Note

Carnosine Modulates Stress-Attenuated Plasma Antioxidative Capacity

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Oral administration of carnosine (β-alanyl-L-histidine) in mice treated with restraint stress moderately alleviated a stress-induced decrease in plasma oxygen radical absorbance capacity (ORAC) activity \((P = 0.075)\). Carnosine treatment also increased the level of plasma glutathione (GSH) and ascorbic acid compared with those in the restraint stress mice. Carnosine and related compounds anserine, histidine and histamine exhibited ORAC activity in vitro, while β-alanine was not effective. These results suggest that carnosine exerts a protective effect against the stress-induced elevation of the oxidative level in plasma.

Keywords: carnosine, oxygen radical absorbance capacity (ORAC), glutathione, ascorbic acid, restraint-stress

Introduction

Carnosine (β-alanyl-L-histidine) is mainly synthesized in the brain, muscles, and other innervated tissues (Zapp et al., 1938; William et al., 1962). In recent years, numerous studies have shown that long-lived cells such as neurons and myocytes, which do not divide, contain high levels of carnosine (Hipkiss et al., 2002). Even though the carnosine content in the brain is high, its function has not been clarified. Early studies indicated that carnosine levels were correlated with the maximum life span of members of an animal species (Hipkiss et al., 1995). Carnosine levels decline with age, which may account for the normal age-related decline in tissues and function (Stuerenburg, 2000). In addition, in the brain, which consumes a large amount of oxygen and glucose, high levels of carnosine may be needed to protect membrane lipids and metals against active oxygen species (Salim-Hanna et al., 1991). Several authors reported that the brain membranes of carnosine-treated mice had significantly lower levels of malondialdehyde (MDA). Carnosine protects against MDA-induced damage (Hipkiss et al., 1997), and several studies indicated that carnosine suppressed protein-sugar interactions leading to advanced glycosylation end-product (AGE) formation (Hipkiss et al., 1998).

Other studies have found that stress increases lipid peroxidation (Kurihara et al., 2002), and decreases the amount of endogenous antioxidative substances (Kurihara et al., 2004), including GSH (Sen et al., 1994) and vitamin E (Zhang et al., 2002). Diminished antioxidant protection and induced oxidative stress may be correlated with life-style related diseases such as cancer (Dreher et al., 1996), diabetes (Maritim et al., 2003), atherosclerosis (Griendling et al., 2003), and Alzheimer’s (William et al., 1999).

Nutritionists have sought to understand the body’s oxidation processes and to prevent damage caused by active oxygen molecules (Mayne, 2003). Several studies have indicated that certain antioxidants might have additional activities, such as quenching free radicals or suppressing the generation of free radicals by interrupting oxidation chain-reactions (Kerr et al., 1996). Antioxidants that trap free radicals and
l IDisposable lipid peroxides may delay the onset of lipid peroxidation, which inhibits further production of free radicals and suppresses damage induced by certain enzymes that can degrade connective tissues (Halliwell et al., 1995).

The oxygen radical absorbance capacity (ORAC) is measured as the loss of fluorescence of disodium fluorescein induced by using 2,2′-azobis (2-amidinopropane) hydrochloride (AAPH) as a peroxyl radical generator (Ou et al., 2002). In recent years, the ORAC assay has been used as a method for the antioxidative evaluation of plasma and other tissues, antioxidative foods and supplements (Cao et al., 1995; Prior et al., 2003).

Early studies indicated that carnosine inhibited lipid oxidation and scavenged superoxide radicals (Gariballa et al., 2000; Decker et al., 2000). However, these findings were obtained by in vitro experiments and the effective doses were rather high. Therefore, we evaluated the anti-stress effects of carnosine by examining plasma ORAC, GSH, and ascorbic acid levels in mice exposed to restraint stress. Restraint, which has an extensive history, has been widely used in investigations of animal physiology, pathology, and pharmacology (Paré et al., 1986). This type of stress promotes lipid peroxidation in liver tissue and decreases natural killer cell levels, which are susceptible to reactive oxygen species and lose their activity as a result of damage incurred by such oxygen species (Kurihara et al., 2002).

Materials and Methods

Chemicals and preparation  L-carnosine, β-alanine, L-anserine, L-histidine, and histamine were purchased from Sigma Chemical (St. Louis, MO, USA), and ascorbic acid, disodium fluorescein, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox, a water-soluble analogue as a control standard), and AAPH were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Ascorbic acid and fluorescein were dissolved directly in an acetone/water mixture (50:50, v/v) and diluted with 75 mM potassium phosphate buffer (pH 7.4) for analysis. Trolox, AAPH, carnosine, β-alanine, anserine, histidine, and histamine were dissolved in potassium phosphate buffer immediately before the ORAC assay.

To examine plasma antioxidative status, carnosine was dissolved in distilled water (20 mg/mL) before use, and the solution was administered orally to animals at 0.1 mL/10 g body weight (200 mg/kg=0.88 mmol/kg) at 24 h and 30 min before exposure to restraint stress. Ten animals in each group were sacrificed for the analysis of antioxidative capacity after restraint-stress.

Animals  Seven-week-old male ICR mice were purchased from Charles River Japan Inc. (Tokyo, Japan). The animals were kept in a specific pathogen-free animal room at 23 ± 1°C with a 12 h light-dark cycle (lights on from 6:00 to 18:00) and were fed standard laboratory chow (CE-2; Clea Japan, Inc., Japan) and tap water. The animals were kept for 1 week before the experiment. For the restraint stress experiment, each mouse was confined to an oval metal restraint cage for 20 h before the assay. As the mice were not fed during the 20 h restraint period, another control group was created, in which mice were not given any food and water for 20 h without restraint stress (starved control). The care and treatment of the animals conformed to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH publication No. 85-23, revised 1985) and the Japanese Society of Nutrition and Food Science (Law No. 105 and Notification No. 6 of the Japanese government).

Assessment of ORAC levels in vivo and in vitro  Under anesthesia with diethyl ether, blood samples were drawn from the heart after 20 h of stress treatment into a tube containing 2% sodium heparin. Each tube was centrifuged at 5,000 rpm for 5 min and the plasma was treated with 3% perchloric acid (PCA) adjusted from 60% PCA. The plasma was centrifuged at 15,000 rpm for 15 min at 4°C. Then, the supernatant was removed as the non-protein plasma fraction and diluted in phosphate buffer (pH 7.4) for ORAC analysis.

An automated ORAC assay was carried out using a Lab systems Fluoroskan Ascent plate reader (Helsinki, Finland) with fluorescent filters (excitation wavelength: 485 nm, emission filter: 527 nm) (Decker et al., 2000). Fluorescein was used as a target for free radicals to attack and the reaction was initiated with AAPH. Trolox was used as a control standard. Final results were calculated based on the difference in the area under the fluorescein decay curve between the blank and each sample.

Plasma GSH and ascorbic acid levels  The concentration of GSH in the plasma was determined by high-performance liquid chromatography (HPLC; column: SC-5 ODS (Eicom Co; 4.6 × 150 mm; i.d. 5 µm); mobile phase: 99% phosphate buffer (pH 2.5)-1% methanol including 100 mg/L SOS and 5 mg/L EDTA; flow rate: 0.5 mL/min) and an electrochemical detection system (ECD-300, Eicom Co) (Haramaki et al., 2001) operated at room temperature. The quantification of GSH was carried out using standard calibration curves. For plasma GSH analysis, blood samples were taken and treated the same as in ORAC analysis. The sample was subsequently stored at −80°C before analysis. Each sample was filtered through a 0.45 µm membrane filter and 10 µl was subjected to HPLC to determine the GSH level.

Plasma ascorbic acid analysis was carried out using an automatic serum analyzer (model 7070, Hitachi Co., Ltd., Japan).
Results

Effect of carnosine on plasma ORAC level  Two hundred mg/kg of carnosine (0.88 mmol/kg) were administered orally twice, at 24 h and 30 min before exposure to stress, and the same volume of water was given to the stressed control animals. These experimental conditions were determined in preliminary experiments. The plasma ORAC value was calculated as the ratio of the area under the fluorescence decay curve for Trolox as a standard. The plasma ORAC level was 905.7 ± 158.2 μM Trolox eq./L for the starved control, while the ORAC value observed after 20 h exposure to stress was significantly decreased (727.7 ± 189.3 μM Trolox eq./L, P = 0.031) compared to the starved control (Fig. 1). The average value for the ORAC of carnosine-treated stressed mice was 886.8 ± 89.2 μM Trolox eq./L, and the total antioxidative capacity of the plasma increased by 21.9% compared with the stressed control (P = 0.075). Likewise, 0.44 mmol/kg carnosine moderately improved the plasma ORAC level, but the difference was not statistically significant.

Effects of carnosine on GSH and ascorbic acid plasma levels  The plasma GSH concentration in the starved control was 2.98 ± 0.26 nmol/mL, while that of the stressed control was 0.77 ± 0.15 nmol/mL (Fig. 2). Stress resulted in a substantial reduction in the plasma GSH concentration. When 0.88 mmol/kg of carnosine was given, the plasma GSH level increased (1.19 ± 0.13 nmol/mL) as compared to the stressed control (P = 0.15). Carnosine (0.44 mmol/kg) moderately increased GSH levels compared to the stressed control (1.01 ± 0.08 nmol/mL).

Likewise, stress also lowered the plasma ascorbic acid level (70.9 ± 6.8 μmol/mL) compared to the starved control (92.8 ± 11.6 μmol/mL), but the decrease in plasma ascorbic acid was smaller than that for the plasma GSH concentration. Compared to the stressed control, 0.88 and 0.44 mmol/kg of carnosine significantly increased plasma ascorbic acid levels (Fig. 3). The mean values of ascorbic acid in the carnosine treated mice were 83.3 ± 9.6 and 82.2 ± 4.9 μmol/mL, respectively.

Radical scavenging activity of carnosine and its related compounds in vitro  ORAC was determined using 10 μM...
Table 1. Radical scavenging activity of carnosine and its related compounds at 10 μM in vitro.

<table>
<thead>
<tr>
<th>Compound</th>
<th>ORAC (μM Trolox equivalent)</th>
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<tbody>
<tr>
<td>Carnosine</td>
<td>0.12</td>
</tr>
<tr>
<td>Anserine</td>
<td>0.24</td>
</tr>
<tr>
<td>β-alanine</td>
<td>No activity</td>
</tr>
<tr>
<td>Histidine</td>
<td>0.28</td>
</tr>
<tr>
<td>Histamine</td>
<td>0.19</td>
</tr>
</tbody>
</table>

Trolox was used as a control standard. The data were calculated from a calibration curve determined using three concentrations and was conducted in triplicate.

of each sample, and the data is given in Table 1. The antioxidative capacity of carnosine was rather weak. However, it is clear that the peptides carnosine and anserine have antioxidative activity. The antioxidative capacities of anserine, histidine and histamine were slightly greater than that of carnosine, while β-alanine did not exhibit antioxidative activity.

Discussion

The effects of restraint as a physiological stressor on the ORAC activity of plasma were examined in mice. Mice subjected to restraint for 20 h showed an approximately 24% decrease in plasma ORAC levels (Fig. 1). The GSH and ascorbic acid contents in the plasma also decreased (Fig. 2 and 3). These results suggest that physiological stress plays a role in increasing oxidative stress. The administration of carnosine ameliorated the stress-induced decrease in plasma ORAC activity ($P = 0.075$) as shown in Fig. 1. The ORAC activity in the plasma reflects the antioxidative capacity of water soluble low molecular weight antioxidants, such as GSH and ascorbic acid, present in the blood (Kurihara et al., 2004; Jiao et al., 2000). The current findings show that carnosine attenuates oxidative stress by changing the oxidative status of plasma in mice subjected to stress, and improves antioxidative processes.

GSH is the most well-known antioxidant found in tissues (Wu et al., 2004). Recent studies have provided clear evidence that decreased liver blood flow induced by stress may attenuate GSH synthesis from circulatory sources via the γ-glutamyl cycle (Deneke et al., 1989) and cause disturbances in GSH homeostasis, such as decreasing its plasma concentration, degrading its cellular redox status, and interfering with GSH transport (Leeuwenburgh et al., 1995). As a result, GSH levels were drastically decreased by restraint stress. The administration of carnosine slightly increased the GSH level compared to the stressed control ($P = 0.15$). Carnosine may have a protective effect on GSH degradation during stress.

Likewise, restraint stress also lowered the plasma ascorbic acid level compared to the starved control, but the differences was smaller than that for the GSH concentration. As mice are capable of synthesizing ascorbic acid, mouse tissues generally have higher levels of ascorbic acid, and were only slightly influenced in the stressed control as compared to the starved control. It was suggested that stress may induce the release of ascorbic acid stores into the plasma (Toutain et al., 1997). The ingestion of carnosine in restrained mice increased plasma ascorbic acid levels compared to the stressed control (Fig. 3), which was thought to suppress the oxidative degradation of plasma ascorbic acid.

Carnosine exhibited only a weak potential for scavenging free radicals generated by AAPH in vitro (Table 1). However, it was shown that carnosine strongly quenches hypochlorite radicals (ClO•) in vitro (Yanai et al., 2004). This effect may be one of the antioxidative effects of carnosine.

Although the protective activities of carnosine against stress-induced plasma ORAC and the reduction of GSH and ascorbic acid levels in vivo are partly due to its antioxidative activity, it is possible these effects may be due to its anti-stress effects (Nagai et al., 2003). Carnosine might exert direct or indirect anti-stress effects via histaminergic neural function in the brain. Carnosine is known to exhibit good oral-absorbability and increases serum levels of L-histidine and carnosine in the carotid artery and hepatic portal vein (Tamaki et al., 1985). L-Histidine is converted to histamine by L-histidine decarboxylase, which affects histaminergic neuronal activation (Tanida et al., 2005). As mentioned above, L-histidine exhibits radical scavenging activity in vitro. It is suggested that the observed modulation of plasma antioxidative capacity induced by restraint stress is due to both carnosine itself and L-histidine generated from carnosine by carnosinase.

Usually, the generation and scavenging of oxygen free radicals is balanced in the human body. If there is an imbalance in the mechanism that regulates antioxidant enzymes, such as superoxide dismutase or catalase, excessive amounts of active oxygen radicals may be generated. Previous studies found that stress can cause free radical reactions (Esch et al., 2002) to produce deleterious modifications in membranes, proteins, enzymes, and DNA (Cooke et al., 2003), which may be correlated with life-style related diseases such as cancer, diabetes, and atherosclerosis. Therefore, it is important to find effective scavengers of active oxygen radicals. Although carnosine is a weak antioxidant in vitro, it was found that this compound exhibited an anti-stress effect in maintaining plasma antioxidative capacity, as indicated by the ORAC assay and GSH and ascorbic acid levels in plasma. Even though the mechanism is still unknown, our results have encouraged us to investigate the pharmacology of carnosine under physiological oxidative conditions, the
examination of which is worthy for its potential benefits to life-style-related diseases and general health.

References


