2-Oxo-histidine–containing dipeptides are functional oxidation products

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Imidazole-containing dipeptides (IDPs), such as carnosine and anserine, are found exclusively in various animal tissues, especially in the skeletal muscles and nerves. IDPs have antioxidant activity because of their metal-chelating and free radical-scavenging properties. However, the underlying mechanisms that would fully explain IDP antioxidant effects remain obscure. Here, using HPLC–electrospray ionization–tandem MS analyses, we comprehensively investigated carnosine and its related small peptides in the soluble fractions of mouse tissue homogenates and ubiquitously detected 2-oxo-histidine–containing dipeptides (2-oxo-IDPs) in all examined tissues. We noted enhanced production of the 2-oxo-IDPs in the brain of a mouse model of sepsis-associated encephalopathy. Moreover, in SH-SY5Y human neuroblastoma cells stably expressing carnosine synthase, H2O2 exposure resulted in the intracellular production of 2-oxo-carnosine, which was associated with significant inhibition of the H2O2 cytotoxicity. Notably, 2-oxo-carnosine showed better antioxidant activity than endogenous antioxidants such as GSH and ascorbate. Mechanistic studies indicated that carnosine monooxygenation is mediated through the formation of a histidyl-imidazole radical, following by the addition of molecular oxygen. Our findings reveal that 2-oxo-IDPs are metal-catalyzed oxidation products present in vivo and provide a revised paradigm for understanding the antioxidant effects of the IDPs.

The mammalian essential amino acid, L-histidine, and its methylated derivatives (1- and 3-methylhistidines) are known to be widely and abundantly distributed in mammalian tissues in their free form or as components of peptides and proteins. Carnosine (β-alanyl-L-histidine), which was first discovered over 100 years ago, is the most well-characterized imidazole-containing dipeptide (IDP). Since then, carnosine and other IDPs, such as anserine (β-alanyl-3-methyl-L-histidine) and homocarnosine (γ-amino-butyryl-L-histidine), have been observed at high concentrations in the skeletal muscles and central nervous systems of many vertebrates (1). The levels of the IDPs are regulated by metabolic enzymes, including carnosine synthase (CARNs) (2), methyltransferase (3), and dipeptidase (4, 5), indicating that IDPs play physiological roles in the muscle and brain. It has been postulated that carnosine contributes significantly to physicochemical buffering in skeletal muscles by neutralizing the lactic acid produced during anaerobic glycolysis (6). Evidence has also been found that the peptide may play a role as a neurotransmitter in olfactory receptor axons (7) and as a regulator of enzymes (8). In addition to these functions, carnosine has attracted a lot of attention as a potential antioxidant because of its reactivity with reactive oxygen and nitrogen species and its potential to form adducts with deleterious aldehydes and ketones (1). Carnosine also shows an efficient metal-chelating property, which may be associated with its antioxidant activity (1). However, the exact antioxidant mechanisms of the peptide remain unknown.

Because of the metal-chelating property of the imidazole ring, histidine is extremely sensitive to metal-catalyzed oxidation reactions. In the presence of oxygen and a reducing agent, such as ascorbate, the binding of metal ions, such as copper ion (Cu2+), to histidine results in the facile oxidation of its imidaz-
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Figure 1. Simultaneous detection of IDPs in the mouse tissues. A, authentic standards were used for the development of a LC-MS/MS method in the separation and quantification of carnosine and anserine using MS/MS fragments and retention times unique to each IDP (Fig. S3). Carnosine, anserine, 2-oxo-carnosine, and 2-oxo-anserine are indicated by red numerals 1, 2, 3, and 4, respectively. B, chemical structure of carnosine, anserine, 2-oxo-carnosine, and 2-oxo-anserine.

ole ring. The reaction involves the reduction of Cu\(^{2+}\) by the reducing agent to generate Cu\(^{+}\). The reduced form of copper ion donates one electron to O\(_2\) to generate an unidentified reactive oxygen species, which immediately oxidizes the ligand itself (histidine). It has been reported that the metal-catalyzed oxidation of histidine generates a number of products, including a unique monooxygenation product, 2-oxo-histidine (9–11). The formation of 2-oxo-histidine has been demonstrated in the peptides and proteins in vitro and implicated in aging and other pathological states associated with oxidative stress (9–11). However, no study has definitively demonstrated the presence of 2-oxo-histidine in vivo.

In the present study, we unambiguously detected 2-oxo-histidine-containing dipeptides (2-oxo-IDPs) in mouse tissue homogenates using HPLC with online electrospray ionization tandem MS (LC–ESI–MS/MS). To the best of our knowledge, this is the first evidence for the presence of oxidized peptides containing 2-oxo-histidine in vivo. In addition, using human neuroblastoma cells stably expressing CARN5, we demonstrated significant inhibition of the H\(_2\)O\(_2\) cytotoxicity and a concomitant increase in the intracellular levels of the 2-oxo-IDPs. We also found that IDPs gain a free radical scavenging activity via the oxygennation. Mechanistic studies of the metal-catalyzed oxidation of IDPs demonstrate that the mono-oxygenation of IDPs may be mediated through the formation of a histidyl radical followed by the addition of molecular oxygen. These results reveal new insights into the antioxidant function of the IDPs.

Results

Comprehensive analysis of IDPs in mouse muscle homogenates

To simultaneously detect carnosine and related peptides in vivo, we sought to identify a common fragment ion from the MS/MS product-ion analysis of the authentic peptides. The product-ion analysis of carnosine gave common fragments at the mass-to-charge ratios (m/z) of 89 and 72, corresponding to the cleavage at the peptide bond, and at the N–Ca bond in the peptide backbone, respectively (Fig. S1). Similarly, anserine gave the same fragment ions (Fig. S2). These characteristic common fragment ions allowed the simultaneous analysis of carnosine and anserine using LC–ESI–MS/MS in the multiple reaction monitoring (Fig. S3). We then applied this method to the tissue samples to detect the IDPs. As shown in Fig. 1A, the IDPs were detected in muscle along with several unknown peptides. Identifications were tentatively made by comparison of the retention time and m/z, indicating that the peptides 1 (m/z 227) and 2 (m/z 241) were identical to carnosine and anserine, respectively. We also detected six other peptides: two (peptides 3 and 4) of them showed molecular ions at m/z 243 and 257 ([M+H]\(^+\)), corresponding to a 16-Da increase in the mass value of carnosine and anserine, respectively. It was speculated that, because this change in molecular weight could originate from the mono-oxygenation of the imidazole groups, the products might contain an oxo-imidazole moiety.

Identification of oxidized IDPs

The formation of 2-oxo-histidine is now known to be due to the metal-catalyzed oxidation of histidine (12). Hence, to confirm the structure of the products, carnosine and anserine were incubated with ascorbate in the presence of Cu\(^{2+}\), and the reaction mixtures were analyzed by LC–ESI–MS/MS. The data (Fig. S4) showed that products 3 and 4 were indistinguishable from β-alanyl-L-2-oxo-histidine (2-oxo-carnosine) and β-alanyl-3-methyl-L-2-oxo-histidine (2-oxo-anserine), respectively (Fig. 1B).

Despite its abundance in the in vitro oxidized proteins, the presence of 2-oxo-histidine in vivo has never been demonstrated. To accurately analyze the endogenous formation of 2-oxo-IDPs in cells and tissues, we established a highly sensitive and specific method for the measurement of 2-oxo-IDPs using LC–ESI–MS/MS coupled with a stable isotope dilution method. Collision-induced dissociation of purified 2-oxo-carnosine showed relevant products at m/z 89.0 and m/z 196.1 (Fig. S5A). The product ions at m/z 89.0 and m/z 196.1 matched values expected to originate from a β-alanine moiety and 2-oxo-carnosine, respectively. The identification of these product ions was supported by the observation that the collision-induced dissociation of the standard isotope-labeled 2-oxo-carnosine, containing [\(^{13}\)C\(_3\),\(^{15}\)N] β-alanine, produced relevant product ions.
ions at $m/z$ 93.0 and $m/z$ 198.1 (Fig. S5B). Fig. S5C demonstrates the result on the standard isotope-labeled carnosine and nonlabeled carnosine using multiple reaction monitoring (MRM) between the transition from the protonated parent ions $[M + H]^+$ to the characteristic daughter ions, $m/z$ 247.1 → 198.1 and $m/z$ 243.1 → 196.1. This allowed detection of both the standard isotope-labeled and nonlabeled carnosine, respectively. The characterization of 2-oxo-anserine and 2-oxo-homocarnosine was also performed in a similar way to 2-oxo-carnosine (data not shown). Parameters for the MRM analysis of the IDPs and 2-oxo-IDPs are summarized in Table S1. The limit of quantification for the 2-oxo-IDPs was 100 fmol on the column with linearity ($r^2 = 0.999$ (100–5000 fmol)).

Using the LC–ESI–MS/MS coupled with a stable isotope dilution method, we analyzed the IDPs and 2-oxo-IDPs in several mouse tissues. Endogenous carnosine and 2-oxo-carnosine were co-eluted with the spiked standards labeled with the stable isotope at the same retention time (Figs. 2, A and B). Results of the quantitative identification of the IDPs and their 2-oxo-products are summarized in Fig. 2C. The data showed that the levels of 2-oxo-carnosine were 0.23, 2.1, and 4.8 pmol/mg protein in the brain, kidney, and muscle, respectively. Carnosine was also detected in the lung, heart, and liver at 86, 224, and 11 pmol/mg protein, respectively. The percentages of 2-oxo-carnosine to carnosine were 0.012, 1.8, and 0.025% in the brain, kidney, and muscle, respectively. 2-Oxo-anserine was detected in all the tested tissues at 0.11, 0.45, 0.36, 7.0, 0.33, and 30 pmol/mg protein in brain, lung, heart, kidney, liver, and muscle, respectively, and the corresponding levels of anserine were 67, 116, 65, 211, 17, and 15,990 pmol/mg protein, respectively. 2-Oxo-homocarnosine was detected only in the brain at 0.89 pmol/mg protein, consistent with the result that homocarnosine was mainly detected in the brain (570 pmol/mg protein).

Enhanced production of 2-oxo-IDPs in a mouse model of sepsis-associated encephalopathy (SAE)

It has been reported that IDPs show an antioxidant activity through metal-chelating and free radical-scavenging mechanisms (1). Therefore, it was speculated that the presence of 2-oxo-IDPs in vivo might reflect the antioxidant activity of the IDPs. To assess the involvement of oxidative stress in the mono-oxygenation of histidine residues in the IDPs, we quantitatively analyzed the 2-oxo-IDPs in the brain of a mouse model of SAE, an acute brain dysfunction resulting from a systemic inflammatory response using LC–ESI–MS/MS coupled with stable isotope dilution methods. After lipopolysaccharide (LPS) injection, symptoms of sepsis, including exhaustion and body-weight loss, were observed (Fig. 3A). The levels of 2-thiobarbituric acid reactive substances (TBARS), a marker of oxidative stress, in the brain significantly increased 24 h after

Figure 2. Quantitative identification of IDPs and oxidized IDPs in mouse tissues. A, LC–ESI–MS/MS analysis of endogenous carnosine in the mouse skeletal muscle. Representative LC–ESI–MS/MS chromatograms of spiked isotope-labeled carnosine (upper trace), and endogenous carnosine (lower trace) are shown. B, LC–ESI–MS/MS analysis of endogenous 2-oxo-carnosine in the mouse skeletal muscle. Representative LC–ESI–MS/MS chromatograms of spiked isotope-labeled 2-oxo-carnosine (upper trace), and endogenous 2-oxo-carnosine (lower trace) are shown. C, quantitative identification of IDPs (upper graph) and oxidized IDPs (lower graph) in the mouse tissues. The IDPs and oxidized IDPs in mouse tissues were quantified using LC–ESI–MS/MS coupled with a stable isotope dilution method. The data are means ± S.D. (n = 3).

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the injection of LPS (Fig. 3B), suggesting that oxidative stress was enhanced in the brains of the SAE mouse. The brain levels of the IDPs (carnosine, anserine, and homocarnosine) were nearly unchanged under these conditions (data not shown). However, the levels of 2-oxo-carnosine were transiently elevated 8 h after injection and returned to the control levels after 24 h (Fig. 3C and D). A similar pattern was also observed for the other 2-oxo-IDPs, including 2-oxo-anserine (Fig. 3E) and 2-oxo-homocarnosine (Fig. 3F). The transient increases in 2-oxo-IDPs may relate to their turnover rates in vivo and may be associated with the low levels of 2-oxo-IDPs in tissues (Fig. 2). These data are in line with the fact that oxidative stress occurs in the early stages of sepsis (13).

Enhanced production of 2-oxo-carnosine in CARNs-overexpressed cells exposed to oxidants

To further assess the relationship between the production of the 2-oxo-IDPs and oxidative stress, we generated SH-SY5Y cells stably expressing CARNs (Fig. 4A) and examined the production of 2-oxo-carnosine following exposure of the cells to \( \text{H}_2\text{O}_2 \). In the presence of \( \text{H}_2\text{O}_2 \), the cell lysates of the SH-SY5Y cells expressing CARNs showed that the levels of carnosine were \( \approx 20 \text{ nmol/mg protein} \), which was 240-fold higher than in the control cell lysates (Fig. 4B). We then examined the effect of the CARNs overexpression on the cytotoxicity caused by \( \text{H}_2\text{O}_2 \). \( \text{H}_2\text{O}_2 \) showed a toxicity to both the control and CARNs-expressing cells; however, the cells overexpressing CARNs were more resistant to the \( \text{H}_2\text{O}_2 \) cytotoxicity than the control cells (Fig. 4C). In addition, concomitant to the reduction of the \( \text{H}_2\text{O}_2 \) cytotoxicity, an enhanced production of 2-oxo-carnosine was observed (Fig. 4D). To assess the involvement of the intracellular \( \text{H}_2\text{O}_2 \) in the enhanced production of 2-oxo-carnosine, we examined the effect of the membrane-permeable PEG-catalase. We observed that the production of 2-oxo-carnosine was completely inhibited by pretreatment with the antioxidant enzyme (Fig. 4E). A similar cytoprotective effect of the CARNs overexpression was observed in the rotenone-induced cytotoxicity (Figs. 4, F–H). These data suggest that the production of the 2-oxo derivatives may reflect the antioxidant activity of the IDPs.

Gain of antioxidant function

To establish that the monooxygenation is involved in the gain of antioxidant function of the IDPs, we examined the free radical scavenging activity of carnosine and its oxidized product (2-oxo-carnosine) using the trolox equivalent antioxidant
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Figure 4. Formation of 2-oxo-carnosine in the SH-SY5Y cells stably expressing CARNS under oxidative stress. A. Western blotting showing CARNS protein levels in the SH-SY5Y cells stably expressing CARNS and in the control cells. Expression of FLAG-tagged CARNS in the SH-SY5Y cells was analyzed using the anti-FLAG antibody. β-Actin was used as a loading control. B. Quantitative identification of carnosine in the SH-SY5Y cells stably expressing CARNS and in the control cells. Carnosine was quantified using LC–ESI–MS/MS coupled with a stable isotope dilution method. The data represent means ± S.D. (n = 3). Student’s paired t test was used for the statistical analysis. ***, p < 0.001 compared with values for the control. C. Cytotoxicity induced by H2O2 in the SH-SY5Y cells stably expressing CARNS (black) and in the control cells (gray). Cell viability was determined by the MTT assay. The data represent means ± S.D. (n = 5). Two-way ANOVA with the Bonferroni post hoc test was used for the statistical analysis. ***, p < 0.001 compared with values for control. D. Quantitative identification of endogenous 2-oxo-carnosine in the SH-SY5Y cells stably expressing CARNS treated with H2O2. 2-Oxo-carnosine was quantified using LC–ESI–MS/MS coupled with a stable isotope dilution method. The data represent means ± S.D. (n = 5). Two-way ANOVA with the Bonferroni post hoc test was used for the statistical analysis. ***, p < 0.001 compared with values for control. E. Inhibition of 2-oxo-carnosine formation by PEG-catalase. The CARNS-expressing cells were pretreated with PEG-catalase. After washing, the H2O2-induced cytotoxicity was assessed. The data represent means ± S.D. (n = 3). One-way ANOVA with the Tukey post hoc test was used for the statistical analysis. ***, p < 0.001 compared with values for 0 h sample; ***, p < 0.001 compared with values for H2O2 treatment. F. Cytotoxicity induced by rotenone in the SH-SY5Y cells stably expressing CARNS (black) and in the control cells (gray). Cell viability was determined by the MTT assay. The data represent means ± S.D. (n = 3). Two-way ANOVA with the Bonferroni post hoc test was used for statistical analysis. *, p < 0.05; ***, p < 0.001 compared with values for control. G. Quantitative identification of endogenous 2-oxo-carnosine in the SH-SY5Y cells stably expressing CARNS treated with rotenone. 2-Oxo-carnosine was quantified using LC–ESI–MS/MS coupled with a stable isotope dilution method. The data represent means ± S.D. (n = 3). One-way ANOVA with the Tukey post hoc test was used for the statistical analysis. ***, p < 0.001 compared with values for control. H. Inhibition of 2-oxo-carnosine formation by PEG-catalase. The CARNS-expressing cells were pretreated with PEG-catalase. After washing, the rotenone-induced cytotoxicity was assessed. The data represent means ± S.D. (n = 3). One-way ANOVA with the Tukey post hoc test was used for the statistical analysis. ***, p < 0.001 compared with values for control; ***, p < 0.001 compared with values for the rotenone treatment.

capacity (TEAC) assay. Carnosine showed a negligible antioxidant activity (TEAC value = 0.0088 μmol/mmol). Strikingly, however, the TEAC of 2-oxo-carnosine was far greater (35,000-fold) than that of carnosine (Fig. 5A). In addition, the oxidized carnosine showed a better free radical scavenging activity than the endogenous antioxidants, such as GSH and ascorbate. Incubation with the stable radical, 1,1-diphenyl-2-picrylhydrazyl (DPPH) resulted in the loss of 2-oxo-carnosine (Fig. 5B), which was accompanied by the formation of a product with a molecular ion at m/z of 620 ([M+H]+) (data not shown). This change in the molecular mass corresponds to a 393-Da increase, accounting for the molecular mass of DPPH, in the mass value of the unmodified carnosine. The detailed mechanism of how 2-oxo-carnosine forms the product with DPPH remains unknown. We also assessed the cytoprotective effect of 2-oxo-carnosine on the rotenone-induced neuronal cell death. In our preliminary experiments, we examined the cytoprotective effect of 2-oxo-carnosine at concentrations ranging from 0 to 400 μM and observed that 2-oxo-carnosine concentrations of as low as 50 μM showed significant effect (data not shown). As shown in Fig. 5C, the oxidized carnosine was slightly more effective than its unoxidized form.
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A mechanism for the conversion of IDPs to 2-oxo-IDPs

To gain an insight into the formation of the 2-oxo-IDPs in vivo, we characterized the mechanism for the conversion of IDPs to the 2-oxo-IDPs by Cu2+/ascorbate in vitro. As shown in Fig. 6A, both Cu2+ and ascorbate were essential for the formation of 2-oxo-carnosine. Similar patterns were observed for anserine and homocarnosine (Fig. S6). To identify the origin of oxygen in the 2-oxo-imidazole ring, we examined the incorporation of 18O from 18O2 and H218O in carnosine. As shown in Fig. 6B, 2-[18O]oxo-carnosine was detected in the reaction mixture containing 18O2, but not H218O, indicating that oxygen at the C2 position of 2-oxo-carnosine is derived from molecular oxygen. This result strongly supports the formation of an imidazole radical as the intermediate.

To detect the imidazole radical intermediate, a spin-trapping reagent, 4-amino-2,2,6,6-tetramethylpiperidinyl-1-oxy (amino-TEMPO), which converts radicals into stable diamagnetic adducts, was used for the MS-based detection of biologically relevant carbon-centered free radicals. When carnosine was incubated with Cu2+/ascorbate in the presence of amino-TEMPO, a putative amino TEMPO-carnosine adduct was detected at m/z 399 (Figs. 7, A and B). Collision-induced dissociation of the adduct produced relevant product ions at m/z 156.0 and 173.1 (Fig. 7C), which were consistent with a histidine moiety and an amino TEMPO moiety, respectively. The formation of the amino-TEMPO-carnosine adduct was accompanied by the reduced formation of 2-oxo-carnosine (Fig. 7, D and E), suggesting that the formation of imidazole radicals in the IDPs might be an intermediate step for the production of the 2-oxo derivatives. These data also support our hypothesis that the mono-oxygenation of the IDPs may be mediated through the formation of an imidazole radical followed by the addition of molecular oxygen (Fig. 7F).

Discussion

A growing body of evidence shows the protective role of IDPs in ischemia/reperfusion damage and in human diseases such as diabetes, cataract, and neurodegenerative disorders (1). However, the underlying molecular mechanism for their beneficial effects remains unclear. In the present study, we adopted a MS-based approach to analyze the IDPs in tissue samples. Taking advantage of the fact that the authentic IDPs we studied, including carnosine and anserine, commonly gave specific fragment ions at m/z 72 and 89, we comprehensively analyzed the IDPs in mouse muscle homogenates and detected two unknown peptides, showing molecular ions at m/z 243 and 257 ([M+H]+), corresponding to a 16-Da increase in the mass value of carnosine and anserine, respectively. Based on the LC–ESI–MS/MS analysis of the synthetic compounds, these derivatives were identical to 2-oxo-carnosine and 2-oxo-anserine. Thus, the comprehensive analysis of tissue IDPs unexpectedly led to the discovery of 2-oxo-IDPs in vivo. To the best of our knowledge, this is the first report demonstrating that the conversion of histidine to 2-oxo-histidine is a naturally occurring reaction in vivo.

Our study on the quantification of IDPs using LC–ESI–MS/MS coupled with a stable isotope dilution method showed that, consistent with previous findings (1), both carnosine and anserine were mainly detected in skeletal muscle tissues. They were also measurable in brain regions and other tissues as well,
but at concentrations 10–1,000-fold lower than in muscle. We also quantified 2-oxo-IDPs in the tissue samples by LC–ESI–MS/MS. This method showed limits of quantitation of $100$ fmol for the oxidized IDPs. The amount of 2-oxo-carnosine was $4.8 \text{ pmol/mg protein}$ in the muscle tissue, which was severalfold higher than in other tissues. This was not surprising in view of the fact that carnosine is the most abundant dipeptide in the skeletal muscle. Similar to 2-oxo-carnosine, 2-oxo-anserine was mainly detected in the muscle tissue. However, the level of 2-oxo-anserine in the muscle tissue was $30 \text{ pmol/mg protein}$, which was significantly higher than that of 2-oxo-carnosine. This may be explained by the fact that the 1-methylimidazole derivatives are much more sensitive to mono-oxygenation than the imidazoles (14). 2-Oxo-homocarnosine was detected only in the brain samples at $0.89 \text{ pmol/mg protein}$. Thus, the quantities of the oxidized IDPs were shown to correlate with those of the original peptides in their respective tissues.

IDPs are believed to function as an antioxidant in muscle and brain. Several studies have indeed claimed that IDPs might exert their cytoprotective effect through their antioxidant activities in neuronal cells (15, 16). However, no direct evidence that can satisfactorily explain this function has been reported. In the present study, we generated SH-SY5Y cells stably expressing CARNS and tested the effect of carnosine overproduction on neuronal cell death induced by oxidative stress. The CARNS-overexpressed SH-SY5Y neuroblastoma cells showing high levels of intracellular carnosine were resistant to cytotoxicity induced by $\text{H}_2\text{O}_2$ and rotenone. More interestingly, following its antioxidant activity, the level of 2-oxo-carnosine was significantly elevated in cells treated with pro-oxidants. Along with the finding that membrane-permeable PEG-catalase inhibited the production of 2-oxo-carnosine, these results support a generally accepted hypothesis that carnosine endogenously produced in cells functions as an antioxidant.

The discovery of 2-oxo-histidine was first reported by Uchida and Kawakishi in 1986 (12) in their attempt to identify oxidized products generated during the metal-catalyzed oxidation ($\text{O}_2$/Cu$^{2+}$/ascorbate) of histidine. They have also shown that 2-oxo-histidine further underwent oxidative degradation to generate ring-opened products such as aspartate, aspartylurea, and formylasparagine (17). Using HPLC with electrochemical detection, the formation of 2-oxo-histidine in oxidized proteins has been established in vitro (18). So far, 2-oxo-histidine has been detected in the in vitro oxidation of proteins, such as copper/zinc–superoxide dismutase (19, 20), human relaxin (21), vanadium bromoperoxidase (22), human growth hormone (23), oxidized low density lipoprotein (24), and prion protein (25). More recently, Traoré et al. (26) provided evidence for the formation of 2-oxo-histidine during the oxidative inactivation of PerR, a metal-dependent sensor of $\text{H}_2\text{O}_2$. It has been
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...proposed that the bound iron coordinates H2O2 and generates a reactive species, which then directly reacts with the nearby histidine (27). The formation of 2-oxo-histidine has been suggested to be an H2O2-sensing mechanism by which PerR uses metal-catalyzed oxidation reactions to regulate the expression of oxidative defense genes. Given the fact that IDPs are efficient metal-chelating agents, the intracellular formation of 2-oxo-IDPs may be the result of metal-catalyzed oxidation reactions involving loosely chelated metal ions. Thus, it is reasonable to speculate that the IDPs show their antioxidant activity when they bind metal ions to form IDP–metal complexes.

It has been shown that the antioxidant activity of IDPs is mediated by different mechanisms involving metal ion chelation and scavenging reactive oxygen species. However, despite the numerous findings and mechanistic insights that revealed the antioxidant functions of IDPs, the exact antioxidant mechanism remained unknown. In the current studies, we characterized the free radical scavenging activity of 2-oxo-carnosine using the TEAC assay and unexpectedly discovered that the oxidized IDP scavenged the free radicals far more efficiently than its original form (Fig. 5). More strikingly, 2-oxo-carnosine showed a better antioxidant activity than GSH and ascorbate, the two main aqueous-phase antioxidants within cells. Thus, the conversion of IDPs into their 2-oxo forms may, at least in part, explain their antioxidant activity. In addition, it can be speculated that the 2-oxo-IDPs may be converted to further oxidized products when they exert their free radical scavenging activity. We indeed detected the product with the molecular mass, corresponding to a DPPH–carnosine complex. However, we observed no oxidized products when they exert their free radical scavenging activity. We indeed detected the product with the molecular mass, corresponding to a DPPH–carnosine complex. However, we observed no oxidized products when they exert their free radical scavenging activity.

In summary, we analyzed IDPs in mouse tissue homogenates and unambiguously detected 2-oxo-IDPs. In addition, we showed that the overexpression of CARNS resulted in a significant inhibition of the H2O2 cytotoxicity and a concomitant increase in the intracellular levels of the 2-oxo-IDPs. Notably, 2-oxo-carnosine showed better antioxidant activity than endogenous antioxidants, such as GSH and ascorbate. Mechanistic studies of the metal-catalyzed oxidation of IDPs revealed that the mono-oxygenation of IDPs might be mediated through the formation of a histidyl radical intermediate, followed by the addition of molecular oxygen. The work described here is the first example of the in vivo detection of 2-oxo-histidine–containing peptides formed during the oxygenation of natural IDPs of high medicinal interest. We thus provide a novel paradigm to understand the antioxidant effects of IDPs. One could also speculate that IDPs showing a beneficial antioxidant effect may gain a new function after converting to 2-oxo derivatives. Studies into this possibility represent an attractive future area of investigation.

Experimental procedures

Materials

l-Histidine, β-alanine, γ-aminobutyric acid, p-toluenesulfonic acid (TsOH), thionyl chloride, and N,N-dimethylacetamide were purchased from Nacalai Tesque (Kyoto, Japan). 3-Methyl-l-histidine, [13C6, 15N] β-alanine, and LPS (from Escherichia coli; O26:B6) were from Sigma–Aldrich. [15N3] L-histidine, [15O] O2, and [18O] H2O were obtained from the Taiyo Nippon Sanso Corporation (Tokyo, Japan). PEGylated catalase (PEG-catalase) was prepared as previously reported (29). Rotenone was purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). Toluene and Dulbecco’s modified Eagle’s medium were purchased from Wako Pure Chemical Industry (Osaka, Japan). Fetal bovine serum was purchased from MultiSer Laboratories (Kumamoto, Japan). All other chemicals and reagents were from common suppliers and were of the highest commercially available grade.

Preparation of stable isotope-labeled IDPs

To prepare the isotope-labeled IDPs, 0.225 mmol of [13C6, 15N] β-alanine was treated with 0.45 mmol of TsOH in water (0.1 ml) for 30 min at room temperature, dried in vacuo, and then dissolved in toluene (0.4 ml). β-Alanine-TsOH was dried in vacuo again. l-Histidine–2TsOH was prepared by the above-mentioned method using 0.45 mmol of l-histidine and 0.90 mmol of TsOH. β-Alanine-TsOH was redissolved in thionyl chloride (0.6 ml) and incubated for 1 h at room temperature,
dried in vacuo, and then dissolved in toluene (0.4 ml). β-Alanine ClTsOH was dried in vacuo again. All the synthesized β-alanine ClTsOH and l-histidine2TsOH were mixed in N,N-dimethylacetamide (0.45 ml), purged with nitrogen, and incubated for 1 h at 4 °C with shaking. An equal amount of water was added to the mixture to hydrolyze the unreacted β-alanine ClTsOH. The obtained [13C3,15N] carnosine was purified by HPLC (JASCO Corporation, Tokyo, Japan) under the following conditions: a Scherzo SS-C18 column (6.0 × 100 mm; Imtakt, Kyoto, Japan) using a linear gradient of solvent A (water containing 0.1% formic acid) and solvent B (water containing 50% acetonitrile and 100 mM ammonium formate) (0% B at 0 min; 75% B at 20 min) at the flow rate of 1.0 ml/min. The elution was monitored by absorbance at 220 nm. 3-Methyl-l-histidine was used for the preparation of [13C3,15N] anserine, instead of l-histidine. γ-Aminobutyric acid and [15N3] L-histidine were used for the preparation of [13C3,15N2] homocarnosine, instead of β-alanine and l-histidine, respectively. The chemical structures of the products were characterized by LC–ESI–MS/MS and NMR analyses.

**Preparation of oxidized IDPs**

Oxidation of the imidazole ring of the IDPs was carried out by the ascorbic acid–copper ion system (12). The reaction mixtures (5 ml) containing 10 mM IDPs, 200 mM sodium phosphate buffer (pH 7.2), 200 mM ascorbate, and 2 mM CuSO4 were incubated at room temperature. Oxygen gas was bubbled into the mixture for 30 min. The oxidized IDPs were purified under the following conditions: a Scherzo SS-C18 column (6.0 × 100 mm) using a linear gradient of solvent A (water containing 0.1% formic acid) and solvent B (water containing 50% acetonitrile and 100 mM ammonium formate) (0% B at 0 min; 90% B at 20 min) at the flow rate of 1.5 ml/min. The elution was monitored by absorbance at 250 nm. The chemical structures of the products were characterized by LC–ESI–MS/MS and NMR analyses. The NMR analyses were performed using a JEOL JNM-ECZ500R (500 MHz) instrument. 2-Oxo-carnosine: 1H NMR (D2O): δH 2.26 (dt, J = 3.5 Hz, 2H), 2.66 (dd, J = 8.2 Hz, 1H), 2.80 (dd, J = 5.5 Hz, 1H), 3.06 (t, J = 6.8 Hz, 2H), 4.41 (dd, J = 4.7 Hz, 1H), 6.12 (s, 1H); 13C δ 31.34, 35.83, 39.70, 56.61, 119.01, 132.18, 159.10, 176.06, 179.17. 2-Oxo-anserine: 1H NMR (D2O): δH 2.26 (dt, J = 3.1 Hz, 2H), 2.60 (dd, J = 10 Hz, 1H), 2.70 (t, J = 6.5 Hz, 2H), 2.84 (m, 1H), 3.05 (s, 3H), 4.26 (dd, J = 4.7 Hz, 1H), 6.12 (s, 1H); 13C δ 31.92, 37.67, 38.87, 55.07, 117.95, 129.67, 155.86, 175.47, 179.10. 2-Oxo-homocarnosine: 1H NMR (D2O): δH 1.72–1.75 (m, 2H), 2.21 (dt, J = 3.5 Hz, 2H), 2.53 (dd, J = 8.2 Hz, 1H), 2.76–2.81 (m, 3H), 4.23 (dd, J = 4.7 Hz, 1H), 6.08 (s, 1H); 13C δ 24.41, 29.46, 33.86, 40.21, 55.46, 116.34, 129.51, 164.25, 175.61, 178.91.

**LC–ESI–MS/MS/MS analysis**

The LC–ESI–MS/MS analyses were carried out using the Xevo TQD triple quadrupole mass spectrometer (Waters, Milford, MA). Chromatography was carried out by an Intrada amino acid column (2.0 × 50 mm, Imtakt) using an Alliance e2695 system (Waters). A discontinuous gradient of solvent A (acetonitrile containing 0.1% formic acid) and solvent B (water containing 100 mM ammonium formate) was used as follows: 0% B at 0 min, 60% B at 0.1 min, 70% B at 5 min, 99% B at 9 min, at the flow rate of 0.3 ml/min. The mass spectrometer operated in the positive mode under the following conditions: capillary voltage at 1000 V and desolvation gas (nitrogen) at 1000 liter/h at 500 °C. The oxidized imidazole dipeptides were identified and quantified in the MRM mode. The MRM parameters are listed in Table S1.

**Animal studies**

This study was performed in accordance with the Guidelines for Animal Experimentation of Osaka Prefecture University (Osaka, Japan). All animal experiments were approved by the Animal Ethical Committee of Osaka Prefecture University. Nine-week-old male C57BL/6J mice (Kiwa Laboratory Animals Co., Ltd., Wakayama, Japan) were reared at 24 ± 1 °C and with a 12-h light/12-h dark cycle with free access to water and a standard diet for 1 week. For quantitative analysis, the mice (n = 3) were sacrificed. Blood was collected, and serum was prepared, flash frozen, and stored at −80 °C until processed. The lung, liver, heart, kidney, brain, and leg muscle were harvested, flash frozen, and stored at −80 °C until processed. For oxidative stress experiments, the mice were divided into four groups (n = 3) and were intraperitoneally injected with LPS (10 mg/Kg of body weight) or quantified by the MRM mode vehicle control (PBS). After injection for 0, 4, 8, or 24 h, the animals were weighed and sacrificed. The brains were harvested, flash frozen, and stored at −80 °C until processed.

**Preparation of mouse tissue samples**

Mouse tissues were homogenized with 10 volumes (w/v) of 80% acetonitrile in water containing 50 pmol of stable isotope-labeled standards using a Heidolph homogenizer (Heidolph, Schwabach, Germany). The homogenates were centrifuged at 18,800 × g for 20 min at 4 °C. The supernatants were collected and concentrated by a vacuum concentrator until the acetonitrile was removed. The samples were mixed with equal amounts of ethyl acetate, an aqueous layer was obtained. The aqueous layer was diluted 4-fold with 100 mM HCl and applied on an Oasis MCX cartridge (Waters) equilibrated with 100 mM HCl. The cartridge was washed with 5 column volumes of methanol, and then the sample was eluted with 3 column volumes of 0.5 M ammonia in methanol. The samples were then dried in vacuo, dissolved in 0.1 ml of 2% formic acid in water, and subjected to an LC–ESI–MS/MS.

**Preparation of SH-SY5Y stable cell line overexpressing CARNS**

The CARNS gene (NP_00159694) was amplified from human brain cDNA using primers: forward, 5′-CACCATTGCCACCATCATCATCATTTCTCTGGTCTCTCCCTGGATCCATCGGGTCCCCG-3′; and reverse, 5′-CTATTTTGAAGTGAGACAGGAAGTGGCCG-3′. The amplified CARNS gene was cloned into the pENTR/d-TOPO vector using the directional TOPO cloning system (Gateway Cloning Technology, Thermo Fisher Scientific). The CARNS gene was subcloned into the pcDNA3.2/nFLAG-DEST expression plasmid by the LR reaction (Thermo Fisher Scientific). The SH-SY5Y cells were cultured at 37 °C in Dulbecco’s modified Eagle’s medium (Wako Pure Chemical Industries, Osaka, Japan) supplemented with the addition of 10% fetal bovine serum.
2-Oxo-IDPs are functional oxidation products

10% fetal bovine serum. The cells were transfected with CARNs/pC DNA3.2/nFLAG-DEST using polyethylenimine Max (Polysciences, Inc., Warrington, PA). Thereafter, the cells were cultured in the medium containing 400 μg/ml G418. Four weeks after transfection, the surviving clones were isolated and grown on a large scale. The expression of the FLAG-tagged CARNs was analyzed by Western blotting using the anti-FLAG antibody (Sigma–Aldrich). Stable cell lines with the overexpression of CARNs were selected and maintained in the medium containing 400 μg/ml G418.

Cell treatment

The SH-SY5Y cells stably expressing CARNs and the control cells were plated at a density of 2.0 × 10⁴ cells/well in 96-well plates for the MTT assay and at 4.0 × 10⁶ cells/dish in a 100-mm dish for the LC–ESI–MS/MS analysis. To investigate the antioxidant capacity of carnosine, the cells were treated with different concentration of H₂O₂ or rotenone for 24 h. The antioxidant capacity of carnosine, the cells were treated with different concentration of H₂O₂ or rotenone for 24 h. The antioxidant capacity was monitored according to the absorbance at 553 nm. Malondialdehyde bis(dimethylacetal) standards. After centrifugation at 18,800 g, 5 min, and the fluorescence intensities were determined by using the MTT method (30).

To analyze the formation of 2-oxo-carnosine, the cells were treated with 150 μM H₂O₂ or 2.5 μM rotenone for the various time periods. To examine the effects of ROS on the formation of 2-oxo-carnosine, the cells were pretreated with or without 200 units/ml PEG-catalase for 1 h. After washing with PBS five times, the cells were then treated with 150 μM H₂O₂ or 2.5 μM rotenone for 2 h. The cells were washed twice with PBS and collected by using a cell scraper in 1 ml of 80% acetonitrile in water containing stable isotope-labeled standards. After centrifugation at 18,800 × g at 4 °C for 20 min, the supernatants were collected, dried, dissolved in 100 mM HCl, and applied on an Oasis MCX cartridge (Waters). The samples were eluted and subjected to LC–ESI–MS/MS by the above-mentioned method.

TBARS

The amount of the TBARS was determined according to the method described by Masaki et al. (31). The brains (~10 mg) were homogenized in 0.4 ml of PBS containing 1% Triton X-100. After centrifugation at 18,800 × g at 4 °C for 20 min, 50 μl of the supernatants were mixed with 0.35 ml of PBS containing 1% Triton X-100 and 0.8 ml of 0.375% 2-thiobarbituric acid, 15% TCA, 2% ethanol, 250 mM HCl, and 0.4% butylhydroxytoluene, then boiled for 15 min. After cooling, the samples were centrifuged (18,800 × g, 5 min), and the fluorescence intensities were analyzed by a fluorescence detector (excitation at 515 nm and emission at 553 nm). Malondialdehyde bis(dimethylacetal) was used as the standard.

Measurement of antioxidant activity

The scavenging effect of 2-oxo-carnosine on a DPPH radical was monitored as previously described (32). Briefly, the reaction mixtures, containing a micromolar range of carnosine, 2-oxo-carnosine, GSH, or ascorbate, were incubated with 100 μM DPPH (Alfa Aesar, Tewksbury, MA) in 12 mM sodium phosphate buffer (pH 7.4), containing 40% ethanol for 30 min at room temperature. The absorbance at 540 nm was measured by a model 680 plate reader (Bio-Rad). Trolox (Merck, Darmstadt, Germany) was used as the standard. The radical scavenging activities were evaluated by μmol of trolox equivalent per mmol of the sample. TEAC was calculated by the equation according to the scavenging percentage of the sample solution to the DPPH radical solution. Consumption of 2-oxo-carnosine and the formation of products were monitored by LC–ESI–MS/MS.

Cytotoxicity

SH-SY5Y cells were plated at a density of 1.0 × 10⁴ cells/well in 96-well plates for the MTT assay. To demonstrate the cytoprotective effect of 2-oxo-carnosine against oxidative stress, the cells were pretreated with 50 μM carnosine or 2-oxo-carnosine for 3 h, and then the cells were treated with 2 μM rotenone for 24 h. The cell viability was determined using the MTT method.

Statistical analysis

All experiments were performed at least three times. The values for the individual experiments are presented as the means ± S.D. Statistical significance was determined by the one-way ANOVA, two-way ANOVA, or Student’s paired t test using GraphPad Prism software. p < 0.05 was considered significant.

References

1. Boldyrev, A. A., Aldini, G., and Derave, W. (2013) Physiology and pathophysiology of carnosine. Physiol. Rev. 93, 1803–1845 CrossRef Medline
2. Drozak, J., Veiga-da-Cunha, M., Vertommen, D., Stroobant, V., and Van Schaftingen, E. (2010) Molecular identification of carnosine synthase as ATP-grasp domain-containing protein 1 (ATPGD1). J. Biol. Chem. 285, 9346–9356 CrossRef Medline
3. Drozak, J., Piecucki, M., Poleszak, O., Kozlowski, P., Chrobok, L., Baede, H. J., and de Heer, E. (2015) UPF0586 protein C9orf41 homolog is anserine-producing methyltransferase. J. Biol. Chem. 290, 17190–17205 CrossRef Medline
4. Lenney, J. F. (1990) Separation and characterization of two carnosine-splitting cytosolic dipeptidases from hog kidney (carnosinase and non-specific dipeptidase). Biol. Chem. Hoppe-Seyler 371, 433–440 CrossRef Medline
5. Teufel, M., Saudek, V., Ledig, J.-P., Bernhardt, A., Boulard, S., Carreau, A., Cairns, N. J., Carter, C., Cowley, D. J., Duverger, D., Ganzhorn, A. J., Guenet, C., Heintzelmann, B., Laucher, V., Sauvage, C., et al. (2003) Sequence identification and characterization of human carnosinase and a closely related non-specific dipeptidase. J. Biol. Chem. 278, 6521–6531 CrossRef Medline
6. Davey, C. L. (1960) The significance of carnosine and anserine in striated skeletal muscle. Arch. Biochem. Biophys. 89, 303–308 CrossRef Medline
7. González-Estrada, M. T., and Freeman, W. J. (1980) Effects of carnosine on olfactory bulb EEG, evoked potentials and DC potentials. Brain Res. 202, 373–386 CrossRef Medline
8. Ikeda, T., Kimura, K., Hama, T., and Tamaki, N. (1980) Activation of rabbit muscle fructose 1,6-bisphosphatase by histidine and carnosine. J. Biochem. 87, 179–185 CrossRef Medline
9. Stadtmann, E. R. (1992) Protein oxidation and aging. Science 257, 1220–1224 CrossRef Medline
10. Stadtmann, E. R., and Levine, R. L. (2003) Free radical-mediated oxidation of free amino acids and amino acid residues in proteins. Amino Acids 25, 207–218 CrossRef Medline
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