TPA- and H_2O_2- induced Apoptosis by Epigenetic Mechanism and Preventive Effect of *L*-Carnosine on TPA- and H_2O_2- induced Apoptosis of v-myc Transformed Rat Liver Epithelial Cells

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Abstract

Apoptosis is characterized by DNA fragmentation, chromatin condensation and plasma membrane blebbing. These apoptotic processes have been mainly associated with genetic mechanisms. Recently, these processes have been also associated with mitochondrial events that include the release of cytochrome c and Diablo/SMAC by modulation of mitochondrial membrane permeability. The present study shows that exogenous cancer-promoting agents, such as hydrogen peroxide (H_2O_2) and 12-O-tetradecanoylphorbol-13-acetate (TPA) induce apoptosis by epigenetic mechanism(s) involving mitochondria. L-carnosine (ß-alanyl L-histidine), occurring abundantly in skeletal muscles, has been suggested to possess antioxidant and anti-aging properties. In the present study, we also investigated whether or not L-carnosine prevents H_2O_2 -or TPA-induced apoptosis of v-myc transformed rat liver epithelial cells. Both TPA- and H_2O_2-induced apoptosis observed by Hoechst 33258 staining and DNA ladder formation. L-carnosine prevented either TPA- or H_2O_2-induced DNA fragmentation. These DNA fragmentations were not associated with p53 expression. Because p53 protein expressions were not different among treatments based upon western blot analysis. Therefore, we hypothesized that both TPA- and H_2O_2–induced apoptosis were associated with mitochondrial events. In order to examine this hypothesis, we observed the changes of mitochondrial membrane potentials using rhodamine 123 fluorescence dye and PARP cleavage. TPA and H_2O_2 significantly changed mitochondrial membrane potential. L-carnosine also protects mitochondrial membrane changes in TPA- and H_2O_2 -treated cells. PARP cleavages were observed in cells treated with either TPA or H_2O_2 and also significantly reduced in L-carnosine-treated cells. The results suggested that TPA and H_2O_2 induced apoptosis through mitochondria-related events, not through nuclear events, and L-carnosine inhibited H_2O_2 - and TPA- induced apoptosis.

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Preventive effect of epicatechin and ginsenoside Rb_2 on the inhibition of gap junctional intercellular communication by TPA and H_2O_2

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Abstract

The anticarcinogenic effects of epicatechin (EC) and ginsenoside Rb_2 (Rb_2), which are major components of green tea and Korea ginseng, respectively, were investigated using a model system of gap junctional intercellular communication (GJIC) in WB-F344 rat liver epithelial cells. 12-*O*-tetradecanoylphorbol-13-acetate (TPA) and hydrogen peroxide, known as cancer promoters, inhibited GJIC in the epithelial cells as determined by the scrape loading/dye transfer assay, fluorescence redistribution assay after photobleaching, and immunofluorescent staining of connexin 43 using a laser confocal microscope. The inhibition of GJIC by TPA and H_2O_2 was prevented with treatment of Rb_2 or EC. The effect of EC on GJIC was stronger in TPA-treated cells than in H_2O_2-treated cells, while the effect of Rb_2 was opposite to that of EC. EC, at the concentration of 27.8 μg/ml, prevented the TPA-induced GJIC inhibition by about 60%. Rb_2, at the concentration of 277 μg/ml, recovered the H_2O_2-induced GJIC inhibition by about 60%. These results suggest that Rb_2 and EC may prevent human cancers by preventing the down-regulation of GJIC during the cancer promotion phase and that the anticancer effect of green tea and Korea ginseng may come from the major respective components, EC and Rb_2.

Keywords: Green tea; Ginseng; Catechins; Ginsenosides; Gap junctional intercellular communication; Connexin 43

1. Introduction

In recent years, considerable interest has been generated on green tea as a health beverage [1]. Several pharmacological properties have been tentatively attributed to green tea that needs further confirmation. Green tea has also been implicated as a chemopreventive agent against the development of various tumors [2–5]. Most of these studies have been carried out with experimental animals [4,5], although some epidemiological data seem to be supportive of such a conclusion [6]. The protective activity of green tea is generally assumed to be due to the free radical-scavenging and/or metal-chelating effects of high concentrations of catechins or their gallates present in green tea [7]. However, the exact

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anticarcinogenic mechanisms of green tea or its components are still elusive and mostly speculative.

Ginseng has been used for traditional medicine in Korea, China, Japan and other Asian countries for the treatment of various diseases, including psychiatric and neurologic diseases, as well as cancer [8–11]. Ginseng saponins (ginsenosides) have been regarded as the principal components responsible for the pharmacological activities of ginseng [12]. The ginsenosides, such as Rb_1, Rb_2, and Rc, have been reported to have anti-tumor effects, particularly on the inhibition of tumor-induced angiogenesis, tumor invasion and metastasis, and the control of phenotypic expression and differentiation of tumor cells [12–15]. However, the mechanisms of anti-tumor effects of ginsenosides have not been fully understood.

Gap junctions are membrane channels that permit the transfer of small water-soluble molecules, including cAMP and inositol triphosphate, from the cytoplasm of one cell to that of its neighbors [16,17]. Most tumor cells have a reduced ability to communicate among themselves and/or with surrounding normal cells, confirming the importance of functional gap junctional intercellular communication (GJIC) in growth control [16,17]. The reversible down-regulation of GJIC between adjacent cells has been hypothesized to be involved in tumor promotion phase of carcinogenesis [18]. 12-*O*-tetradecanoylphorbol-13-acetate (TPA) is a well-known cancer promoter that activates protein kinase C (PKC) [16]. It has been well documented that TPA inhibits GJIC in several kinds of cell lines including WB-F344 rat liver epithelial cells [19–21]. Oxidative stress is also strongly implicated in tumor promotion by epigenetic mechanisms, such as the activation of protein kinases and the inhibition of GJIC [22,23]. H_2O_2, as a tumor promoter, is known to inhibit GJIC. The inhibitory mechanism of H_2O_2 on GJIC is different from that of TPA [23].

In this study, the anticarcinogenic effects of green tea and Korea ginseng were investigated during the promotional phase using TPA or H_2O_2 to inhibit GJIC, which is associated with tumor promotion. The working hypothesis is that green tea, Korea ginseng, or their major components may prevent the blockage of GJIC in tumor promoter-treated cells. Several techniques, including a scrape loading/dye transfer (SL/DT) assay, a fluorescence redistribution assay after photobleaching (FRAP), and an immunofluorescent staining of connexin 43 (Cx43) using a laser confocal microscope were used to assess GJIC. In this study, we report that EC from green tea and Rb_2 from Korea ginseng prevented the inhibition of GJIC by TPA or H_2O_2, indicating that they can be used as anti-tumor or chemopreventive agents, especially acting on cancer promotional stage.

2. Materials and methods

2.1. Materials

Epicatechin (EC), Epicatechin gallate (ECG), Epigallocatechin gallate (EGCG), Greentea extract (GTE), Ginsenoside Rb_2, Ginsenoside Rg_1, Ginseng extract (GSE), and Red ginseng extract (RGE) were obtained from Taepyungyang Chemical Co. Ltd. (Suwon, Korea). TPA and H_2O_2 were purchased from Sigma Chemical Co. (St. Louis, MO). GSE and RGE were dissolved in phosphate buffered saline (PBS) and the other compounds were dissolved in absolute alcohol.

2.2. Cell culture

WB-F344 rat liver epithelial cell lines were obtained from Dr Trosko at Michigan State University (East Lansing, MI) and cultured in D-media supplemented with 5% fetal bovine serum (Gibco Laboratories, Grand Island, NY) and penicillin–streptomycin–neomycin mixture (Gibco Laboratories, Grand Island, NY). The cells were incubated at 37°C in a humidified atmosphere containing 95% air and 5% CO_2. The cells were grown in 35-mm tissue culture plates (Corning Inc., Corning, NY) and the culture medium was changed every other day. Bioassays were conducted with confluent cultures (70–90%).

2.3. Cytotoxicity test

Cytotoxicity was determined by the neutral red uptake assay described by Borenfeud and Puerna [24]. Using a 96-well plate, WB-F344 rat liver epithelial cells (10^4 cells/200 μl/well) were incubated for 24 h at 37°C, followed by the treatment with test compounds such as Rg_1 (0.1, 1, 10, 100, 1000 μg/ml), Rb_2 (0.277, 2.77, 27.7, 277 μg/ml), GTE (0.01, 0.1, 1, 10, 100 μg/ml), EC (0.00278,

0.0278, 0.278, 2.78, 27.8 μg/ml), ECG (0.0072, 0.072, 0.72, 7.2, 72 μg/ml), EGCG (1, 10, 100, 1000 μg/ml), GSE(0.00005, 0.0005, 0.005, 0.05, 0.5 μg/ml) and RGE (0.05555, 0.5555, 5.555, 55.55, 555.5 μg/ml) including TPA (10 ng/ml), H_2O_2 (250 μM) as tumor promoters. The maximum stock concentration of each compound was determined by the maximum soluble concentration in water. From the maximum soluble concentration of each chemical in water, 10-fold serial dilutions were made for each chemical with sterilized-distilled water for the cytotoxicity test. Cells were incubated for 24 h and rinsed three times with PBS, followed by adding 2 ml of fresh growth medium containing 100 ng neutral red (50 ng/ml). After another incubation for 2 h at 37°C, the extracellular neutral red was rinsed off with PBS and the cells were lysed with 200 μl of an aqueous solution containing 1% acetic acid and 50% ethanol. The lysed cells were measured for neutral red at a wavelength of 540 nm using an ELISA reader.

2.4. Scrape-loading dye transfer (SL/DT) assay

GJIC was assessed using the SL/DT technique described by El-Fouly [25]. The WB-F344 rat liver epithelial cells (10^5 cells/ml) in 35 mm cell culture flasks were incubated for 24 h at 37°C and test compounds at various concentrations (consecutively diluted by a factor of 2 or 10 from the maximum non-cytotoxic concentration of the test compounds) were exposed to the WB cells for 4 h with TPA and 1 h with H_2O_2, respectively. The treated liver epithelial cells were rinsed carefully with PBS and then scraped and incubated with 2 ml of 0.05% Lucifer Yellow for 3 min. The cells were then washed with PBS and fixed with 4% paraformaldehyde. The distance that Lucifer Yellow had traveled through gap junctions was observed with an inverted fluorescent microscope.

2.5. Fluorescence recovery after photobleaching (FRAP) assay

The cells (cell confluency, 70–80%) incubated for 24 h in 35-mm cell culture flasks were treated with test compounds including TPA, H_2O_2, EC, and Rb_2. In the combination with TPA, various concentrations of EC or Rb_2 were treated for 4 h to assess their chemopreventive effects against the inhibition of GJIC by TPA. Whereas, in the combination with H_2O_2, various concentrations of EC or Rb_2 were treated for 1 h to assess their chemopreventive effects against inhibition of GJIC by H_2O_2. The cells were rinsed with PBS and loaded with 7 μg/ml of 5,6-carboxyfluorescein diacetate in Ca^{2+}/Mg^{2+}-PBS for 12 min at 37°C [18]. The cells were then rinsed four to five times with 2 ml of Ca^{2+}/Mg^{2+}-PBS to remove extracellular dye and covered with 2 ml of Ca^{2+}/Mg^{2+}-PBS for the FRAP analysis. Dye transfer was monitored at room temperature using an Ultima fluorescence spectrometer (Meridian Instruments, MI, USA). Cells were randomly selected under a microscope with a 40 × objective lens and photobleached to 20–40% of their original fluorescence intensity. They were then examined for recovery of fluorescence after 4 min to obtain the rates of recovery, which were reported as %/min, where % = percent of prebleaching fluorescence. Fluorescence recovery was corrected for fluorescence lost in unbleached controls.

2.6. Western blot analysis for Cx43

WB-F344 rat epithelial cells, grown to the same confluency as in the FRAP assay, were treated with test compounds in the same way as the FRAP assay. Western blot analysis of Cx43 was performed as described previously [22]. Proteins were extracted from the liver epithelial cells, followed by treatment with 20% sodium dodecyl sulfate (SDS) containing 2 mM phenymethylsulfonyl fluoride (PMSF), 10 mM iodoacetoamide, 1 mM leupeptin, 1 mM antipain, 0.1 mM sodium orthovanadate, 5 mM sodium fluoride and then sonicated for three 10-s pulses using a probe sonicator (Branson, Danbury, CT). These cell lysates were centrifuged at 4°C for 30 min to remove insoluble materials. Protein concentration was determined with a DC protein assay kit (Bio-Rad Corp., Richmond, CA), after dilution of samples 1:5 with H_2O. Proteins were separated on 12.5% polyacrylamide gels and transferred to PVDF membranes at 20 V for 16 h. Cx43 was detected using rabbit polyclonal anti-connexin 43 (Zymed Co., San Francisco, CA), followed by incubation with horseradish peroxidase-conjugated secondary antibody and detection with the ECL chemiluminescent detection reagent (Amersham Co., Arlington Heights, IL).

2.7. Immunofluorescent staining for Cx43

Localization of Cx43 in the cells was assessed by immunofluorescent staining as described previously [20]. After treatment with the test compounds, the cells were fixed in methanol/acetate (95:5) for 20 min and then non-specific binding sites were blocked with 10% normal goat serum in PBS for 1 h at room temperature. The cells were incubated overnight at 4°C with mouse monoclonal anti-Cx43 (Transduction Laboratories) diluted 1:500 in PBS and then for 1 h with a rhodamine-conjugated goat anti-mouse IgG $(Fab')_2$ fraction (Jackson Immuno Research Laboratories, PA) diluted 1:200 in PBS at room temperature. Fluorescent staining of Cx43 was viewed and photographed using an Ultima confocal microscope (Meridian Instrument, Okemos, MI).

3. Results

3.1. Cytotoxicity of green tea and Korea ginseng extracts or their components

To select appropriate doses of test compounds to be used in this study, we determined the maximum non-cytotoxic concentrations of test compounds including GSE, RGE, Rg_1, Rb_2, GTE, EC, ECG, and EGCG (Table 1). The various concentrations of these compounds were exposed to the WB cells for 24 h and the cytotoxicity of test compounds was assessed by the uptake of neutral red into viable cells. Cell viability did not change during incubation for 24 h, following the addition of test compounds. The maximum non-cytotoxic concentrations of GSE, RGE, Rg_1, Rb_2, GTE, EC, ECG, and EGCG were 500, 555.5, 1000, 277, 10, 27.8, 72, and 100 μg/ml, respectively (Table 1).

Table 1
Maximum non-cytotoxic concentrations of green tea, Korea ginseng, and their components

Test compounds	Maximum non-cytotoxic concentration (μg/ml)[a]
Ginseng extract (GSE)	500.0
Red ginseng extract (RGE)	555.5
Ginsenoside Rg_1	1000.0
Ginsenoside Rb_2	277.0
Green tea extract (GTE)	10.0
Epicatechin (EC)	27.8
Epicatechin gallate (ECG)	72.0
Epigallocatechin gallate (EGCG)	100.0

[a] Maximum non-cytotoxic concentrations were determined by the neutral red uptake assay.

3.2. Effect of test compounds on GJIC

Based on the cytotoxicity results, various concentrations of these test compounds, which were represented by a consecutive dilution with a factor of 2 or 10 from the maximum non-cytotoxic concentration of the test compounds.

Using the scrape loading/dye transfer technique, the effects of test compounds on GJIC were investigated. As the positive controls, TPA at the concentration of 10 ng/ml or H_2O_2 at the concentration of 250 μm was used. TPA or H_2O_2 markedly inhibited GJIC compared to the untreated negative control (Fig. 1). The photographic image after the treatment of EC or Rb_2 was similar to that of the negative control (Fig. 1B,F). In the combined treatments of test compounds (GSE, RGE, Rg_1, Rb_2, GTE, EC, ECG, and EGCG) with TPA/H_2O_2, only EC and Rb_2 effectively protected against the inhibition of GJIC induced by TPA or H_2O_2 (Fig. 1D,H). Therefore, the following experiments were performed on only EC and Rb_2.

To confirm the protecting effect of EC and Rb_2 on the inhibition of GJIC by TPA or H_2O_2, we also used the FRAP assay (Fig. 2). EC, at the concentration of 27.8 μg/ml, prevented the inhibition of GJIC by TPA to about 60% (Fig. 2A) and Rb_2, at the concentration of 277.0 μg/ml, also prevented the inhibition of GJIC by H_2O_2 by about 60% (Fig. 2B). The effect of EC on GJIC was stronger in the TPA-treated cells than in the H_2O_2-treated cells, whereas the effect of Rb_2 was opposite to that of EC. The protecting activities of EC and Rb_2 were slightly dose-dependent at the concentrations used in this study (Fig. 2).

3.3. Effect of EC and Rb_2 on Cx43 levels and phosphorylation

We examined the changes in Cx43 protein levels, as well as the degree of phosphorylation following the treatment of the cells with EC or Rb_2 by Western blotting, using antibodies specific to Cx43 (Fig. 3).

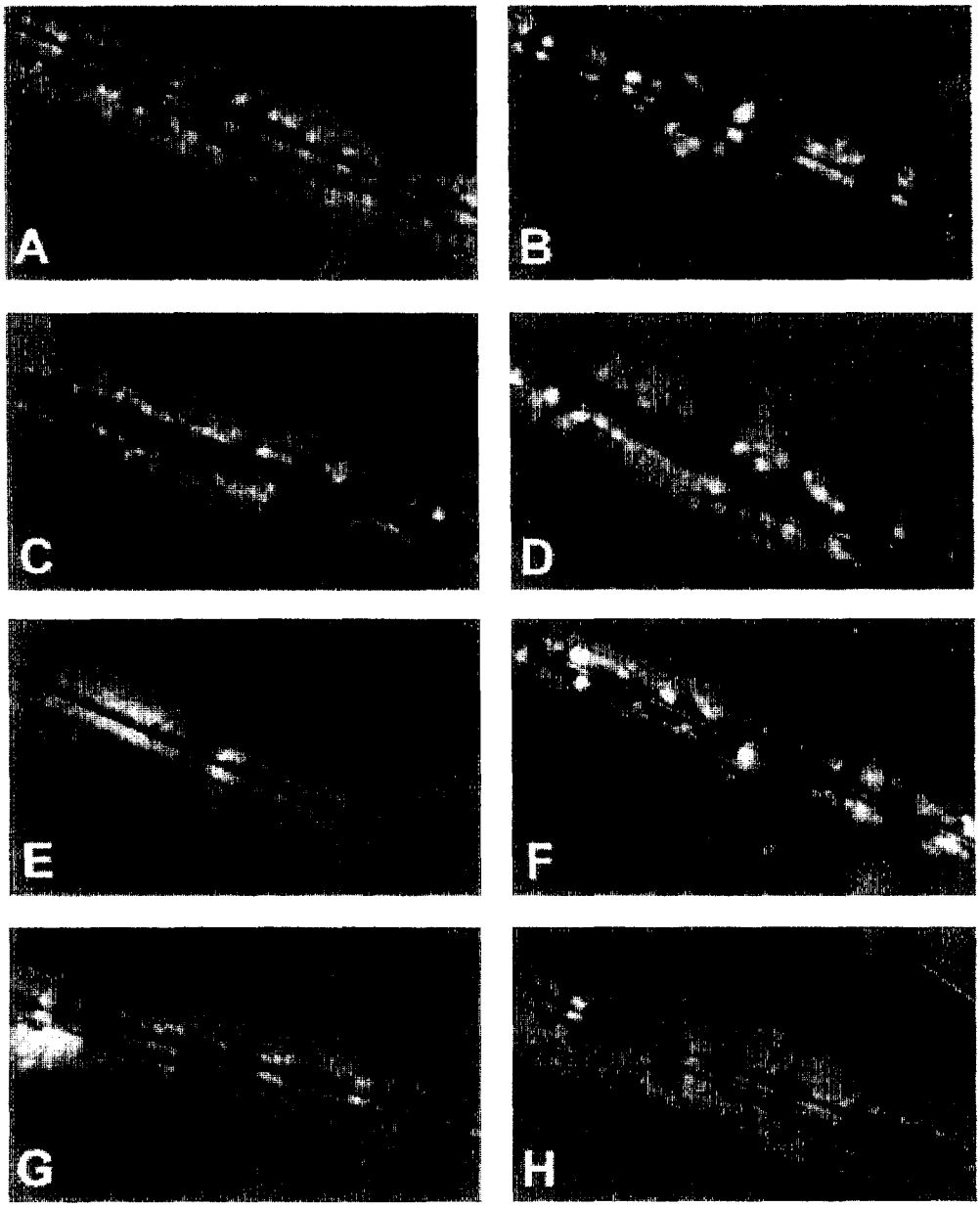

Fig. 1. Scrape loading dye transfer images in the WB-F344 liver epithelial cells treated with test compounds for 4 h against TPA and for 1 h against H_2O_2. Panel A, non-treated (4 h exposure); B, TPA 10 ng/ml; C, EC 27.8 μg/ml; D, TPA 10 ng/ml + EC 27.8 μg/ml, E, non-treated (1 h exposure); F, H_2O_2 250 μM; G, Rb_2 277 μg/ml; H, H_2O_2 250 μM + Rb_2 277 μg/ml.

Three major bands (P0, P1 and P2) were detected in untreated WB cells. The mobility shift in bands from P0 to P1 or P2 indicates phosphorylation of Cx43. Cells treated with TPA displaced a mobility shift in

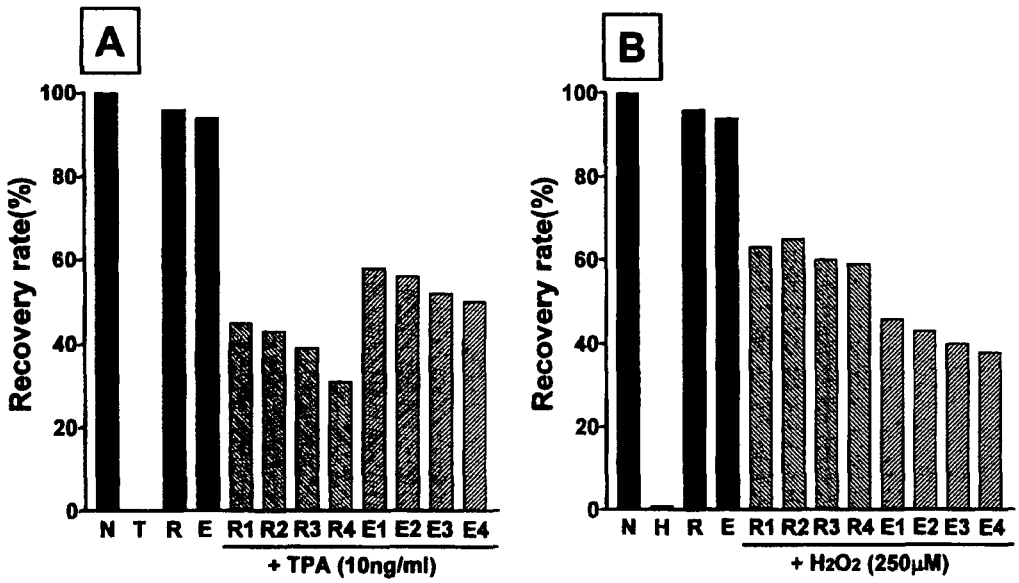

Fig. 2. Recovery by Rb_2 and EC from inhibition of GJIC by TPA (A) and H_2O_2 (B) as determined using a fluorescence redistribution after photobleaching (FRAP) assay. N, non-treated; T, TPA only; H, H_2O_2 only; R, Rb_2 only; E, EC only; R1, Rb_2 277 μg/ml; R2, Rb_2 27.7 μg/ml; R3, Rb_2 2.77 μg/ml; R4, Rb_2 0.27 μg/ml; E1, EC 27.8 μg/ml; E2, EC 13.9 μg/ml; E3, EC 6.95 μg/ml; E4, EC 3.47 μg/ml.

bands to the higher molecular weight P2-band (Fig. 3, band 2). H_2O_2 also shifted the P0 band to P2 band (band 3). The treatment of EC with TPA decreased the phosphorylation ratio (P2/P0) of Cx43 by TPA (band 4). The treatment of Rb_2 with H_2O_2 also affected the phosphorylation ratio of Cx43 by H_2O_2 (band 5).

3.4. Effect of EC and Rb_2 on the distribution of Cx43

The distribution of the Cx43 protein in WB cells was examined by immunostaining after exposure to test compounds for 4 h against TPA and for 1 h against H_2O_2. The typical plaques of Cx43, which are functional when localized on the plasma membrane, were detected in the untreated control (Fig. 4A). The membrane Cx43 proteins were not seen in cells treated with TPA 10 ng/ml (Fig. 4B). The treatment of EC partially prevented the plaques of Cx43 that disappeared after the TPA treatment (Fig. 4C). The membrane Cx43 proteins also disappeared in cells treated with 250 μM of H_2O_2 (Fig. 4D). The treatment of Rb_2 partially prevented the plaques of Cx43 from disappearing after treatment with the H_2O_2 (Fig. 4E).

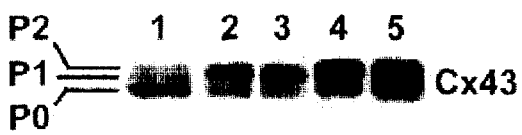

Fig. 3. Western blot analysis for Cx43. Lane 1, non-treated; Lane 2, TPA 10 ng/ml; Lane 3, H_2O_2 250 μM; Lane 4, TPA 10 ng/ml + EC 27.8 μg/ml; Lane 5, H_2O_2 250 μM + Rb_2 277 μg/ml.

4. Discussion

Epidemiological and animal studies have suggested that the intake of ginseng is strongly associated with the low incidence of cancers [10,11]. Its components, such as ginsenosides Rb_1, Rb_2 and Rg_1, may be responsible for its anticarcinogenic effect [8,12,15]. In this study, only Rb_2 among GSE, RGE, Rb_2, and Rg_1, showed a protective effect against GJIC inhibition by the tumor promoters, TPA and H_2O_2, indicating that Rb_2 has an anticarcinogenic activity during the promotional stage of carcinogenesis.

Green tea contains polyphenolic antioxidants that have shown anticarcinogenic properties in animals

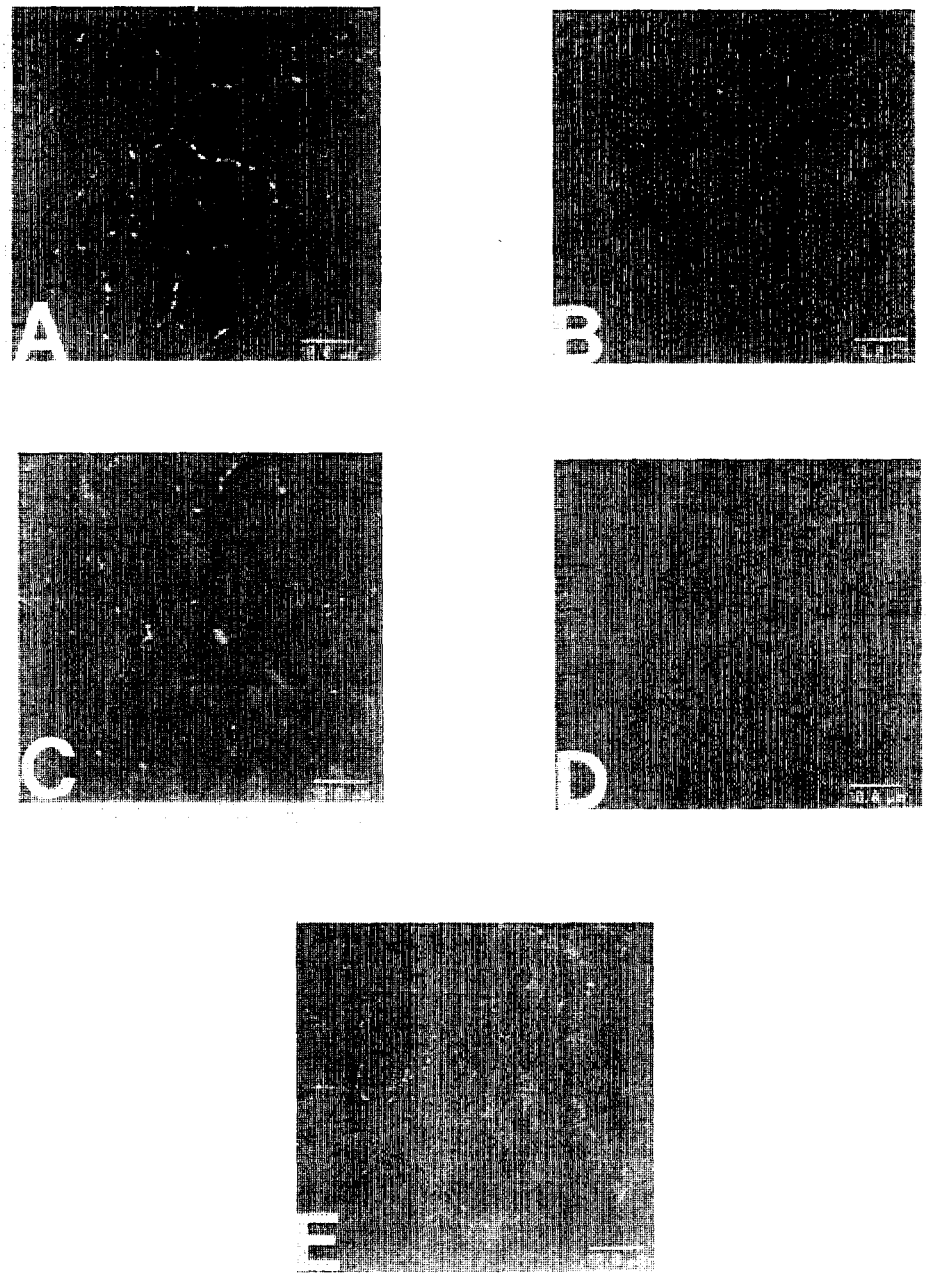

Fig. 4. Immunofluorescent staining for Cx43. A, non-treated control; B, TPA 10 ng/ml; C, TPA 10 ng/ml + EC 27.8 μg/ml, D, H_2O_2 250 μM; E, H_2O_2 250 μM + Rb_2 277 μg/ml.

and in vitro experimental studies [2–5]. The main polyphenolic components are catechins including EC, EGC, ECG, and EGCG. These catechins exert growth inhibition and apoptosis in a variety of tumor cell lines, but the effects vary from the cell lines used in the studies [3,26,27]. Recently, most studies on the anticarcinogenic effect of green tea catechins have been focused on EGCG rather than the others. Although the anticarcinogenic effect of EGCG have been reported in numerous studies, we reported in this study that only EC among the green tea catechins showed an anticarcinogenic activity in the rat liver epithelial cells by preventing the inhibition of GJIC by the promoters, TPA and H_2O_2.

GJIC has been implicated in the regulation of cellular growth and its inhibition to the expression of the neoplastic phenotype [28]. A reversible disruption of GJIC plays a role during the tumor promotion phase of carcinogenesis and a stable down-regulation of GJIC leads to the conversion of a premalignant cell to an invasive and metastatic cancer cell [16]. Therefore, GJIC is frequently reduced in neoplastic and carcinogen-treated cells. In this study, we used a non-tumorigenic WB-F344 rat liver epithelial cell as a model system to screen and verify the anticarcinogenic activity of test compounds. The normal WB cells are known to express high levels of Cx43, to form numerous gap junctions, and to have high percentage of communicating cells (95–100%) [29]. However, the neoplastic transformants of these cells which were generated by *ras* and *neu* oncogene transfection form few gap junctions and have low incidences of communication (20–25%) [29]. The treatment of TPA and H_2O_2, which are known to be cancer promoters, significantly inhibited GJIC as determined by SL/DT assay, FRAP assay and immunostaining of Cx43, thereby resulting in the simulation of the transformed WB cells. The prevention of the inhibition of GJIC by EC, at the concentrations of 27.8–3.47 μg/ml and Rb_2 at the concentrations of 277–0.27 μg/ml might imply that the EC and Rb_2 can be used as anticarcinogenic agents acting on promotional phase.

The phosphorylation state of gap junction proteins seems to plays an important role in the gating of gap junction channels [30]. Many, but not all, exogenous and endogenous chemicals that regulate GJIC also alter the phosphorylation state of the connexins, which are the proteins that form the hexametric connexin doublets that constitutes the gap between cells [31]. In this study, the inhibition of GJIC by TPA in F344 rat liver epithelial cells was correlated with the hyperphosphorylation of Cx43, as measured by mobility shifts (P0 > P2) of the Western blot bands of Cx43. The TPA-induced hyperphosphorylation of Cx43 might be a result of PKC activation, in which PKC is translocated from the cytosolic to particulate fraction of the cell [23,32]. The GJIC is restored concomitantly with the inactivation of PKC: these events were shown to precede the decrease in the mRNA and protein levels [16]. In concert with the hyperphosphorylation by PKC, Cx43 was reported to internalize into the cytoplasm, resulting in the loss of GJIC. In this study, Cx43 plaques in the membrane of WB-F344 rat liver epithelial cells disappeared after treatment of TPA as determined by immunostaining. However, the simultaneous treatment of EC prevented the total disappearance of Cx43 plaques in the cell membranes, resulting in the reduction of inhibition of GJIC. Although EC prevented much of the hyper phosphorylation of Cx43 in the WB cells, a functional change of the Cx43 can not be excluded.

The carcinogenic effect of oxidative stress has primarily focused on the genotoxicity of reactive oxygen species [33]. However, active oxygen is known to play a significant role in the promotion phase of cancer [34]. The promotion phase of cancer is a consequence of epigenetic events involving signal transduction and GJIC [35,36]. Promotion is a reversible or interruptible step in carcinogenesis. Therefore, the underlying mechanism of tumor promotion, such as the hypothesized down-regulation of GJIC, should also be reversible [16]. Similarly to TPA, H_2O_2, at the concentration of 250 μM, also caused a shift in the Cx43 bands to a hyperphosphorylated state, resulting in the inhibition of GJIC. However, the mechanism by H_2O_2 on hyperphosphorylation of Cx43 was different from that by TPA in WB-F344 liver epithelial cells [23]. In this study, Rb_2 prevented the H_2O_2-induced hyperphosphorylation of Cx43 and it also prevented significantly H_2O_2-inhibition of GJIC. H_2O_2-inhibited GJIC is restored after the removal H_2O_2 in WB liver epithelial cells [23]. The preventive effects of Rb_2 on GJIC inhibition by H_2O_2 might be involved in inactivation of the H_2O_2. However, other mechanisms can not be excluded.

In conclusion, the inhibition of GJIC and the disappearance of Cx43 induced by TPA or H_2O_2 in WB-F344 rat liver epithelial cells were prevented with the simultaneous treatment of Rb_2 or EC. These results suggest that the ginsenoside Rb_2 from Korean red ginseng and the epicatechin from green tea may have a cancer preventive effect during promotion phase by the up-regulation of GJIC.

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Carnosine and Related Compounds Protect Against Copper-Induced Damage of Biomolecules

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At concentrations of 1 mM, the protective effects of carnosine and related compounds including anserine, homocarnosine, histidine, and β-alanine were investigated against copper-catalyzed oxidative damage to deoxyribose, ascorbic acid, human serum albumin, liposome, and erythrocytes. Carnosine and anserine reduced Cu (II) to bathocuproine-reactive Cu (I) in a time- and a dose-dependent manner while the others did not. Carnosine reduced 86% of 100 μM Cu (II) in 60 min. Carnosine, homocarnosine, anserine, and histidine inhibited copper-catalyzed deoxyribose degradation by 75, 66, 65, and 45%, respectively. In the presence of 1 μM Cu (II), carnosine and related compounds inhibited ascorbic acid oxidation by 55–85% after incubation for 20 min. In the presence of 0.15 mM ascorbic acid and 0.8 mM H_2O_2, carnosine, anserine, homocarnosine, and histidine inhibited copper-catalyzed oxidation of human serum albumin by 41, 21, 29, and 24%, respectively, as determined by carbonyl formation. These compounds also significantly inhibited copper-catalyzed liposomal lipid peroxidation as measured by malondialdehyde and lipid hydroperoxides. Carnosine, anserine, homocarnosine, and histidine inhibited hemolysis of bovine erythrocytes induced by 0.1 mM Cu (II). These results suggest that histidine-containing dipeptides may play an important role in protecting against free radical-mediated tissue damage.

Keywords: Antioxidant, Ascorbic acid, Carnosine and related compounds, Hemolysis, Lipid peroxidation.

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Introduction

Histidine-containing dipeptides such as carnosine, anserine, and homocarnosine are present in considerable amounts in several vertebrates tissues including skeletal muscle, eye, olfactory system, and brain (Crush, 1970; Flancbaum *et al.*, 1990; Jackson and Lenney, 1996). Several physiological functions of these dipeptides have been postulated. Carnosine has a buffering effect by neutralizing lactic acid produced in skeletal muscle (Harris *et al.*, 1990). Carnosine and anserine are effective copper-chelating agents and may play a role in copper metabolism *in vivo* (Brown, 1981). These dipeptides function as neurotransmitters in the olfactory bulbs, as physiological activators for myosine ATPase, and as regulators of other enzymes (Parker and Ring, 1970; Ikeda *et al.*, 1980). At physiological concentrations, these dipeptides also have anticarcinogenic, antiglycating, and antiaging effects by acting as antioxidants (Hipkiss *et al.*, 1998).

Oxygen radicals have been implicated as an important cause of oxidative modification of biological molecules such as proteins, lipids, carbohydrates, and nucleotides (Koh *et al.*, 1997; Lee and Hendricks, 1997a; 1997b; Kim *et al.*, 1998; Lee *et al.*, 1998a; 1998b). The oxidative degradation of these molecules can be catalyzed by transition metal ions (Lee and Hendricks, 1997a; Kim *et al.*, 1998). Carbonyl formation as an early marker for protein oxidation and lipid peroxidation products is increased in free radical-related diseases such as rheumatoid arthritis, ischemia-perfusion injury, and atherosclerosis (Davies, 1987; Chapman *et al.*, 1989; Cochrane, 1991; Halliwell, 1991; Reznick *et al.*, 1992). Antioxidants, vitamin C and E, and metal-chelating agents such as desferrioxamine protect against these free radical-mediated tissue injuries (Cochrane, 1991; Halliwell, 1991; Reznick *et al.*, 1992; Park and Song, 1994).

Erythrocytes and/or their membranes have been used as a model system to study oxidative damage (Shinar *et al.*, 1989). The production of free radicals is also associated with red blood cell-related diseases such as thalassemia, sickle cell anemia, and glucose-6-phosphate dehydrogenase (G6PDH) deficiency (Hebbel, 1985; Rachmilewitz *et al.*, 1985). Free radicals inflict cellular damage leading to the destruction of red blood cells (RBC). In sickle cell anemia, RBC lesions result from extensive oxidative damage induced by excess iron deposition in the membrane (Kuross and Hebbel, 1988). A wide variety of oxidizing agents have been used to induce changes in normal RBC structure and function. Incubation of RBC with trace metals such as iron and copper causes hemolysis by interfering with the generation of NADPH mediated by G6PDH in normal as well as in G6PDH-deficient cells (Shinar *et al.*, 1989).

Although the antioxidant activity of carnosine and related compounds has been illustrated in several model systems, little is known about their comparitive antioxidant activity in the same model systems. In this study, although the content of carnosine and related compounds varies in tissues or organs of mammals, 1 mM concentrations of test compounds were used because the concentration is representative for most of the tissues or organs. We examined the reducing activity of carnosine and its related compounds on Cu (II) in aqueous solutions. We also investigated the protective effects of these compounds against copper-induced oxidative damage to biomolecules including deoxyribose, ascorbic acid (AA), human serum albumin (HSA), and phospholipid as well as erythrocytes as a whole cellular system.

Materials and Methods

Materials L-carnosine, homocarnosine, L-anserine, L-histidine, β-alanine, glutathione, 2-deoxyribose, 2-thiobarbituric acid (TBA), trichloroacetic acid (TCA), tetraethoxypropane (TEP), ascorbic acid, human serum albumin, and phosphatidylcholine (PC) were purchased from Sigma Chemical Co. (St. Louis, USA). Heparinized bovine blood was obtained from the animal physiological laboratory at Utah State University. All solutions were prepared in chelax-treated phosphate buffer (pH 7.4) using water passed through a four-stage Milli Q system equipped with a 0.2 μm pore-sized final filter.

Cuprous ion measurement The reduction of Cu (II) was assessed by following the appearance of bathocuproine-reactive Cu (I) (Williams *et al.*, 1977). The reaction mixtures in 0.1 M sodium phosphate buffered saline (PBS, pH 7.4) included 25, 50, or 100 μM of Cu (II), test compound solutions, and 0.2 mM bathocuproine sulfonic acid. The reaction was initiated by adding Cu (II) to the reaction mixtures and the absorbance was monitored spectrophotometrically at 485 nm for 60 min at room temperature. The concentration of Cu (I) was calculated using a 100 μM Cu (I) standard reduced by the addition of 1 mM AA as a reductant for Cu (II).

Deoxyribose damage (hydroxyl radical scavenging) Deoxyribose degradation caused by hydroxyl radicals was determined by the formation of thiobarbituric acid reactive substances (TBARS) (Lee and Hendricks, 1997a). The reaction mixtures (1.0 ml) contained 0.1 M phosphate buffer (pH 7.4), 7 mM deoxyribose, 50 μM Cu (II), 100 μM AA, and test solutions. The mixtures were incubated for 60 min at 37°C. One milliliter of a stock solution [1% (w/v) TBA in 50 mM NaOH plus 2.8% (w/v) TCA] was added to the reaction mixture. The mixture was then heated for 10 min in a boiling water-bath, cooled with tap water, and the absorbance of the pink chromogen was read spectrophotometrically at 532 nm. TBARS concentrations were calculated from a standard curve of malondialdehyde (MDA), a breakdown product of TEP.

Ascorbic acid measurement The reaction mixtures included 0.1 M potassium phosphate buffer (pH 7.4), 100 μM AA, 1 μM Cu (II), and 1 mM test solutions. After the final addition of AA to the reaction mixture, AA was monitored directly by reading the absorbance at 265 nm, and the amount of oxidized AA was calculated from the initial value for 100 μM AA.

Lipid peroxidation Liposomes were prepared by diluting 500 mg PC in 5 ml of chloroform. The chloroform solution was dried under vacuum and resuspended in 100 ml of degassed, argon-saturated 0.1 M potassium phosphate buffer (5 mg PC/ml). The suspension was sonicated for 10 pulses of 30 s under argon. The PC liposome preparations were stored at 4°C under an argon atmosphere until used (Lee and Hendricks, 1997a). The pH of the PC liposome was adjusted to 7.4 immediately prior to use. The reaction mixtures (1.0 ml) containing 0.1 M potassium phosphate buffer (pH 7.4), 2.5 mg PC, 10 μM Cu (II), 100 μM AA, and test solutions were incubated for 90 min at 37°C, and 0.8 ml of the incubated mixtures was used for the TBARS determination (Lee and Hendricks, 1997a). Two milliliters of a 15% TCA-0.375% TBA-0.025 N HCl stock solution was added to 0.8 ml of the incubated liposome. The mixtures were then heated for 10 min in a boiling water bath (95–100°C) to develop the pink color, cooled with tap water, and centrifuged for 20 min at 3500 × *g*. The supernatant containing the pink chromogen was quantitated at 532 nm using a UV 2100 U spectrophotometer (Shimadzu Co., Kyoto, Japan). MDA was calculated from a standard curve of MDA, a breakdown product of TEP.

To remove H_2O_2, 0.1 ml of the incubation mixtures described above was mixed with 50 μl (140 IU) of catalase. After standing for 30 min at room temperature, 0.9 ml of a 90% methanol reagent solution (0.1 mM xylenol orange, 0.25 mM ferrous ammonium sulfate, 0.4 mM butylated hydroxytoluene, 25 mM H_2SO_4) was added to the solution. After 30 min standing at room temperature, the absorbance at 560 nm was measured, and lipid peroxides were calculated by a molar absorption coefficient, $4.5 \times 10^4\ M^{-1}cm^{-1}$ (Wolff, 1994).

Protein damage The reaction mixture (1.0 ml) in 0.1 M phosphate buffer (pH 7.4) containing 2 mg HSA/ml, 50 μM Cu (II), 150 μM AA, 0.8 mM H_2O_2, and 10 mM test solutions were incubated for 60 min at 37°C. After 1 ml of 20% TCA (w/v) was added to the reaction mixture, the solution was centrifuged for 10 min at 1000 × *g*, and the supernatant was discarded. After adding 1 ml of 10 mM 2,4-dinitrophenylhydrazine (DNPH) in 2.5 M HCl, the pellet was gently broken with a glass rod and allowed

to stand for 60 min at room temperature, with vortexing every 10–15 min. Then, 1 ml of 20% TCA was added and centrifuged for 10 min at 1000 × *g*. The pellet was obtained and washed with 2 ml of 10% TCA twice and then with 1 ml of ethanol-ethyl acetate (1:1) three times to remove the free DNPH. The protein precipitates were dissolved in 1 ml of 6 M guanidine hydrochloride solution and left for 10 min at 37°C with general vortex mixing. Absorbance at 370 nm was read against a blank of the guanidine hydrochloride solution, and carbonyl content was calculated using the molar absorption coefficient of 22,000 M^{-1} cm^{-1} (Reznick and Parker, 1994).

Hemolysis, Hemoglobin, and Methemoglobin (MetHb)/ Oxyhemoglobin (OxyHb) Bovine blood was centrifuged for 15 min at 1000 × *g*, and the supernatant including the buffy coat was discarded by aspiration. Erythrocytes were washed three times with 5 vol of 10 mM PBS (pH 7.4). The reaction mixtures containing 10% (v/v) bovine erythrocyte suspension in 10 mM PBS (pH 7.4), 100 μM Cu (II), 1 mM AA, and 1 mM test solutions were incubated for 2 or 6 h at 37°C. For hemolysis analysis, 0.1 ml of the incubation mixtures (6 h incubation) was taken, hemolyzed with 5 ml of deionized water, vortexed vigorously, and measured spectrophotometrically at 575 nm as 100% hemolyzed control. After the remaining 0.9 ml of the incubation mixture was centrifuged at 1000 × *g* for 10 min at 5°C, 0.1 ml of the supernatant was diluted with 5 ml of deionized water and the absorbance was measured at 575 nm (Brownlee *et al.*, 1977). For the measurement of hemoglobin release and the ratio of MetHb/OxyHb, the reaction mixture was centrifuged at 1000 × *g* for 10 min. Twenty microliters of the supernatant was used for the determination of the level of hemoglobin using the Sigma commercial kit for plasma hemoglobin. The precipitates were hemolyzed with 8 ml of deionized water, and the absorbances were measured at 540 nm for OxyHb and 630 nm for MetHb (Shinar *et al.*, 1989).

Results

Reduction of Cu (II) Bathocuproine forms a complex with Cu (I) which has maximum absorbance at 485 nm. Carnosine and anserine reduced Cu (II) to bathocuproine-reactive Cu (I) in 0.1 M PBS (pH 7.4) (Fig.1). The reducing rate of Cu (II) by 1 mM carnosine or 1 mM anserine was time-dependent. When the standard of 100 μM Cu (I) reduced by excess ascorbic acid was used, 1 mM of carnosine and anserine reduced 86% and 80% of 100 μM Cu(II), respectively, within 60 min at room temperature (Fig. 1). Homocarnosine, histidine, and β-alanine did not affect the reduction of Cu (II) to Cu (I). The reduction of Cu (II) by carnosine was dose-dependent in the presence of 25, 50, and 100 μM Cu (II) (Fig. 2). The reducing activity of carnosine in PBS (pH 7.4) was similar to that in other biological buffers including HEPES, MOPS, and PIPES. However, carnosine and related compounds could not reduce Fe (III) to Fe (II) in the buffer solutions (data not shown).

Deoxyribose degradation (Hydroxyl radical scavenging activity) Addition of 50 μM Cu (II) in the presence of

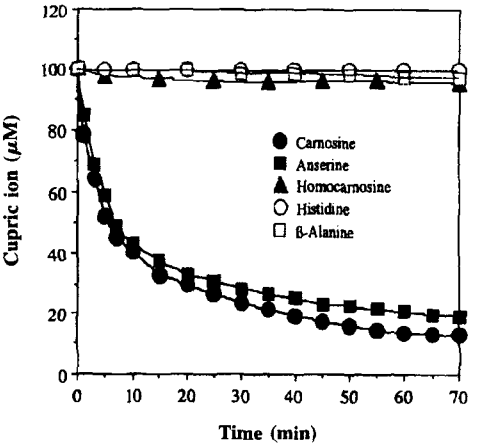

Fig. 1. Reduction of 100 μM Cu (II) to bathocuproine-reactive Cu (I) in 0.1 M PBS (pH 7.4) by 1 mM carnosine and related compounds. The absorbance at 485 nm was monitored for 70 min at room temperature after addition of Cu (II) to reaction mixtures including 1 mM test solution and 0.2 mM bathocuproine sulfonic acid. The Cu (I) concentration was calculated using the absorbance of 100 μM Cu (I) reduced by 1 mM AA. Data points represent the means of three determinations.

100 μM AA to deoxyribose caused degradation of the sugar into an MDA-like compound, which forms a chromogen with TBA (Table 1). Hydroxyl radicals produced via the Fenton reaction are responsible for the breakdown of deoxyribose. Carnosine, anserine, homocarnosine, and histidine effectively inhibited the Cu (II)-catalyzed deoxyribose degradation, indicating a hydroxyl radical scavenging activity of these compounds. β-Alanine did not inhibit the Cu (II)-catalyzed degradation of deoxyribose. At a 1 ml concentration, carnosine, anserine, homocarnosine, and histidine inhibited deoxyribose degradation by 75, 66, 65, and 45%, respectively. The inhibitory effect of these compounds was dose-dependent (data not shown). The histidine-containing dipeptides more effectively inhibited the deoxyribose degradation than did histidine alone. Catalase (50 μg/ml) also effectively inhibited deoxyribose degradation by 62%, probably resulting from removal of H_2O_2 in the solution.

Inhibition of ascorbic acid oxidation The acceleration of AA oxidation by copper is accompanied by the one-electron reduction of molecular oxygen, resulting in the production of oxygen free radicals. When AA concentration was monitored by UV absorbance at 265 nm, 100 μM AA was completely oxidized within 20 min in the presence of 1 μM Cu (II) (Fig. 3). One millimolar carnosine, anserine, homocarnosine, or histidine effectively inhibited the Cu (II)-catalyzed AA oxidation. However, 1 mM β-alanine had no effect on the Cu (II)-catalyzed AA

Table 1. Inhibition by 1 mM carnosine and related compounds of deoxyribose degradation catalyzed by Cu (II) and ascorbic acid (hydroxyl radical scavenging activity).

Reaction mixtures[a]	TBARS (nmol/ml)	% Inhibition
Cu (II) + AA + buffer	4.80 ± 0.25	–
Carnosine	1.21 ± 0.15	74.8
Homocarnosine	1.64 ± 0.16	65.8
Anserine	1.67 ± 0.13	65.2
L-Histidine	2.64 ± 0.14	45.0
β-Alanine	4.87 ± 0.37	0
Catalase (50 μg/ml)	1.83 ± 0.08	61.9

[a]Reaction mixtures containing 7.0 mM deoxyribose in 0.1 M potassium phosphate buffer (pH 7.4), 50 μM Cu (II), 100 μM AA, and 1 mM test solutions were incubated for 60 min at 37°C. TBARS concentrations were determined by the TBA test. Values are the mean ± SD of three determinations.

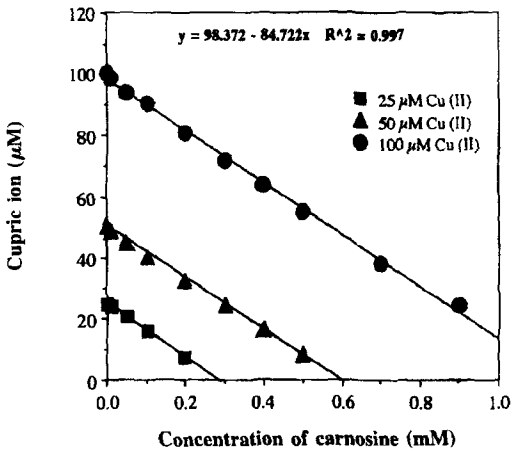

Fig. 2. Effect of various concentrations of carnosine on reduction of Cu (II) to Cu (I) in 0.1 M PBS (pH 7.4) after 60 min standing at room temperature. The absorbance at 485 nm was measured at 60 min after addition of Cu (II) to reaction mixtures including various carnosine concentrations and 0.2 mM bathocuproine sulfonic acid. The Cu (I) concentration was calculated based on the absorbance of 100 μM Cu (I) reduced by 1 mM AA. Data points represent the mean of three determinations.

oxidation. After incubation for 20 min at room temperature, 1 mM carnosine, anserine, homocarnosine, and histidine inhibited AA oxidation by 55, 70, 58, and 85%, respectively. Among these compounds tested, histidine was the most effective for the inhibition of AA oxidation.

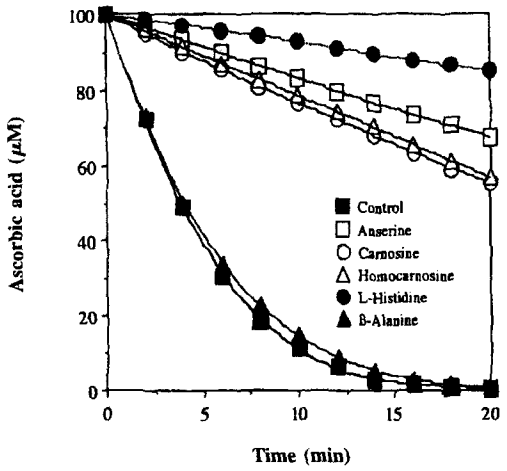

Fig. 3. Inhibition of Cu (II)-catalyzed ascorbate oxidation by 1 mM of carnosine and related compounds. The absorbance of the reaction mixtures in 0.1 M potassium phosphate buffer (pH 7.4), containing 1 μM Cu (II), 100 μM AA, and test solutions was monitored at 265 nm. Data points represent the means of three determinations.

Protection against oxidative protein damage Carbonyl has been used as a marker for the oxidative modification of protein, and metal ions can catalyze protein oxidation. Carbonyl was detected after HSA in 0.1 M phosphate buffer (pH 7.4) was incubated for 60 min at 37°C without reactants. In the presence of 50 μM Cu (II), 150 μM AA and 0.8 mM H_2O_2, carbonyl formation from HSA increased two-fold compared to the control (only HSA) (Table 2). One millimolar carnosine, anserine, homocarnosine, histidine, and glutathione inhibited the Cu (II)-catalyzed oxidation of HSA by 41, 21, 29, 24, and 22%, respectively. Among the tested compounds, carnosine was the most effective inhibitor compound against protein modification. β-Alanine had no effect on the copper-catalyzed protein damage.

Inhibition of liposomal lipid peroxidation In the presence of 10 μM Cu (II) and 100 μM AA, the antioxidant effects of carnosine and related compounds were investigated using PC liposomes (Table 3). Copper catalyzed the liposomal lipid peroxidation, as measured by MDA and lipid peroxides. Without Cu (II) and AA, lipid peroxidation occurred in the PC liposomes, indicating a possible presence of trace amount of metals and preformed lipid peroxides in the reaction mixture. The addition of 10 μM Cu (II) and 100 μM ascorbic acid strongly catalyzed the lipid peroxidation ($p<0.01$). Except for β-alanine, 1 mM carnosine and related compounds strongly inhibited the Cu (II)-catalyzed MDA formation by 64–77 % and the formation of lipid peroxides by 66–88% ($p<0.01$).

Table 2. Protection by 1 mM carnosine and related compounds against Cu (II)-catalyzed oxidative damage to human serum albumin.

Reaction mixtures[a]	Carbonyl (nmol/mg HSA protein)	% vs control
Human serum albumin (HSA)	5.1 ± 0.7	47.2
+ Cu (II) + AA + H_2O_2 + buffer	10.8 ± 1.7	–
+ Carnosine	6.4 ± 0.8	59.3
+ Homocarnosine	7.7 ± 0.9	71.3
+ Anserine	8.5 ± 0.6	78.7
+ L-Histidine	8.2 ± 1.2	75.9
+ β-Alanine	11.0 ± 1.1	101.9
+ Glutathione	8.4 ± 0.4	77.8

[a] Reaction mixtures in 0.1 M phosphate buffer (pH 7.4) containing 2 mg HSA/ml, 50 μM Cu (II), 150 μM AA, 0.8 mM H_2O_2, and test solutions were incubated for 60 min at 37°C and then carbonyl content was spectrophotometrically determined. Data represent the mean ± SD of three determinations.

Table 3. Effect of 1 mM carnosine and related compounds on lipid peroxidation of phosphatidylcholine liposomes.

Reaction mixtures[a]	MDA (nmol/mg PC/ml)	Lipid peroxides (nmol/mg PC/ml)
Liposomes only	0.23 ± 0.07^{b}	3.02 ± 0.36^{b}
+ Cu (II) + AA + buffer	4.61 ± 0.38^{c}	30.93 ± 2.71^{c}
+ Carnosine	1.07 ± 0.16^{d}	4.89 ± 0.64^{d}
+ Homocarnosine	1.64 ± 0.17^{e}	10.67 ± 1.44^{e}
+ Anserine	1.22 ± 0.14^{d}	9.16 ± 1.01^{e}
+ Histidine	1.10 ± 0.10^{d}	3.76 ± 0.57^{bd}
+ β-alanine	4.22 ± 0.41^{c}	31.2 ± 2.28^{c}

[a] Reaction mixtures (final volume, 1.0 ml) in 0.1 M potassium phosphate buffer (pH 7.4) containing 2.5 mg PC, 10 μM Cu (II), 100 μM AA, and test solutions were incubated for 90 min at 37°C. MDA was calculated using a standard curve of TEP. Lipid hydroperoxides contents were calculated by the molar absorption coefficient, $4.5 \times 10^4\ M^{-1}cm^{-1}$, at 370 nm. Data represent the mean ± SD of three determinations.
[bcde] Means within the same column bearing different superscripts are significantly different ($p<0.01$).

Carnosine and histidine were the most effective inhibitors against the formation of MDA and lipid peroxides, respectively.

Protection against hemolysis Oxygen radicals can be produced in solution in the presence of Cu (II) and ascorbic acid. These oxygen radicals can damage erythrocyte membranes, thereby releasing hemoglobin into solution. In the presence of 100 μM Cu (II) and 1 mM AA, bovine erythrocytes released hemoglobin into the reaction solution after incubation for 2 h at 37°C ($p<0.01$). Carnosine and related compounds inhibited membrane breakdown in this preparation (Table 4). Bovine erythrocytes were 30% hemolyzed in the presence of Cu (II) and AA after incubation for 6 h at 37°C. One millimolar carnosine, anserine, homocarnosine, or histidine significantly inhibited the hemolysis ($p<0.01$). The addition of copper and ascorbic acid also induced MetHb formation after incubation for 2 h at 37°C. One millimolar carnosine, anserine, or histidine concentrations significantly increased the MetHb formation compared to the control ($p<0.01$).

Discussion

Carnosine is one of the most abundant nitrous compounds in the non-protein fraction of vertebrate skeletal muscle (ranging 1–20 mM), olfactory epithelium and bulbs (0.3–5.0 mM), and eye lens (Crush, 1970; Boldyrev and Severin, 1990). Carnosine forms a bicyclic ring chain tautomeric structure, resulting in the release of hydrogen ions in solution (Boldyrev *et al.*, 1993). The tautomeric carnosines possess strong biological activity as a quencher of free radicals and as a reducing agent. Carnosine has an anodic cyclic voltammetry response, indicating possible activity as a reducing agent (Kohen *et al.*, 1988). In this study, carnosine and anserine slowly but effectively reduced Cu (II) to Cu (I) in several buffer solutions, whereas homocarnosine, histidine, and β-alanine did not.

Table 4. Effect of 1 mM carnosine and related compounds on Cu (II) + ascorbic acid-induced Hb release, MetHb formation, and hemolysis.

Reaction mixtures[a]	Hemoglobin (mg/dl)	MetHb/OxyHb (%)	Hemolysis (%)
RBC only	2.1 ± 0.7^{b}	3.5^{b}	0.3^{b}
Control (Cu + AA)	29.4 ± 2.7^{c}	33.1^{c}	29.6^{c}
Carnosine	8.1 ± 1.7^{d}	38.4^{cd}	2.5^{d}
Homocarnosine	9.1 ± 1.6^{d}	32.7^{c}	3.5^{d}
Anserine	7.8 ± 1.3^{d}	37.7^{cd}	2.4^{d}
L-Histidine	8.7 ± 1.7^{d}	43.7^{d}	3.3^{d}
β-Alanine	25.2 ± 2.1^{c}	33.5^{c}	26.2^{c}

[a] Reaction mixtures containing 10% bovine erythrocyte suspension in 10 mM PBS (pH 7.4), 100 μM Cu (II), 1 mM AA, and 1 mM test solutions were incubated at 37°C for 2 h. Hemolysis was determined after 6 h incubation against a 100% hemolyzed control. Data represent the mean 6 SD of three determinations.
[bcd] Means within the same column bearing different superscripts are significantly different ($p<0.01$).

The reducing activity of carnosine and anserine was specific for Cu (II) but not for Fe (III), since they could not reduce Fe (III) to Fe (II) in the solutions. The reducing potential of carnosine was about one-tenth that of ascorbic acid. The action of carnosine and anserine may be involved in several enzyme reactions involving copper in biological systems. Further studies will be needed to investigate the role of carnosine in copper-involved enzyme reactions.

Free radicals are involved in the etiology of a number of degenerative diseases such as cancer, arthritis, atherosclerosis, and diabetes (Cochrane, 1991; Halliwell, 1991). Aging is also accompanied by the accumulation of oxidized proteins (Stadtman and Oliver, 1991). Both membrane and intracellular biomolecules including phospholipids and proteins are readily attacked by free radicals, creating oxidized products such as MDA and carbonyls (Davies, 1987; Halliwell and Chirico, 1993, Lee and Hendricks, 1997a). In this study, both Cu (II) and AA were used to catalyze oxidative damage to deoxyribose, phospholipid, and erythrocytes. Copper can catalyze the generation of the most reactive oxygen species, hydroxyl radicals, from H_2O_2 via the Fenton reaction (Minotti and Aust, 1992).

$$Cu^{+} + H_2O_2 \longrightarrow {}^{\cdot}OH + OH^{-} + Cu^{2+}$$
$$Cu^{2+} + \text{ascorbate} \longrightarrow Cu^{+} + \text{semidehydroascorbate}$$

Carnosine, anserine, homocarnosine, and histidine effectively inhibited deoxyribose degradation and lipid peroxidation in the Cu (II) + AA-dependent system. Several mechanisms might be involved in the inhibitory effects of these compounds. First, carnosine, anserine, and homocarnosine are known to scavenge superoxide anions and peroxyl radicals (Kohen *et al.*, 1988; Boldyrev and Severin, 1990). In addition, histidine-containing dipeptides can scavenge hydroxyl radicals produced in the Fenton reaction (Chan *et al.*, 1994). In this study, the inhibitory effect of these compounds on deoxyribose degradation may be related to their hydroxyl radical scavenging activity, because hydroxyl radicals are known to break deoxyribose into MDA-like compounds (Lee and Hendricks, 1997a; 1997b). Second, carnosine and anserine are known to be very effective copper-chelating agents (Brown, 1981). When the concentration (physiologically relevant) of carnosine is 100–1000 times that of the cupric ion, four molecules of carnosine are bound to one molecule of copper (Brown, 1981). The complexes of carnosine and related compounds with copper may be very stable and catalytically inactive in liposomal lipid peroxidation. Third, carnosine and anserine act as reducing agents, thereby maintaining the reduced form of copper in the aqueous solution (Kohen *et al.*, 1988). In this study, carnosine and anserine were effective agents for the reduction of Cu (II) to Cu (I). The ratio of Cu (II) to Cu (I) was reported to be important to stimulate lipid peroxidation (Minotti and Aust, 1992). Therefore, the reduction of Cu (II) by carnosine and anserine might affect the liposomal lipid peroxidation and deoxyribose degradation. Fourth, histidine-containing dipeptides may have an enzyme-like activity. Carnosine diminished the amount of the already-formed lipid peroxidation products, so it could act as a lipid peroxidase (Babizhayev *et al.*, 1994). The complexes of copper:carnosine and copper:homocarnosine can also dismute superoxide radicals, indicating an SOD-like activity (Kohen *et al.*, 1991). Carnosine has been reported to inhibit lipid peroxidation catalyzed by iron, hemoglobin, lipoxidase, and singlet oxygen (Decker *et al.*, 1992). These results indicate that the inhibitory effects of carnosine and related compounds on lipid peroxidation or deoxyribose degradation may be due to the involvement of several mechanisms and not one single mechanism.

Carnosine, homocarnosine, anserine, and histidine also protected against metal-catalyzed HAS oxidation, as determined by carbonyl formation. Kim *et al.* (1998) reported that iron and heme-mediated Fenton-like reactions produced oxidative protein modification via formation of hydroxyl radicals and ferryl iron, respectively. In addition, lipid peroxidation products are associated with DNA damage and mutagenicity (Koh *et al.*, 1997). Therefore, the protection by histidine-containing dipetides against oxidative protein and lipid damage can be implicated in the retardation of aging or prevention of degenerative diseases such as cancer and atherosclerosis.

Ascorbic acid can be a pro-oxidant or antioxidant. The actions of AA may depend on the concentrations of AA and transition metal ions. Many *in vitro* metal/ascorbate systems have been used for free radical studies where AA acts as a pro-oxidant. A small amount of AA can increase lipid peroxidation, while high concentrations of AA inhibit the reaction (Miller and Aust, 1989; Lee and Hendricks, 1997b). Ascorbic acid, present in high concentrations in mammalian tissues, can not only scavenge active and stable oxygen radicals but can also regenerate vitamin E (Niki, 1991; Winkler *et al.*, 1994). Copper is a well-known catalyst for AA oxidation. Carnosine, anserine, homocarnosine, and histidine effectively inhibited 1 μM Cu (II)-catalyzed AA oxidation. Although the mechanism of these compounds to inhibit the AA oxidation is uncertain, it may be due to chelating copper, thereby generating less-active complexes for AA oxidation, or competing with AA for reducing Cu (II). In addition, they may inhibit AA oxidation by breaking the cascades of oxygen radical production. Carnosine and related compounds may preserve the antioxidant potential of AA, especially in the eye lens where AA prevents cataract formation (Meister, 1994).

Metal-catalyzed oxidation reactions in biological membranes impair membrane functioning, change fluidity, inactivate membrane-bound receptors and enzymes, and

increase nonspecific permeability to ions such as Ca^{2+} (Cochrane, 1991; Halliwell, 1991). The erythrocyte is a unique biological structure containing high concentrations of polyunsaturated fatty acids, cellular oxygen, and ferrous iron in the ligand state. These conditions might be expected to make it highly susceptible to oxidative damage. Copper catalyzes hemolysis of RBC by the formation of free radical and MetHb formation, depletion of glutathione levels, and interference with the reactive thiol groups of G6PDH (Hebbel, 1985; Rachmilewitz *et al.*, 1985; Kuross and Hebbel, 1988; Shinar *et al.*, 1989). Free radicals generated in the presence of Cu (II) and AA might cause hemolysis by inducing the chain oxidation reactions of lipids and proteins in the RBC membrane. Copper + ascorbic acid also enhances the oxidation of hemoglobin (Shinar *et al.*, 1989). However, MetHb formation might inhibit lipid peroxidation in the RBC membrane and eventually might prevent hemolysis because MetHb is not an effective catalyst for lipid peroxidation in the presence of H_2O_2 (Clemens *et al.*, 1985). The protection conferred by carnosine and related compounds against hemolysis may be due to either the formation of copper-complexes and reduction of Cu (II) to Cu (I) or scavenging reactive oxygen species such as superoxide anions and hydroxyl radicals. In addition, the increase in MetHb by carnosine, anserine, and histidine may be related to the protection against hemolysis, perhaps resulting from a decrease in RBC membranal lipid peroxidation.

The antioxidant activity of carnosine and related compounds may be due to the imidazole moiety of the molecules. The proton on the nitrogen of the imidazole ring is especially important for antioxidant activity. Our results suggest that histidine-containing compounds including carnosine, anserine and homocarnosine, which can be endogenously synthesized and supplied by a histidine-supplement diet, may effectively protect against free radical-mediated damage of biomolecules or cells thereby preserving their biochemical and physiological functions.

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