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Interactions between Carnosine and Captopril on Free Radical Scavenging Activity and Angiotensin-converting Enzyme Activity *in vitro*

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Interactions between carnosine (β-alanyl-L-histidine), being plentiful in skeletal muscles and neuronal tissues, and captopril, a widely used angiotensin-converting enzyme (ACE) inhibitor, were examined concerning free radical scavenging activity and ACE activity *in vitro*. Not only captopril, but also carnosine, at concentrations less than those ordinarily found in muscles and neuronal tissues, significantly scavenged 2,2′-azinobis(3-ethylbenzothiazoline-6-sulphonate) (ABTS) radical cations, and inhibited ACE activity. Cupric ions reversed the ABTS scavenging activity of carnosine and captopril, whereas cupric ions strengthened the inhibitory action of carnosine on ACE activity. In contrast, cupric ions antagonized the inhibition of ACE activity induced by ethylenediaminetetraacetic acid, indicating that the inhibitory effect of carnosine on ACE activity is not related to the chelating action of carnosine. On the other hand, carnosine and captopril synergistically enhanced the free radical scavenging activity, but not the inhibitory effect on the ACE activity. These data suggest that carnosine in its concurrent use with captopril could act as a beneficial free radical scavenger, with less danger of overdose, in the inhibition of ACE activity.

Key words——carnosine; captopril; angiotensin-converting enzyme; free radical scavenging activity

INTRODUCTION

Hazardous interactions between food ingredients and medicines are currently serious matters in public health because adverse effects often occur through unintentional intake of foods and chemicals. Grapefruit juice, a common beverage, increases the effects of calcium blockers and β-hydroxy-β-methylglutaryl CoA reductase inhibitors, for example.[1] Although food-drug interactions frequently relate to the metabolic processes common to these substances, the precise mechanisms of their interactions have not been elucidated for the most part.

Several peptides from foods materials such as milk, fish meat, and soybeans have been reported to have an antihypertensive effect due to their inhibition of angiotensin-converting enzyme (ACE) activity.[2,3] Carnosine (β-alanyl-L-histidine) are present at high concentrations in skeletal muscles and neuronal tissues.[4] Since the antioxidative effects of carnosine have been reported,[4—8] this peptide is used in so-called health foods or dietary supplements. Additionally, carnosine has been documented to inhibit ACE activity.[9] On the other hand, captopril, which has a sulfhydryl (SH) group in its chemical structure, has a role as a free radical scavenger[10,11] in addition to its use as a prevailing ACE inhibitor to treat hypertension. In the present study, we examined interactions between carnosine and captopril on free radical scavenging activity and ACE activity *in vitro*.

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MATERIALS AND METHODS

ACE from bovine lung, 2,2′-azinobis(3-ethylbenzothiazoline-6-sulphonate) (ABTS), carnosine, reduced glutathione (GSH), N-[3-(2-furyl)acryloyl]-Phe-Gly-Gly (FAPGG) were obtained from Wako Pure Chemicals, Osaka, Japan. Other chemicals were of reagent grade.

ABTS Radical Cation Decolorization Assay The radical scavenging activity of antioxidants for ABTS radical cations was determined according to the method of Re *et al.*[12] as described in a previous report.[13] Shortly, two ml of ABTS solution, whose absorbance was 0.70 at 734 nm, were mixed with 20 μl of a test compound solution in a cuvette. At 4 min after mixing, the absorbance was measured at 734 nm. Free radical scavenging activity was expressed as a % of control based on the absorbance measured.

Assay for ACE Activity The ACE activity was spectrophotometrically measured according to the

method of Hou *et al.*[9] with a slight modification. Forty microliters of commercial ACE (0.5 units/ml, from bovine lung) were mixed with 200 μl of a sample solution, which was prepared with 50 mM Tris-HCl buffer (pH 7.5) containing 0.3 M NaCl, and 200 μl of 50 mM Tris-HCl buffer (pH 7.5) containing 0.3 M NaCl in the cuvette of a spectrophotometer maintained at 30 ℃. The reaction was started by adding 0.5 ml of FAPGG dissolved in 50 mM Tris-HCl buffer (pH 7.5) containing 0.3 M NaCl to the incubation medium, followed by monitoring the decrease in the absorbance (ΔA_{sample}) for 5 min at 345 nm. In a blank experiment, the decrease in the absorbance (ΔA_{blank}) was determined by the addition of 50 mM Tris-HCl buffer (pH 7.5) containing 0.3 M NaCl instead of the FAPGG solution and a sample solution. The inhibitory effect of sample was expressed as % inhibition calculated as follows: $[1-(\Delta A_{sample}-\Delta A_{blank})/(\Delta A_{control}-\Delta A_{blank})]\times 100$ (%).

Statistical Analysis All values were expressed as the mean±S.E. Statistical analyses were performed by one-way analysis of variance (ANOVA) followed by Tukey-Kramer's multiple comparison test or Dunnett's multiple comparison test at a level of significance of $p<0.05$.

RESULTS AND DISCUSSION

Captopril is an ACE inhibitor used in the treatment

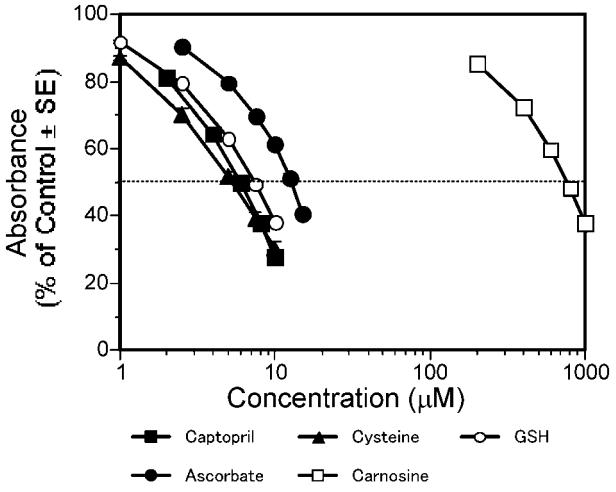

Fig. 1. ABTS Radical Cation Scavenging Activity of Antioxidants

Each point represents the mean±S.E. from five experiments.

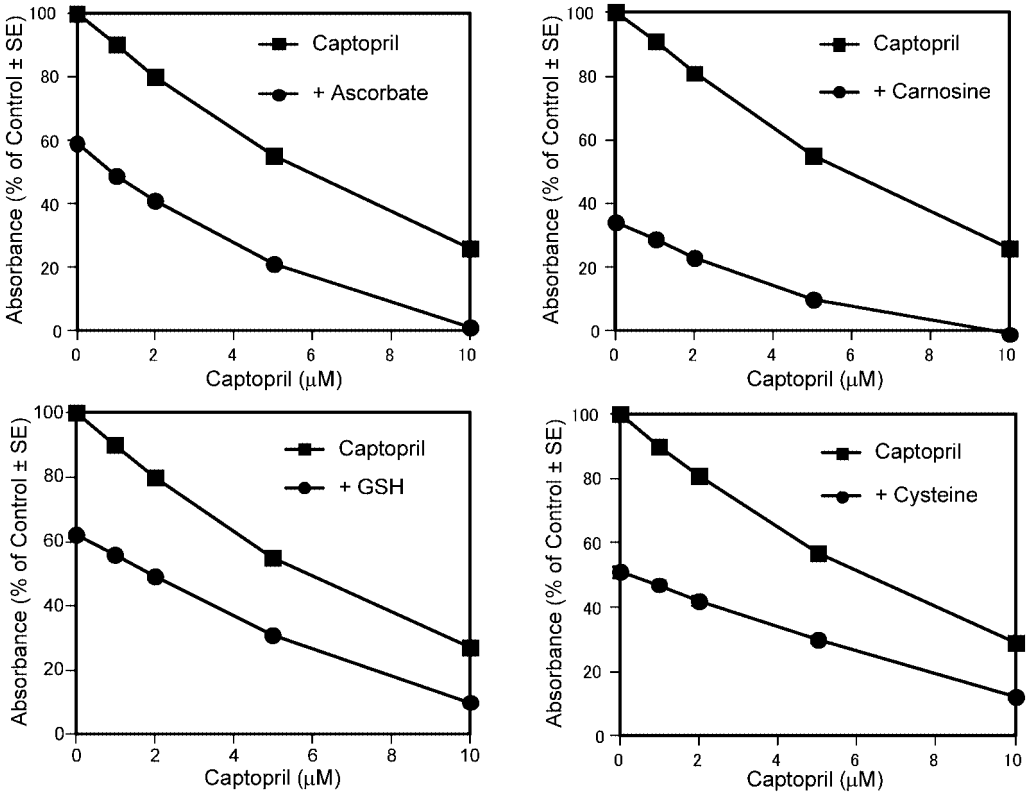

Fig. 2. ABTS Radical Cation Scavenging Activity of Captopril in Combination with Antioxidants

ABTS radical cation scavenging activity of captopril was determined in the presence of captopril, and in combination with 10 μM ascorbic acid, 1 mM carnosine, 5 μM reduced glutathione (GSH), or 5 μM cysteine. Each point represents the mean±S.E. from four experiments.

of hypertension and congestive heart failure. This drug has been reported to have free radical scavenging activity[10,11] probably owing to the SH group in its chemical structure. This property is beneficial, especially in the treatment of cardiovascular diseases accompanying atherosclerosis. We first compared the ABTS radical scavenging activities of captopril and antioxidants widely distributing in foods and living cells (Fig. 1). The potency of ABTS radical scavenging activity was decreased in the following order; cysteine≥captopril≥GSH>ascorbic acid≫carnosine. It is obvious that substances having the SH group were effective to a similar extent. Furthermore, the combination of captopril with each of the antioxidants synergistically ($p<0.05$) increased the radical scavenging activity for ABTS radical cations (Fig. 2). It is notable that SH-containing substances like cysteine and GSH enhanced radical scavenging activity of captopril presumably without producing disulfides, because oxidized glutathione exhibited no effects on the radical scavenging activity of captopril (data not shown). Since GSH, ascorbate, and carnosine can exist naturally in the mM range in tissues, while the maximum concentrations of captopril in human plasma after a clinical use of 100 mg have been recognized to be around 5 μM,[14,15] these synergisms at concentrations used in the present study could occur in living cells.

Carnosine (0.5—1.0 mM) significantly inhibited ACE activity (Fig. 3A), and Lineweaver-Burk plots of ACE activity indicated that carnosine did not largely alter the affinity of the enzyme for a substrate, FAPGG; apparent Km were 0.761 mM and 0.757 mM for FAPGG in the absence and in the presence of 0.5 mM carnosine, respectively (Fig. 3B). The present results are comparable with those of Hou et al.[9] on the point that the inhibition was not in a competitive manner. In addition, no synergistic effects on ACE activity were found in the presence of carnosine and captopril (Fig. 4). Carnosine, being abundant in meats and some dietary supplements, is absorbed intact from the small intestine.[16] In addition, dietary supplementation of carnosine attenuated the ischemia /reperfusion-induced renal dysfunction.[17] Hence, our findings suggest that carnosine and captopril may be synergistic antioxidants, and could thus pose less danger of overdose in the inhibition of ACE activity. Deleterious interactions between medicines and food ingredients, including so-called health foods and

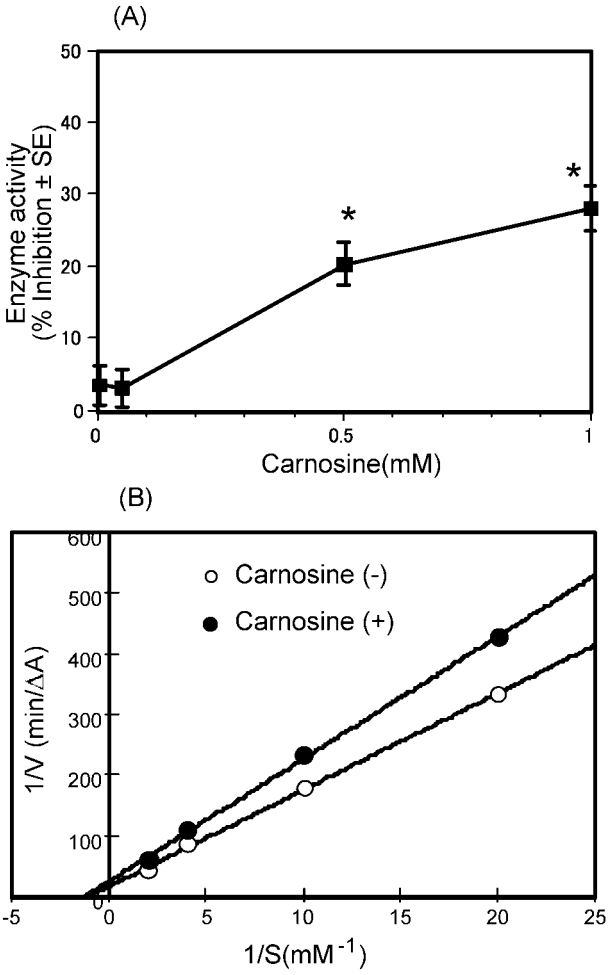

Fig. 3. Effect of Carnosine on Angiotensin-converting Enzyme (ACE) Activity

A: The ACE activity was indicated as % inhibition. Each point represents the mean±S.E. from seven experiments. The differences between the control group and each of the groups treated with carnosine were analyzed by Dunnett's multiple comparison test; *, statistically different at $p<0.05$. B: Lineweaver-Burk plots of the ACE activity in the presence or absence of 0.5 mM carnosine. Each point represents the mean±S.E. from four experiments.

functional foods, are currently increasing. Further *in vivo* experiments and clinical trials are needed to clarify the advantage of carnosine intake.

Heavy metals such as copper and zinc are essential elements for many enzymes. Captopril inhibits ACE activity by interacting with Zn^{2+} in the prosthetic groups of ACE.[18] Additionally, captopril and carnosine have been documented to chelate cupric ions.[19,20] In the present study, cupric ions, but not zinc ions, significantly ($p<0.05$) antagonized the ABTS radical scavenging activity of captopril (Fig. 5), as well as that of carnosine (Fig. 6). In contrast, cupric ions and zinc ions synergistically enhanced the inhibitory effect of carnosine on ACE activity, whereas these

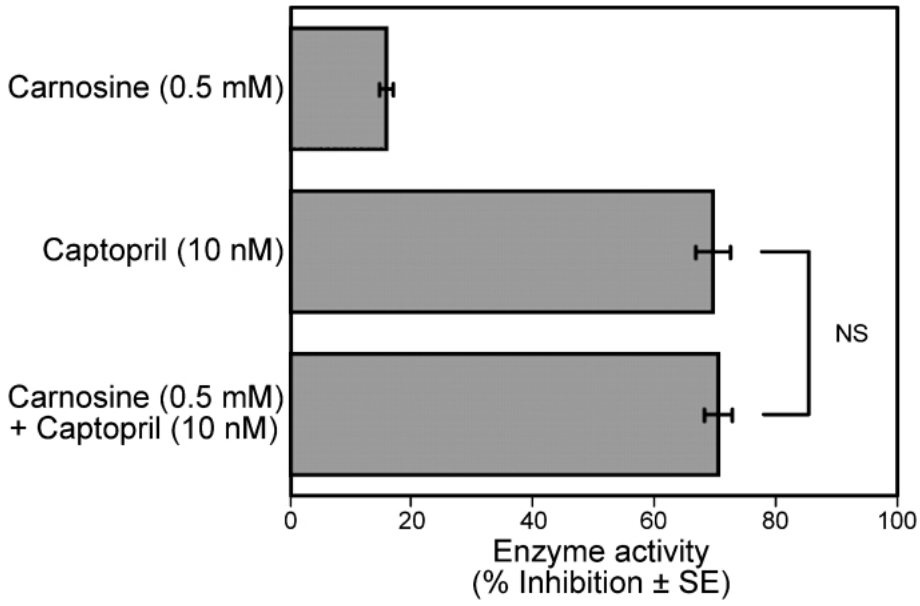

Fig. 4. Interaction between Carnosine and Captopril on the Angiotensin-converting Enzyme (ACE) Activity

The ACE activities were determined by the addition of 0.5 mM carnosine and 10 nM captopril, and expressed as % inhibition. Each bar represents the mean± S.E. from six experiments. NS: not significant according to Tukey-Kramer's multiple comparison test.

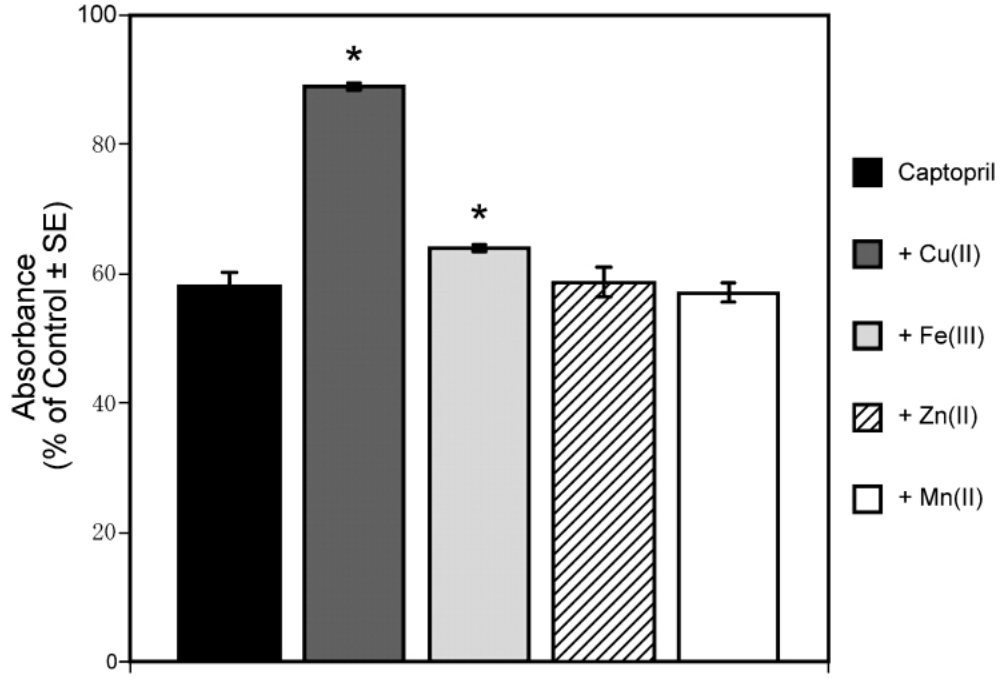

Fig. 5. Effects of Metal Ions on ABTS Radical Cation Scavenging Activity of Captopril

ABTS radical cation scavenging activities were determined in the presence of 5 μM captopril, and in combination with each of metal salts at 5 μM. Each bar represents the mean±S.E. from four-nine experiments. Statistical analyses were performed by one-way ANOVA followed by Dunnett's multiple comparison test, * : statistically different from captopril alone at $p<0.05$.

metal ions antagonized the inhibitory effect of ethylenediaminetetraacetic acid (EDTA) on ACE activity (Table 1). Since EDTA, a distinguished chelating agent, interacts with Zn^{2+} in ACE, additional cupric ions and zinc ions could reverse the interference from EDTA on ACE. The inhibitory effect of carnosine, whose molecular mechanisms are unclear at present, may not be related to the co-factor Zn^{2+} in

Table 1. Effects of Metal Ions on the Inhibition of Angiotensine-converting Enzyme (ACE) Activity Induced by Carnosine and EDTA

	ACE activity (% Inhibition±SE)		ACE activity (% Inhibition±SE)
Experiment A		Experiment C	
10 μM EDTA	73.5±0.9[a)]	1 mM Carnosine	21.8±1.8[a)]
10 μM Cu^{2+}	15.6±2.1[b)]	10 μM Cu^{2+}	24.5±0.4[a,b)]
10 μM EDTA+ 10 μM Cu^{2+}	17.5±7.2[b)]	1 mM Carnosine+ 10 μM Cu^{2+}	32.4±2.4[b)]
Experiment B		Experiment D	
10 μM EDTA	73.3±0.9[a)]	1 mM Carnosine	23.1±1.1[a)]
10 μM Zn^{2+}	0.5±0.1[c)]	100 μM Zn^{2+}	3.7±1.1[c)]
10 μM EDTA+ 10 μM Zn^{2+}	4.8±2.7[b)]	1 mM Carnosine+ 100 μM Zn^{2+}	34.3±4.4[b)]

The differences among the groups were analyzed by the Tukey-Kramer's test. *a,b,c*): Different letters indicate significantly different observations ($p<0.05$, $n=6$).

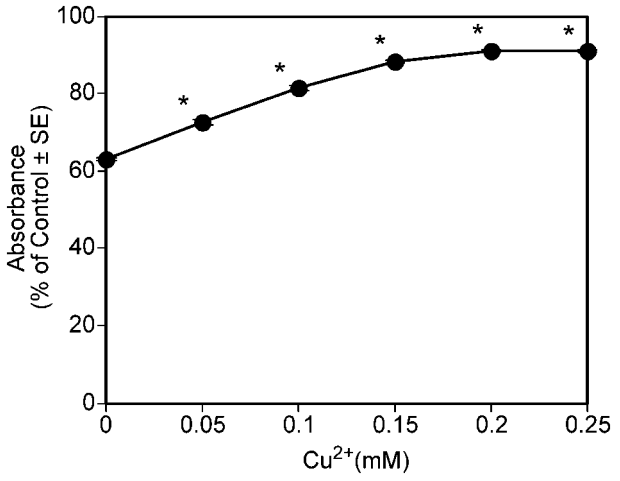

Fig. 6. Effect of Cupric Ions on ABTS Radical Cation Scavenging Activity of Carnosine

ABTS radical cation scavenging activity of 0.5 mM carnosine was determined in combination with cupric ions. Each point represents the mean± S.E. from six experiments. Statistical analyses were performed by one-way ANOVA followed by Dunnett's multiple comparison test, * : statistically different at $p<0.05$.

ACE.

In conclusion, carnosine significantly scavenged ABTS radical cations, and inhibited ACE activity *in vitro* at concentrations less than those ordinarily found in muscles and neuronal tissues. The concomitant addition of carnosine with captopril, a prevalent ACE inhibitor used as an antihypertensive drug, synergistically enhanced the free radical scavenging activity, but not the inhibitory effect, on ACE activity. The present findings suggest that carnosine in concurrent use with captopril could act as a beneficial free radical scavenger with less danger of overdose in the inhibition of ACE activity.

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