS, fixed with 4% paraformaldehyde for 15 min at room temperature. After stained with 5 µg/mL Hoechst 33342 (Sigma Chemical Co.) at 37°C for 15 min, samples were observed under a DMR fluorescence microscope (Carl Zeiss Imager A1, Germany) with UV illumination. The dye was excited at 340 nm, and fluorescence emission was filtered with a 510 nm barrier filter. To quantify the apoptotic process, cells with fragmented or condensed DNA and normal DNA were counted respectively. Data were expressed as the ratio of apoptotic cells to total cells.

**Cell Apoptosis Assay by FACS** BAECs were cultured in 6-well plates at 5×10⁴ cells/well. After pretreated with 0.1–1 µM carnosol with or without 10⁻⁸ M estrogen receptor specific inhibitor MPP or PHTPP for 24 h, cells were exposed to 1 mM H2O2 for 30 min as oxidative stress stimulation. Subsequently, cells were collected and washed with PBS. Cell apoptotic activities were detected by Annexin V-FITC (fluorescence isothiocyanate) apoptosis detection kit (BD Biosciences, San Diego, CA, U.S.A.). Cells were resuspended in Binding Buffer and stained with 5 µL Annexin V-FITC and 5 µL propidium iodide (PI) for 15 min at RT (25°C) in the dark. After adding additional 400 µL Binding buffer to each tube, cell apoptosis was analyzed by Fluorescence Activated Cell Sorter (FACS) (FACS Caliber, BD Bioscience, CA, U.S.A.) within 1 h.

**Measurement of Intracellular Reactive Oxygen Species (ROS) Formation by DCF Production** Production of ROS was measured by oxidation sensitive fluorescent probe, 2’7’-di-chlorofluorescein diacetate (DCF-DA) (Sigma Chemical Co.) method, based on the ROS dependent oxidation of DCF-DA to DCF. 1×10⁵ BAECs were plotted into 24 well plates. Cells were grown in DMEM and pretreated with 0.1–1 µM carnosol in the presence or absence of 10⁻⁸ M estrogen receptor specific inhibitor MPP or PHTPP for 24 h. Subsequently, medium was removed and cells were washed with PBS twice, followed...
by incubation with 30 µm DCF-DA (Sigma Chemical Co.) for 30 min at 37°C in the dark. After washed with PBS to remove excess DCF-DA, the oxidative stress stimulation was performed by 1 mM H2O2 for 30 min. After washed two times with PBS, ROS generation was measured by microplate reader as the fluorescence intensity of each well at an excitation wavelength of 495 nm and an emission wavelength of 530 nm.

Plasmid Transfection and Luciferase Reporter Gene Assay in HEK 293 Cells One day before transfection, HEK 293 cells were plotted into 24-well plates at a concentration of 2×10^4 cells/well. For transfection, maintenance media was aspirated and 100 µL transfection mix was added. Cells were cotransfected with 0.1 µg ERα or 0.1 µg ERβ, 0.8 µg ERE-luc and 0.1 µg β-gal control using lipofectamine 2000 (2 µL/well) (Invitrogen, Carlsbad, CA, U.S.A.). After an incubation of 4 h for transfection according to the manufacturer’s instructions, experimental medium was added without removal of the transfection media. The experimental medium was prepared with phenol red-free DMEM, 10% charcoal-stripped fetal bovine serum (HyClone) and carnosol with the final concentrations from 10^{-6} to 10^{-11} M. Each experiment also included an estradiol standard curve with 10-fold dilutions from 10^{-8} to 10^{-13} M. A vehicle control containing 0.1% ethanol, which was the maximal level of ethanol used in the treatment media, was included in the test. Cells were incubated for 24 h at 37°C. Then media was aspirated and cells were lysed with 100 µL Glo Lysis Buffer (Promega, Madison, WI, U.S.A.). Lysates were transferred to microcentrifuge tubes and stored at -70°C. For the luciferase assay, 50 µL lysate was added to 50 µL steady Glo luciferase assay reagent (Promega). Luminescence was measured with multilfunctional microplate reader (Tecan). A β-galactosidase assay kit (Promega) was used to test enzyme

![Fig. 2. Carnosol Inhibited Loss of Cell Availability Induced by H2O2 Stimulation](image-url)
activity. The final result of luciferase activity was standard by β-gal value in order to correct for well-to-well variation. The maximal achievable response to 10^{-8} M estradiol was arbitrarily set at 100%, luciferase activity in response to carnosol was measured by relative light units (RLUs).

**Data Analysis** All data were represented as mean values±S.D. Experimental results were analyzed by using factorial analysis of variance (ANOVA) with appropriate post hoc test (SPSS version 13.0). Differences were considered statistically significant at p values of 0.05 or less. Median effect concentration (EC_{50}) of all samples tested was estimated by nonlinear regression analysis. The computer program Origin was used for the analysis of sigmoidal dose–response curves to obtain estimates of EC_{50} values.

**RESULTS**

**Carnosol Inhibited Reduction of Cell Viability** MTT assay showed that 1 mM H_2O_2 treating induced a time-dependent cell viability reduction in BAECs. After exposure to 1 mM H_2O_2 for 30 or 60 min, the result indicated a significant reduction of 49.0% and 45.6% viability (relative to control, respectively) (Fig. 2). Pretreatment with different concentrations of carnosol attenuated H_2O_2 induced cell number reduction. As for 30 min in 1 µM carnosol treating group, cell availability remained 69.5% of control. However, if ERα or ERβ antagonist was added in the pretreatment together with carnosol, the attenuation of the reduction in cell availability was inhibited.

**Carnosol Inhibited Cell Apoptosis by Morphological Study and FACS Test** Hoechst 33342 staining indicates the condensation in the typical apoptotic cell nucleus. As shown in Fig. 3, control cells had normal round nuclei with dispersed...
DNA molecule and chromatin. After treated with 1 mM H$_2$O$_2$ for 30 min, BAECs showed a 20.8 percentage of chromatin condensation and nuclear shrinkage. In the carnosol pretreated group, percentage of apoptosis cells was decreased significantly.

In response to H$_2$O$_2$ stimulation, a statistically increased population of apoptotic cells was observed in FACS test. By pretreatment with carnosol for 24 h, apoptotic cell percentage was effectively inhibited compared with that in H$_2$O$_2$ treating group (Fig. 4).

Furthermore, in both morphological study and FACS test, ER$\alpha$ or ER$\beta$ antagonist suppressed the inhibitory effect of carnosol towards cell apoptosis. The difference between MPP and PHTPP treated groups in FACS test was significant. It implies that ER$\alpha$ plays more important roles than ER$\beta$ in reduction of cell apoptosis induced by carnosol.

Fig. 4. Carnosol Inhibited Cell Apoptosis Induced by H$_2$O$_2$

BAECs were treated with 1 mM H$_2$O$_2$ for 30 min alone or incubated for 24 h with 0.1 $\mu$M or 1 $\mu$M carnosol (with or without ER$\alpha$/ER$\beta$ selective inhibitor) before H$_2$O$_2$ exposure. (A) FACS analysis of BAECs labeled with Annexin V and propidium iodide (PI). (B) Histogram showing the percentage of apoptotic cells elevated in H$_2$O$_2$ treating group compared with that in control group. Pretreatment with carnosol effectively reduced the apoptotic cell population. But such reductive activity of carnosol was attenuated by ER antagonist, especially ER$\alpha$ selective antagonist. CON: control; CAR: carnosol. **$p<0.01$ represents significant differences compared with control group. ††$p<0.01$ represents significant difference compared with same dose of carnosol treating group without any ER antagonist. §§$p<0.01$ represents significant difference compared with same dose of carnosol treating group with ER$\alpha$ antagonist. All data were expressed as mean±S.D. from three experiments.
**ROS Going Up Is Alleviated by Carnosol** After stimulation with 1 mM H$_2$O$_2$ for 30 min, ROS level in BAECs showed a significant increase (Fig. 5). Carnosol pretreating could effectively reduce the H$_2$O$_2$ induced intracellular ROS elevation in BAECs. By DCF fluorescence measure, 1 µM carnosol led to a significant ROS decrease to 67.7, much lower than the H$_2$O$_2$ treating group. ER antagonists, especially ERα antagonist, caused a blockage of the reductive effect of carnosol on H$_2$O$_2$ induced intracellular ROS elevation. And there is significant difference between MPP and PHTPP pretreating groups.

**ERE-Mediated Luciferase Expression Could Be Induced by Carnosol via Both ERα and ERβ** Luciferase activities induced by carnosol at various doses are shown in Fig. 6 as a percentage of the maximal response to estradiol treatment. Carnosol could elicit a dose-dependent response in ERE-mediated gene expression at a range of 10$^{-11}$–10$^{-6}$M, especially through ERα. The EC$_{50}$ for ERα and ERβ was estimated with a full dose–response curve. All these values are summarized in Table 1. The results also indicate that carnosol has a weaker sensitivity to activate luciferase transcription via ERα or ERβ compared with estradiol.

**DISCUSSION**

In circulatory system, endothelial cells are initial site of injury because it reacts with physical and chemical stimuli within the circulation directly. The endothelium is now recognized to be a crucial homeostatic organ, fundamental for the regulation of the vascular tone and structure. Endothelial function is an essential part of the normal cardiovascular system. Studies have shown that the severity of endothelial dysfunction relates to the risk for an initial or recurrent cardiovascular event. It could be present even before actual changes occur in the vasculature. Estrogen has often been shown to prevent vascular dysfunction and injury. It affects vascular reactivity through direct effects on endothelial cells (ECs). It has been reported that a growing number of interventions known to reduce cardiovascular risk also improve endothelial function. Vascular endothelial cell is an important target of estrogen action through ER pathway. 17β-Estradiol shows the ability to mediate antioxidant effects on the oxidative stress induced by H$_2$O$_2$ in BAECs and thus contribute to maintain cardiovascular integrity. The active components from herbs as antioxidant agents including carnosol are being paid great attention and under careful research. But there has been no report connecting antioxidant effects of carnosol to the ER pathway.

**Table 1. The EC$_{50}$ Values of 17β-Estradiol and Carnosol for ERα and ERβ**

<table>
<thead>
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<th>Compound</th>
<th>EC$_{50}$ for ERα (M)</th>
<th>EC$_{50}$ for ERβ (M)</th>
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<tbody>
<tr>
<td>17β-Estradiol</td>
<td>5.77 × 10$^{-11}$</td>
<td>1.55 × 10$^{-10}$</td>
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<tr>
<td>Carnosol</td>
<td>1.94 × 10$^{-8}$</td>
<td>5.23 × 10$^{-8}$</td>
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*a* A dose–dependent curve shown in Fig. 6 to determine EC$_{50}$ was fit to the data.
demonstrated in our experiment to possess a significant action on BAEC proliferation in vasoprotective antioxidant events during cell oxidative stress triggered by H$_2$O$_2$. Carnosol showed a BAEC protective action towards oxidative damage. Specific ER subtype antagonist could effectively attenuate the anti-proliferative action of carnosol. That is to say, carnosol is able to display a phytoestrogenic action in BAECs.

There are two isoforms of estrogen receptors: ER$\alpha$ and ER$\beta$. Both are found in endothelial cells. Through activation of ERs and alternation of related gene expression, estrogen exerts multiple vascular effects including antioxidant properties. Actions of estrogen are primarily mediated by these two molecular targets. Nuclear ER signaling has been also considered one major mechanism for regulating the growth of endothelial cells. Through both ERs, estrogen exerts the function of preventing vascular dysfunction and injury and maintaining the integrity of vascular endothelium in postmenopausal woman. ER$\alpha$ and ER$\beta$ show different effects during that procedure. In several cases, ER$\alpha$ has been reported as the main factor in antioxidative process. As mentioned in the beginning, much more attention has been paid to phytoestrogens in recent years, especially some active components in Chinese medicine that show estrogenic effects. As a multifunctional compound, phytoestrogen resveratrol has attracted much attention to exert endothelial improving function through both ER$\alpha$ and ER$\beta$. Our cell proliferation results showed that the antioxidative action of carnosol is also mediated through ER pathway.

At the meantime, it has been reported that ER$\alpha$ and ER$\beta$ display different functions in mediating cell proliferation. ER$\beta$ is reported to play much important roles in reducing cell proliferation rate in ER positive cells. Comparing with estrogen, phytoestrogen also possesses the ability to activate ER pathway and always shows different binding ability to ER$\alpha$ and ER$\beta$ due to their similar but different structure or binding site. Thus the downstream effects of phytoestrogen are always different from estrogen.

According to the morphological observations by Hoechst staining, carnosol showed antioxidant ability towards the going up cell apoptosis rate induced by H$_2$O$_2$. The result of FACS assay also indicated an antioxidant effect of carnosol towards cell apoptosis. And in both tests, a descending of protective effects of carnosol towards oxidant stress was observed with EC$_{50}$ values of 5.77×10$^{-11}$M and 1.55×10$^{-10}$M respectively. The affinity of carnosol upon ER$\alpha$ and ER$\beta$ is also different. Like estradiol, carnosol induced greater luciferase expression via ER$\alpha$ than ER$\beta$. It has been reported that some kinds of phytoestrogen possess relatively higher affinity with ER$\beta$ than ER$\alpha$. But our results showed that ER$\alpha$ is a more effective target point for carnosol than ER$\beta$. And the results also demonstrate that the affinity of phytoestrogen with ER$\alpha$ and ER$\beta$ differed from that of estradiol owing to their diversity in conformation and the low homology in ligand binding regions of ERs. So the ER-ligand complex with specific conformation would bind to ERE and activate certain ERE-induced gene expressions in various degrees.

Above all, our study focused on the antioxidant effects of carnosol via ER pathway in BAECs. The ER subtype, especially ER$\alpha$ dependent estrogenic effect of carnosol has been further demonstrated by reporter gene assay performed in HEK 293 cells. The results contribute to explain the protective function and its pathways of Chinese medicine in treating or alleviating postmenopausal cardiovascular symptoms.

Furthermore, several animal studies have suggested that daily oral administration of carnosol is well tolerated. A good prospect of its clinical application can be predicted.

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