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Potential of Rosemary (*Rosemarinus officinalis* L.) Diterpenes in Preventing Lipid Hydroperoxide-Mediated Oxidative Stress in Caco-2 Cells

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The effects of 24 h supplementation of Caco-2 cells with carnosic acid and carnosol, and their activities against 5 μM oleic acid hydroperoxide (OAHPx)-mediated oxidative stress, were investigated. At 24 h of incubation, under nonstressed and stressed conditions, both compounds at 25, 50, and 100 μM supplement concentrations reduced catalase activity, whereas changes in glutathione peroxidase and superoxide dismutase activities varied depending upon the concentrations. Relative to control cultures, carnosic acid and carnosol reduced membrane damage by 40−50% when stressed by OAHPx. Carnosic acid and carnosol inhibited lipid peroxidation by 88−100% and 38−89%, respectively, under oxidative stress conditions. Both compounds significantly lowered DNA damage induced by OAHPx. Results of this study suggest that antioxidant activities of carnosic acid and carnosol could be partly due to their ability to increase or maintain glutathione peroxidase and superoxide dismutase activities.

KEYWORDS: Oxidative stress; antioxidants; lipid hydroperoxides; rosemary; carnosol; carnosic acid; Caco-2 cells

INTRODUCTION

Lipid oxidation is a common, but undesirable chemical change, which impacts flavor, aroma, nutritional quality, and texture of food. It also leads to the production of toxic compounds that negatively affect many vital biological reactions. It has been suggested that reactive oxygen species (ROS), free radicals, and oxidative products, such as lipid hydroperoxides, participate in tissue injuries and on the onset and progression of degenerative diseases in humans (*1*−*3*). We have previously reported that lipid hydroperoxides are capable of disrupting cell membranes and inducing cellular lipid peroxidation and DNA damage in human intestinal cells at 5−25 μM concentrations (*4*). It was also revealed that the existing antioxidant enzyme mechanisms in the intestine were not capable of overcoming cell toxicities mediated by lipid hydroperoxides, even at a low concentration of 5 μM (*4*). Physiologically relevant levels of lipid hydroperoxides (1−5 μM) have also been known to induce mild oxidative stress in the intestine (*5*). Therefore, supplementing the diet with natural antioxidants is very important to protect cells from unwanted free radical attacks.

The search for new antioxidants has been an undying interest of researchers, and the methods used to evaluate the antioxidant potential of these compounds have also captured continuous attention. This study investigated the potential of two dietary antioxidants, carnosic acid and carnosol, in reducing lipid hydroperoxide-mediated cell injury in human intestinal cells.

Carnosol and carnosic acid are phenolic diterpenes that can be found in sage and rosemary leaves. Carnosic acid and carnosol account for over 90% of the antioxidative activity of commercially available rosemary extracts (*6*). In addition to antioxidative activities, carnosic acid is a promoter of synthesis of nerve growth factor (NGF), which is vital for the function and growth maintenance of nerve tissues (*7*). This compound has also been gaining interest as a mean to manage weight in humans due to its ability to inhibit lipid absorption activities in the digestive system (*8*). Carnosic acid has also exhibited anticancer activities in leukemic cells by inhibiting cell proliferation (*9*). Topical application of rosemary extract containing carnosol decreased mouse skin tumor formation in another study (*10*). In stimulated mouse peritoneal cells, carnosol decreased inflammation-induced nitrite production, which indicates its antiinflammatory properties (*11*).

Rosemary extracts exhibit strong synergistic effects with α-tocopherol by donating hydrogen atoms to regenerate α-tocopherol from the α-tocopheroxyl radical (*12*). Carnosic acid is at least partially responsible for this effect (*13*). Both compounds possess strong hydroxyl- and peroxyl-radical scavenging properties, and in addition, carnosic acid scavenges hydrogen peroxide, and hypochlorous acid, which is a strong inflammatory agent in biological systems (*14*). Other studies have shown that carnosic acid and carnosol inhibit superoxide radical generation in a xanthine/xanthine oxidase system (*15*)

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and prevent LDL oxidation by scavenging lipid free radicals and superoxide radicals *in vitro* (*16*).

Haraguchi et al. (*15*) reported that carnosol and carnosic acid were powerful antioxidants in inhibiting lipid peroxidation in rat liver microsomal and mitochondrial systems and also protected blood cells against oxidative hemeolysis. The discovery of *in vivo* glutathione-S transferase activity enhancement in rat liver cells by carnosol suggested its capability in protecting cells against oxidation by affecting cellular antioxidant defense systems (*17*). Carnosol has been effective in protecting hepatocytes against carbon tetrachloride-induced liver damage by improving their structural integrity (*18*). Others have shown that carnosic acid is effective in scavenging intracellular ROS in human leukemia cells (*19*). The mechanism of the radical scavenging activity of carnosic acid is analogous to that of antioxidants, such as α-tocopherol, and is caused by the presence of two *O*-phenolic hydroxyl groups found at C_{11} and C_{12} of the molecule (*20*).

The results of above studies have shown that carnosol and carnosic acid are promising antioxidants to protect biological systems against oxidative stress, not only as free radical scavengers but also as compounds that are capable of affecting antioxidant enzymes. However, no studies have been conducted to evaluate the potential of these compounds in preventing oxidative stress in a gastrointestinal system. The current study provides information on the effectiveness of carnosic acid and carnosol (25, 50, and 100 μM) in reducing lipid hydroperoxide-induced oxidative stress in the intestine using a human colon carcinoma cell line, Caco-2.

MATERIALS AND METHODS

A human colon carcinoma cell line (Caco-2) was obtained from the American Type Culture Collection (Rockville, MD). Dulbecco's modified Eagle medium (DMEM) and fetal bovine serum (FBS) were purchased from Invitrogen Corp. (Carlsbad, CA). L-Glutamine, penicillin with streptomycin, trypsin with ethylenediaminetetraacetic acid (EDTA), and phosphate-buffered saline (PBS) were purchased from Fisher Scientific (Fair Lawn, NJ). Trypan blue, nonessential amino acid solution, xanthine, hypoxanthine, nitro blue tetrazolium, diethylenetriaminepentaacetic acid, lactate dehydrogenase (LDH)-based TOX-7 kit, and glutathione peroxidase cellular activity assay kit CGP-1 were obtained from Sigma Chemical Co. (St. Louis, MO). Hydrogen peroxide, chloroform, cyclohexane, and ethanol were purchased from VWR International (Bridgeport, NJ). The Micro BCA protein assay kit was purchased from Pierce Biotechnology (Rockford, IL). Carnosol was donated by Kalsec Inc. (Kalamazoo, MI). Carnosic acid was donated by Dr. Cliff Hall of North Dakota State University. Purities of these compounds were >99%.

Culture and Oxidation of Caco-2 Cells. Caco-2 cells were cultured using standard conditions as described in Wijeratne and Cuppett (*4*). Briefly, Caco-2 cells were grown in DMEM supplemented with 20% FBS, 1% L-glutamine, 1% nonessential amino acids, and 50 units/mL penicillin with 50 μg/mL streptomycin. The cell cultures were maintained at 37 °C in a humidified atmosphere with 5% CO_2 and were seeded onto collagen-coated 25 or 75 cm^2 area culture flasks. At the end of the fourth day, the media was replaced with fresh DMEM supplemented carnosic acid or carnosol and further incubated at 5% CO_2 at 37 °C for another 24 h. Stock solutions of 10000 μM carnosic acid and carnosol were prepared in 100% ethanol and further diluted in DMEM to attain concentrations of 25, 50, and 100 μM for carnosic acid and carnosol before incubation with Caco-2 cells. The final ethanol concentration in DMEM did not exceed 1%. At the end of the fifth day, half of the cultures were used to quantify effects of 24 h supplementation of carnosic acid and carnosol, and the other half of cultures were subjected to oxidative stress by 5 μM oleic acid hydroperoxides in FBS-free DMEM or PBS, supplemented with 1% L-glutamine and 1% nonessential amino acids, for 30 min. Cells were harvested by a brief (6 min) trypsinization and then centrifuged (Beckman GS-15R centrifuge, Beckman, Palo Alto, CA) at 200*g* for 5 min. The effects of 24 h supplementation of carnosic acid and carnosol and their effects on oxidative stress were quantified by measuring membrane leakage, DNA damage, conjugated dienes, and antioxidant enzyme activities.

Preparation of OAHPx Solutions. Lipid hydroperoxides were generated by oxidation of oleic acid as described previously (*4*). The total hydroperoxide content was quantified using the peroxide value of oxidized oleic acid (*21*). Preliminary studies showed that oxidation products other than lipid hydroperoxides were extremely low and were not in significant amounts in the oxidized oleic acid samples. Stock solution of peroxidized lipid emulsions was prepared by sonicating 80 μmol of oxidized oleic acid in PBS at pH 7.4 containing 19 mmol L^{-1} sodium taurocholate (*22*). The stock emulsion was diluted with FBS-free DMEM before exposure to cells to attain needed OAHPx concentrations. The final sodium taurocholate content of the medium in all treatments was adjusted to be the same.

Cell Membrane Damage. Cells were grown to confluence in 25 cm^2 culture flasks and washed with PBS prior to use. Different concentrations of OAHPx (0−25 μM) in PBS supplemented with 1% L-glutamine and 1% nonessential amino acids were used to induce oxidation. After 30 min of exposure, PBS from each flask was collected. Damage to cell membrane by OAHPx was studied by measuring the release of lactic acid dehydrogenase (LDH) from injured cells. LDH leakage into PBS and total LDH activity (LDH leakage to PBS plus LDH in remaining cells) were measured with an *in vitro* cytotoxicity assay kit, lactate dehydrogenase-based TOX-7 (Sigma Chemical Co.), and corrected by the activity already present in the medium of untreated cells. The assay is based on the reduction of NAD to NADH by LDH. NADH is utilized to convert a tetrazolium dye in the assay kit to a colored compound with an absorption maximum at 490 nm. The intensity of the color is indicative of LDH activity in the assay medium, and the LDH activity was measured spectrophotometrically (Beckman Coulter DU800 spectrophotometer, Beckman Coulter Inc., Fullerton, CA) at 490 nm. LDH activity in the PBS supernatant was determined as a percentage of the total LDH activity.

Preparation of Cell Lysates. Culture medium was decanted, and cells were washed with 5−10 mL of PBS. The cells were harvested by a brief trypsinization. Cell suspensions were centrifuged at 200*g* for 5 min and washed twice with 5 mL of PBS. Supernatants were discarded and cell pellets resuspended in 5 mL of PBS at 0 °C and then placed on ice. Cells were lysed using a minibead beater (Biospec Products, Bartlesvillle, OK) for 10 s at 4200 rpm. The lysates were centrifuged (Beckman GS-15R centrifuge) at 14000*g* for 10 min at 4 °C and supernatants immediately used for lipid peroxidation and antioxidant enzyme assays.

Lipid Peroxidation Assay. Lipid peroxidation was assayed by measuring conjugated dienes in cell lysates. Conjugated dienes were quantified according to the method described by Buege and Aust (*23*). One milliliter of cell lysate in PBS was mixed thoroughly with 5 mL of chloroform/methanol (2:1) solution, followed by centrifugation (Beckman GS-15R centri-

fuge) at 1000*g* for 5 min until phase separation was achieved. Most of the upper layer was removed by suction, and 3 mL of the lower chloroform layer was transferred to a test tube. The chloroform layer was removed under nitrogen infusion, and the lipid residue was dissolved in 1.5 mL of cyclohexane. The absorbance of the solution at 233 nm was measured (Beckman Coulter DU800 spectrophotometer) against a cyclohexane blank at 233 nm. Conjugated dienes were reported as absorbance at 233 nm.

Catalase Assay. Catalase was assayed spectrophotometrically at 25 °C by following the extinction of H_2O_2 at 240 nm (*24*). The catalase activity per milliliter of the cell lysate was calculated as the reduction of H_2O_2 (mmol min^{-1} mL^{-1}). Nonenzymatic H_2O_2 decomposition (baseline) was subtracted from each determination.

Superoxide Dismutase (SOD) Assay. A modified version of the Nishikimi et al. (*25*) method was used to detect SOD activity in cell lysates. In this method, superoxide radicals were generated using a xanthine oxidase/hypoxanthine system, and the potential of the cell lysates to scavenge superoxide radicals was measured spectrophotometrically. The reaction mixture contained 1 mL of 3 mM hypoxanthine, 1 mL of 100 mIU xanthine oxidase, 1 mL of 178 μM nitro blue tetrazolium, 1 mL of 12 mM diethylenetriaminepentaacetic acid, and 1 mL of the cell lysate. All solutions were prepared in PBS. The absorbance of the mixtures at 560 nm was recorded initially at 0 min and thereafter at 5 min intervals up to 30 min. Superoxide radical-scavenging capacities of the cell lysates at the end of 30 min were calculated with the equation:

$$Y = [1 - (A/B)] \times 100$$

where Y = percentage of superoxide radicals scavenged, A = absorbance of the medium containing cell lysate at 30 min, and B = absorbance of the medium without cell lysate at 30 min (blank).

Glutathione Peroxidase (GPx) Assay. GPx activity was measured using the GPx cellular activity assay kit CGP-1 (Sigma Chemical Co.). This kit uses an indirect method, based on the oxidation of glutathione (GSH) to oxidized glutathione (GSSG) catalyzed by GPx, which is then coupled with recycling GSSG back to GSH utilizing glutathione reductase (GR) and NADPH. The decrease in NADPH at 340 nm during oxidation of NADPH to NADP is indicative of GPx activity. The activity of GPx per milliliter of the cell lysate was calculated as the decrease in NADPH (μmol min^{-1} mL^{-1}).

DNA Damage by Comet Assay. The comet assay was performed using Trevigen's comet assay reagent kit for single-cell electrophoresis assay (Trevigen Inc., Gaithersberg, MD). Cells (1×10^5) were suspended in 1 mL of ice-cold PBS. Fifty microliters of the cell suspension was combined with 500 μL of prewarmed low melting point (LMP) agarose, and 75 μL of this mixture was immediately pipetted onto a CometSlide. Slides were placed flat at 4 °C in the dark for 30 min for gelling. After completion of gelling, slides were transferred into a prechilled lysis solution (2.5 M sodium chloride, 100 mM EDTA, pH 10, 10 mM Tris base, 1% sodium lauryl sarcosinate, and 1% Triton X-100) and placed at 4 °C for 50 min. Slides were then incubated in a fresh electrophoresis buffer (0.3 M NaOH, 1 mM EDTA, pH 13) for 40 min at room temperature to allow unwinding of DNA. Electrophoresis was carried out at room temperature in fresh electrophoresis buffer for 40 min at 1 V/cm and 300 mA. After electrophoresis, slides were gently rinsed by dipping several times in distilled water and then immersed in 70% ethanol for 5 min and air-dried. Slides were stored with desiccant at room-temperature prior to analysis. Slides were stained with SYBR green and viewed by an Olympus AX70TRF microscope digital camera system (Olympus Optical Co. Ltd.). Digital images of DNA were analyzed, and DNA damage was quantified by measuring the tail moment using NIH Image software available at http://rsb.info.nih.gov/ij/. About 100−150 cells were scored per sample. Damage is represented by an increase of DNA fragments that have migrated out of the cell nucleus in the form of a characteristic streak similar to the tail of a comet (*26*). DNA damage can be assessed using different parameters, such as tail length, relative tail fluorescence intensity, and tail moment (*26*, *27*).

Statistical Design. A general linear fixed effects model blocked by cell passage number, which corresponds to the age of cells, was used in all test systems. All experiments were repeated four times. Statistical analysis was conducted using Statistical Analysis System (SAS) software version 8.02 (SAS Institute Inc., Cary, NC) with analysis of variance (ANOVA) followed by Tukey's HSD test for significant differences. A p value of ≤ 0.05 was considered to be statistically significant unless stated otherwise.

RESULTS

The nonstressed and oxidatively stressed Caco-2 cell cultures responded differently depending on type and concentration of diterpene used to supplement the confluent cultures. The first sector of results deals with changes observed in cell cultures after supplementing the culture medium for 24 h with carnosol and carnosic acid. The second part describes changes occurring after exposing cell cultures to 5 μM oleic acid hydroperoxide for 30 min following 24 h supplementation with the diterpenes. In the text 'control' always refers to cell cultures that were not supplemented with diterpenes and/or not subjected to oxidative stress treatment by OAHPx.

Cellular Responses after 24 h Incubation with Carnosic Acid and Carnosol. Cellular Damage. Carnosic acid and carnosol significantly reduced base level LDH leakage (**Figure 1**) that occurs during normal cell culturing and handling procedures. There were no significant changes in cell lipid peroxidation or DNA damage in cultures supplemented with either of the two diterpenes compared to that of the control (**Figure 1**). In general, cell damage determinations revealed that neither carnosic acid nor carnosol, by themselves, at 25, 50, and 100 μM levels, initiated cellular damage within confluent Caco-2 cells during 24 h incubation.

Antioxidant Enzyme Activities. Diterpene supplementations caused significant changes to antioxidant enzyme profiles. Both compounds lowered the catalase activity compared to those of the control, but the decreasing effect of the lowest level of carnosic acid (25 μM) was less evident (**Figure 2**). GPx activity was significantly elevated in cultures supplemented with 25 and 50 μM carnosic acid, whereas the remaining supplements did not cause any notable changes (**Figure 2**). In contrast, SOD activity was significantly reduced by carnosic acid at 25 and 50 μM levels, whereas carnosol, at 25 and 50 μM, resulted in SOD activities comparable to those of the control cultures. Carnosic acid and carnosol at 100 μM significantly increased SOD activity, compared to those of the control (**Figure 2**).

Cellular Responses after Oxidative Treatment following 24 h Supplementation with Diterpenes. Cellular Damage. Exposing cultures to 5 μM OAHPx caused a 17% leakage of total cell LDH compared to 7% by control cultures (**Figure 3**). Carnosic acid and carnosol supplementation showed a 12−13% LDH activity in the culture medium, which is a 40% reduction

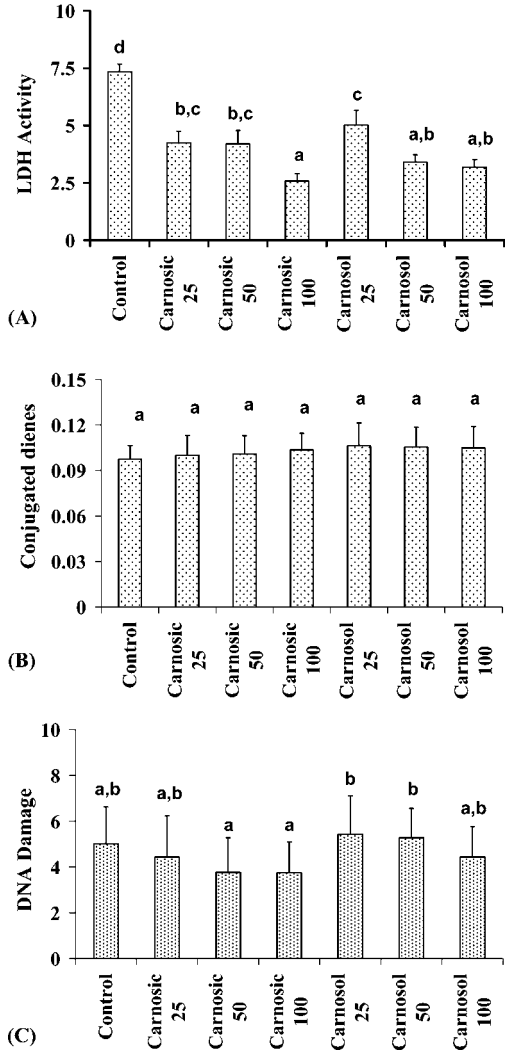

Figure 1. LDH activity in the culture medium (% of total cell LDH activity) (A), conjugated dienes (absorbance at 233 nm) (B), and DNA damage (tail moment in arbitrary units) (C), in cell cultures supplemented with carnosol and carnosic acid at 25, 50, and 100 μM for 24 h compared to control that had no supplements.

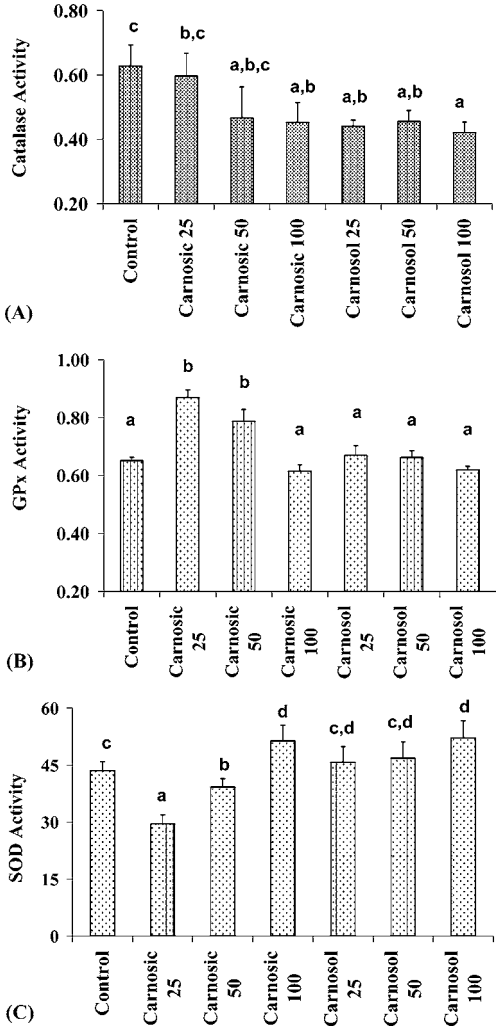

Figure 2. Activities of catalase (decrease in H_2O_2 mmol L^{-1} min^{-1} mL^{-1}) (A), glutathione peroxidase (decrease in NADPH μmol L^{-1} min^{-1} mL^{-1}) (B), and superoxide dismutase (% scavenged superoxide radicals) (C), in cell cultures supplemented with carnosol and carnosic acid at 25, 50, and 100 μM for 24 h, compared to control that had no supplements.

of the leakage caused by OAHPx exposure of Caco-2 cells. Conjugated diene measurements indicated that all levels of the two diterpenes protected Caco-2 cells against lipid peroxidation induced by OAHPx (**Figure 3**). Carnosic acid and carnosol at 25, 50, and 100 μM levels inhibited 89, 96, and 100%, and 38, 68, and 88%, respectively, of the conjugated dienes formed when the nonsupplemented cultures were exposed to OAHPx. A similar pattern of protection occurred against DNA damage (**Figure 3**). Both compounds at the tested concentrations significantly lowered DNA damage to levels comparable to those of control cultures.

Antioxidant Enzyme Activities. Oxidative stress induced by 5 μM OAHPx did not alter catalase or GPx activity profiles in Caco-2 cells. However, all the tested diterpene supplementations caused a significant decrease in catalase activity compared to nonsupplemented cultures (**Figure 4**). GPx activity was markedly elevated in cultures supplemented with 25 and 50 μM carnosic acid, but the rest of the supplement treatments showed no effect. OAHPx caused a significant decrease of 20% in SOD activity from that of the control. Carnosic acid and carnosol, at 25 μM, restored 36% and 52%, respectively, of the reduction in SOD activity caused by OAHPx, whereas 50 and 100 μM levels of both diterpenes completely restored the SOD activity up to levels that were the same as or slightly higher than that of control cultures (**Figure 4**).

Comparison of Responses of Diterpene Supplemented Caco-2 Cultures under Oxidatively Stressed and Nonstressed Conditions. As depicted by the **Figures 1** and **3** protections offered by carnosol and carnosic acid showed different patterns of activity between stressed and nonstressed cell cultures. There was a significant difference in LDH activity between nonstressed and stressed conditions, at all tested concentrations of the two diterpenes, which denotes the inability of either of these compounds to completely protect cell membranes from disruption. Under stressed conditions, carnosic acid was highly effective in reducing lipid peroxidation up to levels observed under nonstressed state, even at 25 μM concentrations, whereas carnosol showed the same activity at the 100 μM levels. In oxidatively stressed cultures, both compounds decreased DNA damage to levels analogous to those at nonstressed conditions, and 25 μM concentrations were as effective as 100 μM for both carnosic acid and carnosol in protecting DNA against lipid hydroperoxides mediated toxicity. Under nonstressed and oxidatively stressed conditions, catalase showed similar responses to both compounds (**Figures 2** and **4**). The decreasing effect

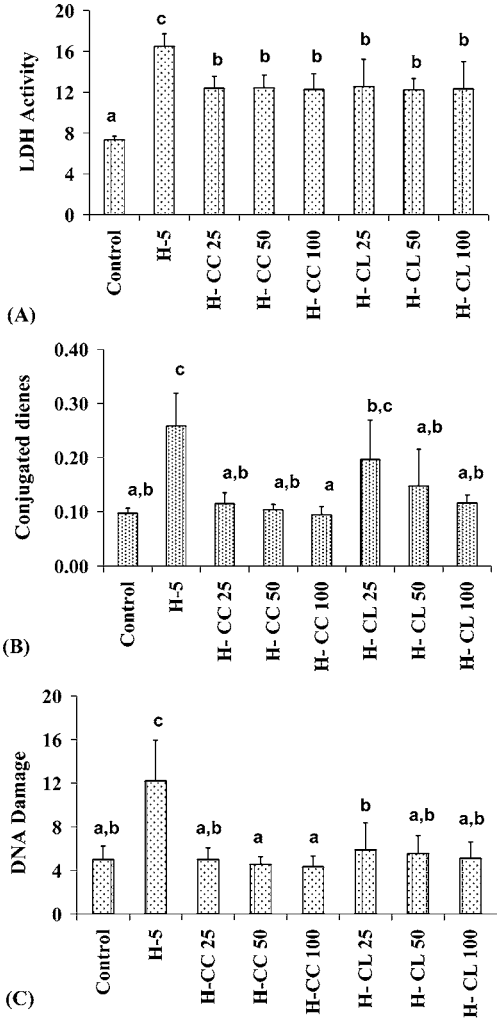

Figure 3. Changes in LDH activity in culture medium (% of total cell LDH activity) (A), conjugated dienes (absorbance at 233 nm) (B), and DNA damage (tail moment in arbitrary units) (C), in cell cultures supplemented with carnosol and carnosic acid at 25, 50, and 100 μM for 24 h and treated with 5 μM oleic acid hydroperoxide for 30 min. Symbols: H, hydroperoxide treated; CC, carnosic acid; CL, carnosol.

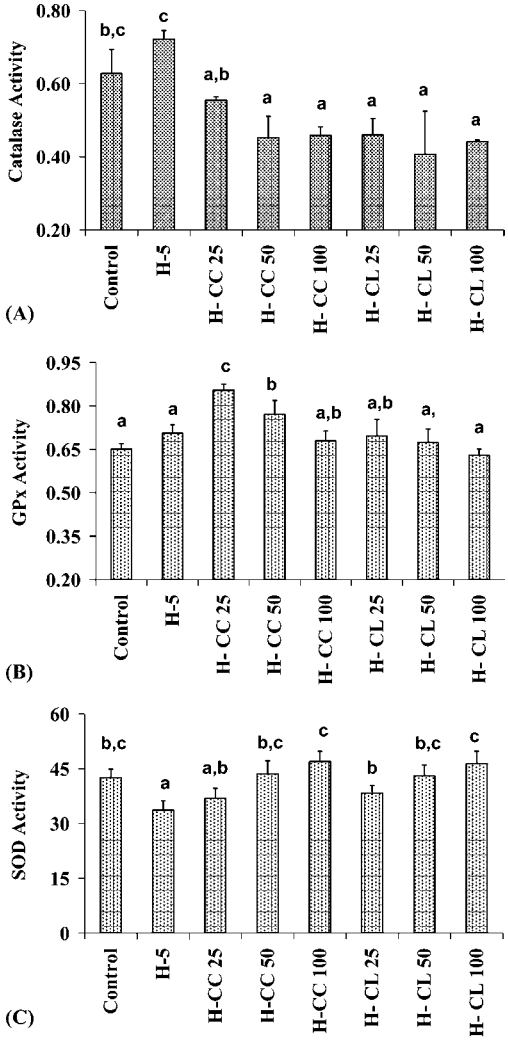

Figure 4. Activities of catalase (decrease in H_2O_2 mmol L^{-1} min^{-1} mL^{-1}) (A), glutathione peroxidase (decrease in NADPH μmol L^{-1} min^{-1} mL^{-1}) (B), and superoxide dismutase (% scavenged superoxide radicals) (C), in cell cultures supplemented with carnosol and carnosic acid at 25, 50, and 100 μM for 24 h and treated with 5 μM oleic acid hydroperoxide for 30 min. Symbols: H, hydroperoxide treated; CC, carnosic acid; CL, carnosol.

on GPx activity was more pronounced with increasing concentrations of carnosic acid compared to those of carnosol at both nonstressed and stressed conditions (**Figures 2** and **4**). The GPx activities between nonstressed and stressed conditions, for a given concentration of a diterpene, were not significantly different from each other.

Carnosol and carnosic acid increased SOD activity with increasing concentrations under both stressed and nonstressed levels (**Figures 2** and **4**). Under nonstressed conditions, the rate of increase of SOD activity was more pronounced with increasing carnosic acid concentrations compared to those at stressed conditions, whereas for carnosol, the change in the rates were not distinguishable. As observed with cellular protection, the effects on antioxidant enzymes were more pronounced by carnosic acid compared to carnosol.

DISCUSSION

This study assessed the effects of carnosol and carnosic acid supplementation on nonstressed and oxidatively stressed human colon cells. Both carnosic acid and carnosol protected cells against OAHPx-mediated cell toxicity by reducing membrane disruption and lipid peroxidation. Membrane lipids are highly susceptible to oxidation because of their high polyunsaturated fatty acid content. Peroxidized membranes become rigid, lose selective permeability, and under extreme oxidative stress conditions can lose their integrity (*28*). Lipid peroxidation, which can be measured by conjugated diene formation, occurred in Caco-2 cells when exposed to 5 μM OAHPx for 30 min. Carnosic acid and carnosol significantly inhibited the formation of conjugated dienes mediated by OAHPx, albeit carnosic acid was the stronger protector. In contrast, cell-mediated LDL oxidation was inhibited more effectively by carnosol compared to carnosic acid in a human aortic endothelial cell model system (*29*). A previous study showed that complete lipid peroxidation inhibition in rat liver mitochondrial and microsome systems induced by NADH or NADPH oxidation was attained by both carnosic acid and carnosol at 9 μM (*15*). Carnosic acid is more hydrophilic than carnosol due to its free carboxylic acid group (*13*). Hydrophilic antioxidants are expected to exert their effects in the aqueous environment in cells (e.g., cytoplasm and plasma), whereas lipophilic antioxidants are more aggregated, thus effective in lipid-rich cell components (e.g., membranes). However, carnosic acid also has an affinity toward the oil phase

in unbuffered oil−water mixtures with no emulsifiers (*30*). Therefore, carnosic acid could have oriented both in and around cell membranes, which are basically lipid bilayers consisting of a hydrophobic core and a hydrophilic exterior, thus preventing oxidative attack by OAHPx more effectively compared to carnosol.

As demonstrated by Aruoma et al. (*14*), carnosol and carnosic acid can also exert pro-oxidant effects at higher concentrations. Their studies showed both carnosic acid and carnosol, at concentrations between 10 and 2000 μM, promoted bleomycin-dependent DNA damage possibly by reducing ferric-bleomycin−DNA to the DNA-degrading ferrous form. These authors also reported that both compounds prevented site-specific deoxyribose damage by chelating iron and inhibiting hydroxyl radical formation. Our study showed that carnosic acid and carnosol prevented OAHPx-induced DNA damage in Caco-2 cells and did not induce DNA damage when incubated with nonstressed Caco-2 cells for 24 h at 25−100 μM concentrations. Therefore, it is evident that carnosol and carnosic acid activities vary depending on the tissue as well as the oxidative agent of interest. Because inhibition of DNA damage by carnosic acid and carnosol at 25 μM were not significantly different from those at 100 μM concentrations, antioxidant activity could be attained by using low levels of carnosic acid and carnosol, such as 25 μM, *in vivo*, while avoiding higher levels that may produce adverse effects.

Antioxidant activity of carnosic acid and carnosol is mainly due to the presence of *o*-dihydroxyl groups in their structures and to the ability of these groups to donate hydrogen atoms to neutralize free radical activity (*31*, *32*). Structure−activity relationship studies of different constituents in rosemary extracts showed that the hydroxyl group at C-11 is more important than other hydroxyl groups for their antioxidant activity (*33*). During lipid peroxidation inhibition by carnosic acid, first the hydroxyl group at C-11 undergoes hydrogen donation to a lipid radical species to form a carnosate radical, and then radical termination takes place by a radical−radical coupling reaction of carnosate radical with another lipid peroxyl radical (*31*). The coupling reaction takes place at the 12- or 14-positions, which are ortho- and para-positions, respectively, to the oxygen radical (*31*) forming epoxides and other intermediary products. The C-12 and C-14 positions are considered as the radical-stabilizing positions according to the captodative (electron-withdrawing and -releasing) effect (*34*).

In addition to radical trapping activities, carnosic acid and carnosol may also have affected lipid hydroperoxide decomposition by causing alterations in antioxidant enzyme activities. Diterpene supplementation of Caco-2 cell cultures for 24 h led to changes in catalase, SOD, and GPx activity patterns in both stressed and nonstressed conditions. Catalase activity was decreased significantly to below basal levels in cells treated with both carnosic acid and carnosol. In biological systems, catalase is responsible for removing H_2O_2, while GPx is responsible for the removal of hydrogen peroxides and other organic peroxides (*35*). Under physiological conditions, where a maximum H_2O_2 concentration is not greater than 75−100 μM, the more important enzyme in removing H_2O_2 is GPx compared to catalase (*36*). This fact is also confirmed by the low K_m (0.2 mM) of GPx (*37*) compared to that of catalase, which is around 1 M (*38*). However, decreasing antioxidant enzyme activity renders the cells more susceptible to free radical attacks, and therefore the mechanisms underlying the decreasing effect of diterpenes on catalase should be investigated further.

Carnosic acid and carnosol supplementation either increased or maintained GPx activity at base levels in the Caco-2 cells. Therefore, these results suggest that one of the mechanisms underlying lipid hydroperoxide detoxification by carnosic acid and carnosol is by increasing the level of activity of the GPx, which is a major enzyme responsible for the removal of lipid hydroperoxides in biological systems. Carnosol has also been reported to increase glutathione-S-transferase activity, which is another enzyme involved in decomposing hydroperoxides (*17*).

SOD is responsible for removing superoxide radicals in living tissues (*35*). In nonstressed Caco-2 cultures, depending on the supplement concentration, carnosic acid and carnosol increased, decreased, or had no effect on SOD activity compared to those of control cultures. However, when stressed by OAHPx, both compounds at all three supplement concentrations elevated the SOD activities higher than those of nonsupplemented oxidatively stressed cell cultures. This indicates that carnosic acid and carnosol could be responsible in preventing SOD from inactivation induced by lipid hydroperoxides.

In summary, our studies showed that incubating Caco-2 cells for 24 h with 25, 50, and 100 μM carnosic acid or carnosol significantly reduced 5 μM OAHPx-mediated cell toxicity. The protective effect imposed by carnosic acid at 25 μM was attained by carnosol at 100 μM, indicating that carnosic acid was more effective compared to carnosol in inhibiting OAHPx-induced lipid peroxidation and DNA damage. The effects on catalase, SOD, and GPx enzymes were dependent upon the compound and their concentrations at supplementation. It is evident that lipid hydroperoxide detoxification by carnosic acid and carnosol is partly due to their ability to increase GPx activity (by carnosic acid at 25 and 50 μM) or to maintain it at basal levels (carnosic acid 100 μM, carnosol all three levels). Further investigations should be conducted to determine whether the observed changes in the antioxidant enzyme activities brought about by carnosic acid and carnosol are associated with changes to active sites of the enzymes or whether it is a result of direct effects on their gene expressions. The underlying protective effects of carnosic acid and carnosol on the intestine could ultimately be determined by understanding these mechanisms.

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