Article

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The Effect of Serum Carnosinase on the Tissue Distribution of Imidazole Dipeptides After their Oral Administration in Golden Hamsters

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Abstract

Background/Objectives: Imidazole dipeptides (IDPs), carnosine and anserine, are endogenous antioxidants. The metabolism and functions of IDPs have mainly been investigated in rodents. However, the blood of primates, such as humans, contains carnosinase (CN1), which hydrolyzes IDPs. In non-primates, CN1 is absent, allowing IDPs to be distributed throughout tissues. There are concerns about whether the results of animal experiments can be directly applied to humans. Therefore, we aimed to investigate the blood kinetics and tissue distribution of IDPs following their oral administration to golden hamsters, the only non-primates known to possess CN1. **Methods**: Plasma CN1 activity was compared between hamsters and humans. Hamsters were administered IDPs (an anserine/carnosine mixture) purified from chicken meat at a dose of 1,000 mg/kg. Blood samples were collected at time points up to 6 h after administration. Tissue samples were collected at 6 h after administration to measure the concentrations of IDPs and related substances. Additionally, IDP levels in human and mice tissues from previous studies were compared with that of hamster tissues in this study. **Results**: Hamster plasma CN1 activity was more than 10 times higher than that in humans. Although IDPs were not detected in IDP-treated hamster plasma, constituent amino acids of IDPs increased up to 1–2 h and Nπ-methyl-histidine (m-His) remained at high levels up to 6 h after administration. IDP levels in control tissues (vehicle) were similar to those in human tissues. In the IDP group, tissue IDPs were higher than those in the vehicle and m-His increased in all tissues. **Conclusions**: This study suggests that IDPs and m-His levels increase in human tissues following a single oral administration of IDPs, and that m-His may serve as a substitute for IDPs.

Keywords: carnosinase; imidazole-dipeptides; anserine; carnosine; Nπ-methyl-histidine; golden hamster

1. Introduction

Imidazole dipeptides (IDPs) are endogenous antioxidants synthesized from β-alanine (b-Ala) and L-histidine (His), that is, their synthesis and degradation occur in vivo [1,2]. Carnosine (β-alanyl-L-histidine, Car) is first synthesized by carnosine synthase (Carns-1) and then methylated by carnosine methyltransferase to form anserine (β-alanyl-Nπ-methyl-L-histidine, Ans) [3]. IDPs are abundant in the skeletal muscles of vertebrates, but the types of IDPs present in skeletal muscles differ depending on the species [4]. In humans, Car is predominant. Ans is predominant in mice, rats, dogs, cats, (Ans-to-Car ratio of 10:2 to 3) and chickens (Ans-to-Car ratio of 3:1). In fish, such as tuna and bonito, Ans is predominant, whereas Car is not detected in salmon. However, with improvements in the accuracy of IDP analyses, IDPs other than Car have been detected in trace amounts in human tissue [5].

IDPs exhibit pH-buffering [6], metal-chelating [7], and antioxidant activities [8] that inhibit advanced glycation end product formation [9], prevent neurodegenerative diseases [10], protect

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tissues from diabetic nephropathy [11], and enhance cardiac function [12]. Therefore, IDPs are considered beneficial substances potent activity for preventing age-related disorders and maintaining overall health. However, many of these studies used animals lacking serum carnosinase (CN1) as test subjects. Therefore, it remained unclear whether the effects of IDPs observed in animal experiments could be replicated in humans, where IDPs are degraded in the blood by CN1. This is because, while oral administration of Ans or Car to mice has been observed to increase levels of Ans and Car respectively in both blood and major tissues [13], in humans, only trace amounts of Ans are detected in the blood, and Car is not detected at all [14].

Despite the above, administration of chicken extract-derived IDPs (Ans/Car = 3/1) reduces oxidative stress in healthy individuals [15]. Additionally, chronic supplementation with the Ans-Car complex derived from chicken extract and Ans derived from salmon extract improves cognitive decline in the elderly and reduces the risk of developing Alzheimer's disease [16]. These findings suggest that IDPs exert health benefits, particularly as antioxidants, even after being degraded in the blood. IDPs may undergo repeated synthesis and degradation by Carns-1 and carnosinase.

In the blood of primates, such as humans, a carnosine-degrading enzyme called carnosinase 1 (CN1) is always present [17]. However, CN1 is lacking in the blood of other mammals, which only contains carnosinase 2 (CN2), a cytosolic nonspecific dipeptidase, in their tissues. Therefore, in human blood, orally ingested Car is rapidly degraded into b-Ala and His, which are then transported and distributed to various tissues via peripheral circulation. It is hypothesized that Car is resynthesized by Carns-1 in the skeletal muscle, brain, and other tissues, where it exerts various biological functions [4,18,19].

Ans is a methylated form of Car and has a lower affinity for CN1 than for Car, resulting in slower degradation in the human blood following oral administration [20,21]. However, the distribution of Ans in the human body after oral administration remains unclear. Additionally, there is limited knowledge regarding the metabolism of Ans in experimental animals. Therefore, whether the effects of IDPs observed in animal experiments can be replicated in humans in unclear. To the best of our knowledge, no previous studies have used hamsters as model animals for IDP research. In this study, we orally administered IDPs to golden hamsters (*Mesocricetus auratus*), a non-primate mammal that uniquely possesses CN1 in their blood, to investigate the blood levels and distribution of IDPs and related substances in major tissues. This study can help improve the understanding the physiological impact of IDPs in humans, given their diverse biological functions.

2. Materials and Methods

2.1. Reagents

L-carnosine, L-anserine nitrate, and 3-methyl-L-histidine (Nπ-methyl-L-histidine, m-His) were purchased from Sigma-Aldrich Japan (Tokyo, Japan). The b-Ala, L-histidine (His), trichloroacetic acid, sodium 1-heptanesulfonate, acetonitrile (HPLC grade), and other reagents were purchased from Fujifilm Wako Pure Chemical Corporation (Osaka, Japan). The purified IDP derived from chicken meat was AC-10(IK)LF (Lot No. 22061301; Tokai Bussan Co. Ltd., Tokyo, Japan), which is a commercially available ingredient in processed foods. It was purified from chicken meat using ion-exchange chromatography and nanofiltration membrane treatment [22], and had a solid Ans/Car content of 91.0%, with a weight ratio of Ans:Car of 2.74:1 (Ans:Car molar ratio of 2.58:1).

2.2. Human Plasma

Human plasma samples were prepared from the blood of four healthy middle-aged male volunteers (46.5 ± 4.1 years old) who provided written informed consent. The volunteers fasted overnight and blood was drawn into heparin sodium-containing blood collection tubes. The obtained blood was centrifuged (3,000 ×*g*, 4°C, 10 min) to collect plasma, which was then stored at -80°C until CN1 activity was measured.

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2.3. Administration of IDPs to Hamsters and Collection of Blood and Tissue Samples

Eight-week old male hamsters (Slc: Syrian) were purchased from Japan SLC Co. Ltd. (Shizuoka, Japan) and that had been acclimatized for 1 week. They were individually housed in plastic cages (W 18.2 × D 26.0 × H 12.8 cm) and provided with solid feed (Labo MR Stock, Nippon Nogyo Kogyo Co. Ltd., Tokyo, Japan) and tap water ad libitum. The hamsters were randomly divided into two groups of four, fasted for approximately 16 h, and administered IDPs dissolved in water for injections at a dose of 1,000 mg/kg body weight (Ans, 733 mg; Car, 267 mg) via forced oral administration (IDP group). The vehicle group was administered water for injections.

Blood samples were collected sequentially from the jugular vein under isoflurane inhalation anesthesia at time points before IDPs administration, and 30 min, 1 h, and 2 h after administration. After 6 h of administration, the abdomen was opened under isoflurane inhalation anesthesia, and blood was collected from the posterior vena cava. Blood was collected into heparin sodium-containing blood collection tubes. The obtained blood samples were centrifuged at 13,600×*g* for 1 minute at 4°C to recover plasma. The recovered plasma was stored at -80°C until use. Following blood collection, systemic perfusion was performed using heparin-containing physiological saline solution. The brain, soleus muscle, kidneys, lungs, and liver were harvested and weighed. The tissues were frozen in liquid nitrogen and stored at -80°C.

2.4. Measurement of CN1 Activity

CN1 activity was measured by performing an enzymatic hydrolysis reaction in a 1.5-mL microtube according to the method of Teufel et al. [23], and the residual substrate concentration after the reaction was determined using the HPLC method described below. The reaction mixture (100 µL) contained heparinized human plasma sample (10 µL), 50 mM Tris-HCl buffer (pH 7.5), and 1 mM substrate (Car or Ans). The tubes were pre-warmed at 30°C in a water bath for 5 min prior to substrate addition. The reaction was initiated by adding the substrate, followed by incubation at 30°C for 60 min. The reaction was terminated by adding 50 µL of 1% (w/v) trichloroacetic acid solution to the reaction mixture. For control samples, trichloroacetic acid solution was added before substrate addition. After termination, the mixture was centrifuged at 10,000 × *g* at 4°C for 15 min, and the supernatant was filtered through a disposable filter (pore size 0.2 µm) to obtain the test solution for HPLC. Based on preliminary tests indicating that hamster plasma has higher CN1 activity than that of human plasma, 5 µL of the hamster plasma sample added to the reaction solution and the reaction time was shortened to 5 min.

To calculate the CN1 activity in the sample, the following equation was used:

$$\text{CN1 activity} = (\mu mol_{CTR} - \mu mol_{HYD}) / (V \times t)$$

Here, CN1 activity is the plasma hydrolysis rate measured in micro-moles [µmol/(mL x h)] of substrate hydrolyzed per 1 mL of serum over 1 h, μmol_{CTR} is the substrate concentration determined in the control sample, μmol_{HYD} is the residual substrate concentration determined in the hydrolysis sample, V is the plasma volume (mL), and t is the reaction time (h).

2.5. Quantification of IDPs-Related Compounds

Plasma was obtained by mixing with a cooled trichloroacetic acid solution and centrifuging (10,000 x g, 15 min, 4°C) to obtain the supernatant. Each tissue sample was weighed, mixed with a cooled 5% trichloroacetic acid solution, homogenized, and centrifuged as described above to obtain the supernatant. These supernatants were filtered through a disposable filter (pore size 0.2 µm) and used as test solutions. The content of the constituent amino acids of IDP (b-Ala, His, and m-His) was measured using a Hitachi high-speed amino acid analyzer L-8900 (Hitachi High-Tech Corporation, Tokyo, Japan). Ans and Car contents were measured using the modified reverse-phase ion-pair HPLC-ultraviolet detection method described by Kumagai et al. [24]. The column used was TSK gel ODS-80Ts (150 × 4.6 mm; particle size, 5 µm; Tosoh Corporation, Tokyo, Japan). The HPLC system

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used was a LC-2030D (Shimadzu, Kyoto, Japan), with a flow rate of 1.0 mL/min, wavelength of 220 nm, and sample injection volume of 10 μL. Buffer A consisted of 50 mM potassium dihydrogen phosphate and 6 mM sodium 1-heptanesulfonate (pH 3.4), whereas buffer B consisted of acetonitrile. The gradient conditions were as follows: 0–4 min: B 0%, 4–12 min: B 4%, 12–17 min: B 40%, and 17–32 min: B 0%. Using this analytical method, it was confirmed that the peaks of Ans and Car did not overlap with those of balenine and homocarnosine.

2.6. Protein Degradation Assay for Evaluating Antioxidant Activity Against Reactive Oxygen Species

The protein degradation inhibition rate of antioxidants against three types of reactive oxygen species was measured according to the method described by Yanai et al. [25]. A solution of the target protein (egg white albumin), 175 μL) was placed in a 1-mL centrifuge tube, and 25 μL of each antioxidant solution (final concentration 5 mM) was added and mixed. The mixture was left to stand at room temperature for 30 min, then 25 μL of each reactive oxygen species solution (final concentration, 5.0 mM) was added. Hypochlorous acid (HClO) was incubated at 37°C for 30 min, hydroxyl radical (HO·) for 60 min, and peroxynitrite radical (ONOO·) for 120 min. Reaction solution (10 μL) was mixed with an equal volume of a 20% 2-mercaptoethanol and glycerol-containing polyacrylamide gel electrophoresis sample solution (two-fold concentrated) to terminate the reaction. These samples were loaded onto a 10–20% concentration gradient SDS-polyacrylamide gel (First Chemical, Tokyo, Japan) and electrophoresed at 40 mA for 50 min. After electrophoresis, the gel was stained with Coomassie Brilliant Blue R250 solution (Sigma-Aldrich). The band intensity of ovalbumin was quantified using the image processing software Image J (NHI) and the inhibition rate of protein degradation for each test substance was calculated using the following formula:

$$\text{Inhibition rate of protein degradation (\%)} = (A - B) / (C - B)$$

where A is the band intensity of the protein treated with the test substance, B is the band intensity of the protein without the test substance, and C is the band intensity of the untreated protein.

2.7. Statistical Analysis

Data are presented as mean ± standard error (SE), except where otherwise indicated. Differences between groups were evaluated using the free statistical software EZR [26] with the Student's t-test or one-way analysis of variance (ANOVA) and Tukey's multiple comparison test. All tests were considered statistically significant at $p < 0.05$.

3. Results

3.1. CN1 Activity in Human and Hamster Plasma

To evaluate CN1 activity in hamster plasma, they were measured and compared with CN1 activity in human plasma (four healthy males, mean age 46.5 ± 4.1 years). CN1 activity in human plasma was 2.73 ± 0.54 μmol/mL/h for Car and 1.08 ± 0.20 μmol/mL/h for Ans. In contrast, CN1 activity in hamster plasma was approximately 20 times higher for Car and approximately 12 times higher for Ans (53.1 ± 1.8 μmol/mL/h and 12.9 ± 0.4 μmol/mL/h, respectively) compared with those in human plasma (Table 1). Additionally, a positive correlation was observed between the activity of CN1 on each substrate in both human and hamster plasma samples (human: $R^2 = 0.969$, hamster: $R^2 = 0.975$).

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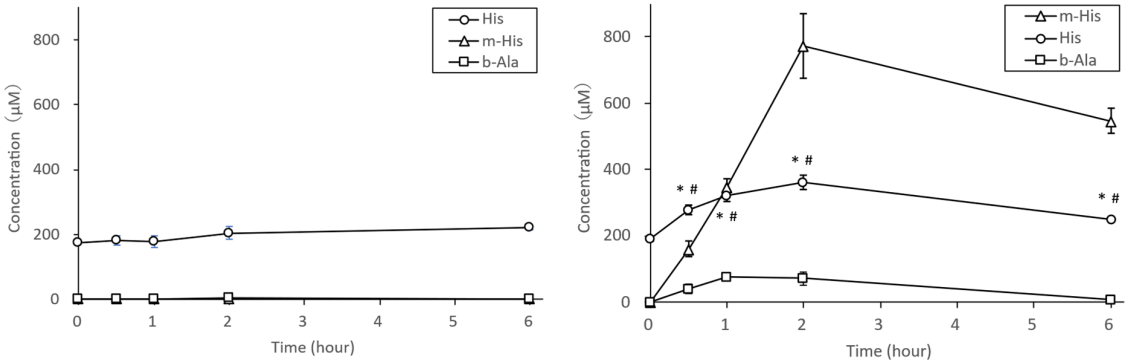
Table 1. CN1 activity in human and hamster plasma.

Substrate	Degradation activity (μmol/mL/h) [1]		*P* value (Hamster vs. Human)	Activity ratio (Hamster/Human)
	Human	Hamster		
Carnosine	2.73 ± 0.54	53.1 ± 1.8	< 0.001	19.5
Anserine	1.08 ± 0.20	12.9 ± 0.4	< 0.001	11.9

[1] The average values ± standard error of plasma CN1 activity were obtained from four male subjects (aged 46.5 ± 4.1 years) and four male hamsters (8 weeks old).

3.2. Changes in the Concentration of Ans-Car-Related Compounds in Hamster Plasma after IDP Administration

After administering IDPs at a dose of 1,000 mg/kg body weight via forced oral administration to hamsters, we investigated the plasma concentration profiles of IDP-related compounds for up to 6 h after administration. In the vehicle group, neither Ans, Car, m-His, nor b-Ala were detected in the plasma; only His was detected (Figure 1a). In contrast, in the plasma of the IDP group, Ans and Car were not detected at 6 h after administration, but b-Ala, His, and m-His, which are the constituent amino acids of Car and Ans, were detected 30 min after administration (Figure 1b). The time to reach peak plasma concentration (T_{max}) was 1 h for-Ala, and 2 h for both His and m-His. The maximum plasma concentration (C_{max}) was 773.3 ± 21.1 μM for m-His, which was significantly higher than those of b-Ala and His (77.5 ± 10.0 and 361.2 ± 21.1 μM, respectively), and the plasma increase rate was notably high. Furthermore, m-His remained at high concentrations up to 6 h after IDP administration (545.5 ± 37.9 μM, approximately 70% of C_{max}), and the half-life of plasma concentration ($T_{1/2}$) was estimated to be approximately 9 h.



(**a**) (**b**)

Figure 1. Concentration changes of IDP-related compounds in the hamster plasma after administration of water for injection (**a**) or IDPs 1,000 mg/kg body weight (**b**) (μM, n=4). Circles, His; triangles, m-His; squares, b-Ala. * Significant difference from 0 time ($p<0.01$), # Significant difference from the vehicle group ($p<0.01$). b-ala, β-alanine; His, L-histidine; IDP, imidazole dipeptide; m-His, Nπ-methyl-histidine.

3.3. Contents of Ans-Car-Related Compounds in Hamster Tissues 6 h after IDP Administration

As described above, neither Ans nor Car were detected in the hamster plasma up to 6 h after IDPs administration, and only these constituent amino acids were detected (Figure 1b). Hamster tissues were examined for IDP levels. IDPs were constantly present in the soleus muscle and heart, but Car was dominant, with a concentration approximately 17–60 times higher than that of Ans (Figure 2). Additionally, b-Ala and His, the constituent amino acids of IDPs, were present in all tissues. However, m-His, the constituent of Ans, was not detected, except in the soleus muscle; it was detected at significantly higher concentrations in all tissues 6 h after IDP administration (Figure 2). It was particularly high in the soleus muscle and kidneys, followed by the liver, brain, heart, and lungs in descending order. The levels of constituent amino acids of Car, b-Ala and His slightly increased in

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all tissues, compared with that of m-His. Notably, in the soleus muscle and heart, where Ans and Car are typically present, increases in Ans and Car levels were observed; in particular, Car levels significantly increased in the heart ($p < 0.05$). Although at trace levels, significant increases in Ans and Car were observed in the kidneys (both $p < 0.01$), whereas no increases in IDP levels were detected in the brain or lungs. Other IDP constituent amino acids, b-Ala and His, increased in all tissues following IDP administration, with the most pronounced increases observed in the lungs ($p < 0.01$ and $p < 0.05$, respectively).

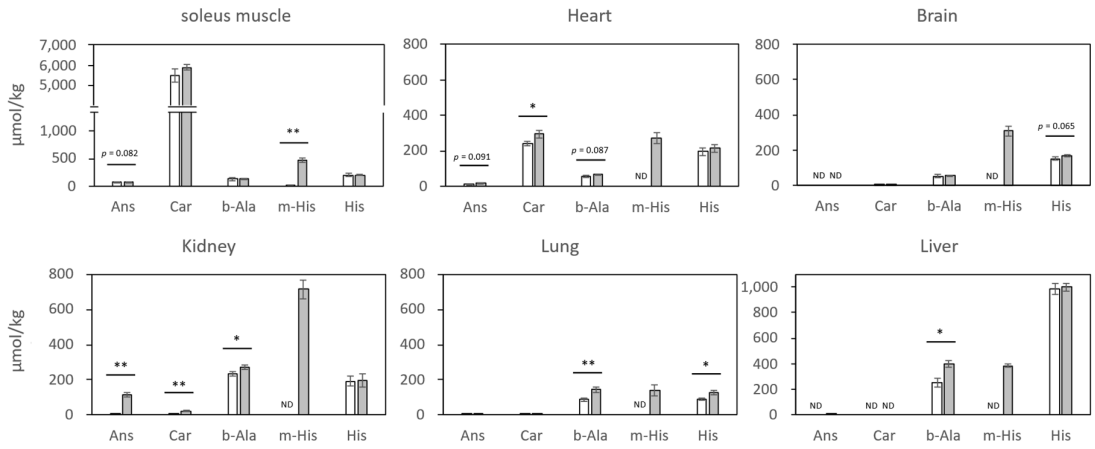

Figure 2. Concentration of IDP-related compounds in hamster tissues 6 h after IDP administration (unit: µmol/kg, n=4 in each group). White bars: control group; gray bars: IDP-administered group. Significant differences from the control group by T-test: * $p < 0.05$; ** $p < 0.01$; ND, not detected. Ans, anserine; b-ala, β-alanine; Car, carnosine; His, L-histidine; IDP, imidazole dipeptide; m-His, Nπ-methyl-histidine.

m-His, a constituent amino acid of Ans, was detected only in trace amounts in the soleus muscle but was below the detection limit in other tissues of the control group. However, following IDP administration, the m-His concentration in each tissue significantly increased, which was attributed to the distribution of m-His generated in the plasma in each tissue. Therefore, we summarized the tissue transfer rate of m-His based on the concentration ratios of m-His in each tissue and plasma sample 6 h after IDP administration (Figure 3). In the kidneys, m-His was not detectable before IDP administration (control group); however, 6 h later, it was 1.33 times that in the plasma, indicating the accumulation of m-His. Subsequently, the tissue-to-plasma concentration ratio in the soleus muscle was 0.85, while that in the liver, heart, and brain were 0.55–0.75, which was comparable that in the soleus muscle. The tissue-to-plasma concentration ratio in the lung was only 0.3.

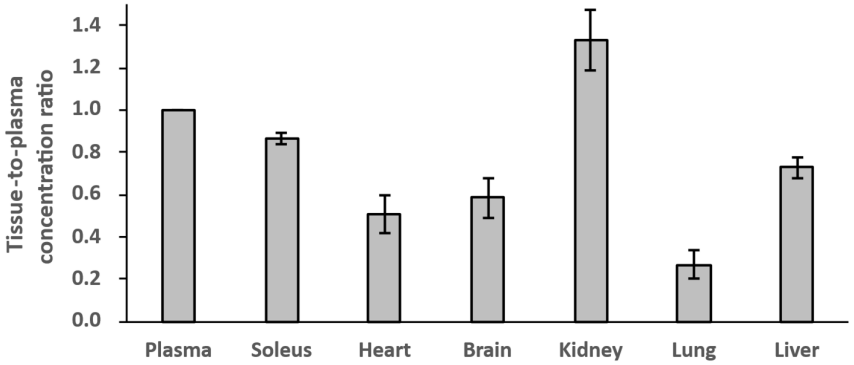

Figure 3. The tissue-to-plasma ratio of m-His 6 h after IDP administration (units: %, n=4). The plasma concentration of m-His 6 h after IDPs administration was 545 ± 38 µM. IDP, imidazole dipeptide; m-His, Nπ-methyl-histidine.

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3.4. Antioxidant Activity of Ans-Car-Related Compounds Against Hypochlorous Radicals

Specific antioxidants have previously been found to target particular reactive oxygen species. For example, IDPs (Car and Ans) neutralize hypochlorite, while ascorbic acid targets peroxynitrite and ferulic acid scavenges hydroxyl radicals [25]. Therefore, we compared the antioxidant activities of Ans, Car, and their constituent amino acids against each of these three types of reactive oxygen species (Table 2). The protein degradation inhibition rates against hypochlorous acid were in the order Car > Ans > m-His > His > b-Ala, confirming that the antioxidant activity of m-His against hypochlorous acid was comparable to that of Ans. In contrast, the protein degradation inhibition rates of m-His against both peroxynitrite radicals and hydroxyl radicals were low, similar to other Ans-Car-related compounds.

Table 2. Inhibitory effects of Ans-Car-related compounds on protein degradation by hypochlorous acid, hydroxyl radicals, and peroxy nitric acid radicals (n = 2, mean).

Test substance	Protein degradation inhibition rate (%)		
	HClO	HO・	ONOO・
Car	58	5.2	1.1
Ans	49	5.0	0
m-His	47	2.2	0
His	39	4.4	0
b-Ala	38	0	0

Ans, anserine; b-ala, β-alanine; Car, carnosine; His, L-histidine; IDP, imidazole dipeptide; m-His, Nπ-methyl-histidine; HClO, hypochlorous acid; HO·, hydroxyl radical; ONOO·, peroxynitrite radical.

4. Discussion

Experiments involving the oral administration of IDPs to animals have been conducted to investigate their physiological functions in vivo. However, the IDP-degrading enzyme CN1 is present in the blood of primates such as humans, causing IDPs to be rapidly degraded into constituent amino acids during absorption from the intestinal tract and entry into the blood. In contrast, CN1 is absent from the blood of non-primate mammals, meaning that administered IDPs are distributed throughout the body without degradation [13]. Therefore, in the investigation of the in vivo functions of IDPs by oral administration, it was concerned that the findings from animal experiments would be directly extrapolated to humans.

Among non-primate mammals, only the golden hamster has CN1 activity in its blood, which is stronger than that found in humans [17]. In our study, CN1 activity in hamster plasma was approximately 20 times higher for Car and around 12 times higher for Ans than in healthy humans (Table 1). As these results reflect the effect of CN1 in the blood, hamsters are considered the most appropriate model for functional studies involving the oral administration of IDPs and extrapolation to humans. Previously, no studies using hamsters as model animals for IDP research have been reported. For this reason, we orally administered IDPs to hamsters and investigated their distribution in the blood and other major tissues.

Previous studies have reported IDP levels in mouse and human tissues. Therefore, we measured the IDP levels in hamster tissues without administering IDPs and compared them with the previously reported values in mice and humans (Figure 4).

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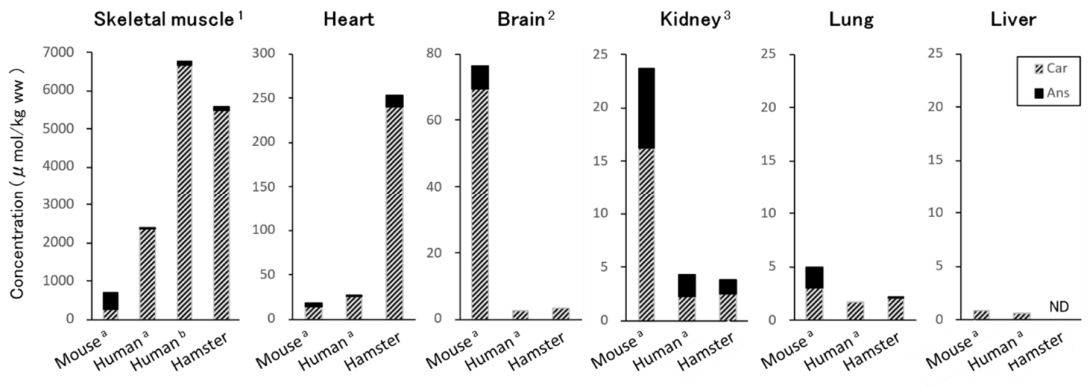

Figure 4. Comparison of anserine and carnosine content (μmol/kg wet weight) in hamster tissue with values reported in the literature for mice and humans. Shaded bars: carnosine; black bars: anserine. [a] Values reported by Van der Stede et al. [5] (reprinted with permission from John Wiley and Sons). [b] Values reported by Hoetker et al. [27]. [1] Mice and hamsters: soleus muscle; humans: lateral vastus muscle. [2] Mice and humans: cerebral cortex (white matter); hamsters: entire brain. [3] Mice and hamsters: entire kidney; humans: kidney (medulla). ND, not detected; Ans, anserine; Car, carnosine.

According to Van der Stede et al. [5] and Hoetker et al. [27], although IDP levels are lower in mice than in humans, the Ans:Car ratio in mouse skeletal muscles significantly differs from that in humans. In the mouse soleus muscle, Ans is dominant and the Ans:Car ratio is 1.87/1 [5], whereas in the human lateral vastus muscle, Car is dominant and the Ans:Car ratio is 1/68.5 [5] or 1/60.2 [27]. In contrast, Car is dominant in the soleus muscle of hamsters, with an Ans:Car ratio of 1/65.4, similar to that in humans. Furthermore, the total IDP content in the soleus muscle of hamsters is 5,587 ± 349μmol/kg ww and is comparable to the human lateral vastus muscle (6,791 ± 206 μmol/kg ww) [27].

In hamster hearts, the Ans:Car ratio (1/17.4) is closer to that in humans (1/21.0) [5] rather than mice (1/2.8) [5]; however, the total IDP content (253.6 ± 13.2 μmol/kg ww) is approximately 10 times higher than those of mice and humans (18.4 ± 8.6 μmol/kg ww and 27.7 ± 22.5μmol/kg ww, respectively [5]). In hamster brains, only Car was detected at 3.26 ± 1.44 μmol/kg ww, which was nearly identical to the levels in the human cerebral cortex (2.56 ± 1.77 μmol/kg ww [5]. In hamster kidneys and lungs, both the total IDP content and Ans:Car concentration ratio were closer to those in humans than to those in mice. In the liver, although Car was reported to be present only in humans and mice, neither Ans nor Car was detected in hamsters.

Notably, the ratio and total content of IDPs in hamster tissues were more similar to those in humans than those in mice (Figure 4). In this study, we did not investigate the expression levels of *CARNS1* in hamster tissues; however, it its expression levels in humans, mice, and rats are proportional to the tissue IDP content at the systemic level [5]. Based on these findings, despite differences in the intensity of CN1 activity in the plasma, both the expression levels of *CARNS1* and tissue IDPs in various tissues may be similar between hamsters and humans.

Following IDP administration, only b-Ala, m-His, and His were detected in the plasma of hamsters. Furthermore, m-His remained at high concentrations in the plasma even 6 h after IDP administration (Figure 1; $T_{1/2}$ was approximately 9 h), and its concentrations in various tissues were found to be comparable to those in the plasma (Figure 3). We previously investigated the plasma concentration profile of an IDPs derived from chicken extract (1,000 mg, Ans:Car = 3:1) administered orally in a single dose to healthy middle-aged human males. Ans was detected in small amounts in plasma (C_{max} of Ans was approximately 1/14 of m-His), the increase in m-His concentration was significant, and the $T_{1/2}$ of m-His was estimated to be 6.5 h (Figure 5, [14]). Similarly, the $T_{1/2}$ of m-His in human blood after a single oral administration of Ans was 7.3 h [28] and $T_{1/2}$ of m-His in human plasma after chicken meat consumption was 5.9 h, indicating that m-His is a potential biomarker for chicken meat consumption [29]. In contrast, the b-Ala is likely to be preferentially used in other

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metabolic pathways, such as transamination or energy supply [30]. Additionally, His is not only a precursor for Car but essential for protein synthesis, and its surplus is converted into other substances, such as histamine, and catabolized [31].

The urinary excretion of Ans following the consumption of chicken meat was 6.3% of the ingested Ans amount. In contrast, the urinary excretion of m-His was 80% of the ingested Ans amount [32]. Together, these findings suggest that in humans, where CN1 activity is lower than that in hamsters, the degradation of Ans is delayed after IDPs intake. However, the high concentration and prolonged retention of m-His compared to those of Ans, b-Ala, and His is a common phenomenon in hamsters and humans.

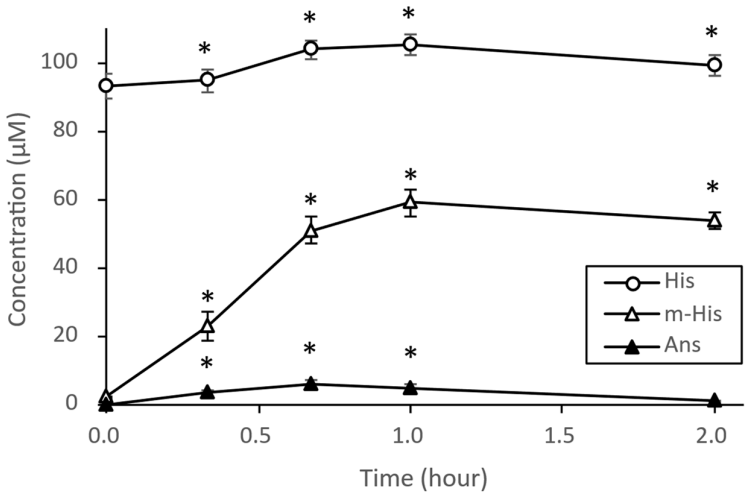

Figure 5. Changes in plasma concentrations of IDP-related compounds in healthy middle-aged men after oral administration of 1,000 mg of IDPs (μM, n = 10). Modified from the report by Shiotani et al. [14]. White circles: His, white triangles: m-His, filled triangles: Ans.* Significant difference from 0 time ($p<0.01$). Ans, anserine; His, L-histidine; IDP, imidazole dipeptide; m-His, Nπ-methyl-histidine.

Various biochemical properties and physiological functions of IDPs have been reported; however, reports on m-His are limited. m-His exhibits copper ion-chelating activity equivalent to or greater than that of Car and Ans [33]. *In vitro* studies have demonstrated that IDP and m-His may reduce serum uric acid levels by inhibiting uric acid uptake by renal cells and suppressing xanthine oxidase activity [34]. Additionally, m-His and Ans were recently reported to restore the loss of electrical resistance caused by methylglyoxal in epithelial and endothelial cells [35]. Furthermore, in the current study, m-His exhibited hypochlorous radical-scavenging activity equivalent to that of Car, Ans, and His (Table 2). Most of the functional characteristics of Car and Ans are believed to be associated with the His component (primarily the imidazole ring) of the dipeptide [4], therefore, m-His may be a substitute for Car and Ans. It is suggested that the health benefits of ingesting Ans in humans may be due to the contribution of m-His.

At 6 h after IDP administration, significantly higher levels of Ans and Car were observed in the hamsters' soleus muscle, heart, kidneys, and liver than those in the vehicle group (Figure 2). These increments are likely due to either the re-synthesis of IDPs from constituent amino acids or the migration of the administered IDPs into the tissue without degradation. Previous studies have suggested that red blood cells may protect against Car degradation by CN1 [5,36]; therefore, even if hamsters have strong CN1 activity, some IDPs may migrate to tissues without degradation.

In contrast, excitatory tissues, such as the soleus muscle and heart, are expected to have high CARNS1 expression owing to the inherently high tissue concentrations of Ans and Car (Figure 2). Although the administered amount of Car was approximately one-third of the amount of Ans and was more sensitive to CN1 than Ans, the concentration of Car showed a greater increase in the heart

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at 6 h after IDP administration compared to that of Ans. Thus, the increase in Car concentration in the heart was likely due to re-synthesis rather than IDP migration to the tissue without degradation.

In the brains of hamster at 6 h after IDP administration, Ans was below the detection limit and no increase in Car was observed compared with those in the vehicle group. Although His in the brain showed a tendency toward a higher concentration than in the vehicle group, m-His concentration was markedly increased in the soleus muscle, heart, kidney, lung, and liver (Figure 2). In human studies, sustained supplementation with IDPs derived from chicken extract (Ans:Car = 3:1) or Ans alone derived from salmon improved cognitive function in elderly people and reduced the risk of Alzheimer's disease [37,38]. Accordingly, the concentrations of m-His and His in the cerebrospinal fluid of individuals at high risk of Alzheimer's disease are lower than those in healthy individuals with normal cognitive function [39].

Although we have no findings indicating that supplementation with IDPs increases the levels of IDPs or related substances in the human brain, results from hamsters suggest that m-His or His may be involved in the improvement of cognitive function following IDP ingestion. Furthermore, although it is still unclear whether IDPs accumulate in the brain when administered to hamsters for a long time, previous studies have suggested that the molecular mechanism underlying the beneficial effects of IDP supplementation on the brain may involve Car inducing intestinal cells to secrete exosomes, which activate neurons [40]. Car may play a role in regulating oxidative stress and inflammation in the kidney-brain axis [41]. However, in this study, we observed that m-His accumulated at higher concentrations in the kidneys and heart than that of Car in hamsters.

5. Conclusions

When IDPs were orally administered to hamsters expressing CN1, their blood distribution markedly differed from that observed in mice. IDPs were not detected; instead, an increase in the constituent amino acids of IDPs, especially m-His derived from Ans, was observed. The blood profile was similar to that in humans. Consequently, the increased blood m-His levels in hamsters is distributed to major tissues throughout the body via the blood. If the tissue distribution of m-His also occurs in humans, it was considered that the health functions observed with the oral intake of IDPs might not be due to the IDPs themselves, but rather m-His as a substitute. In the future, additional animal experiments using hamsters will be important for surveying the function of IDPs in humans. The findings indicate that hamsters are suitable for investigating possible IDP functions in humans.

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Institutional Review Board Statement: This study was conducted in accordance with the Declaration of Helsinki and approved by the Ethics Committee of Tokai Bussan Co., Ltd. (Protocol Code 2018-01, Approval Date: February 21, 2018). The animal experiment protocol was approved by the Facility Animal Management and Utilization Committee of Biosafety Research Center Co., Ltd. (Shizuoka Prefecture) (Protocol Code M179 (716-010), Approval Date: June 4, 2024).

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: The original contributions presented in this study are included in the article. Further inquiries can be directed to the corresponding author.

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Conflicts of Interest: All authors are employees of Tokai Bussan Co., Ltd., and the research funding was supported by Tokai Bussan Co., Ltd. However, this study does not describe the efficacy or effects of imidazole dipeptides (IDPs). The purpose of this study was to conduct basic research aimed at elucidating the reasons behind the various health effects demonstrated by oral administration of IDPs, which are degraded by blood carnosine-degrading enzymes. Furthermore, this research has already been presented at the 2025 Annual Meeting of the Japanese Society of Nutrition and Food Science, and no patent application based on this research has been filed. Therefore, there is no conflict of interest between all authors and Tokai Bussan Co., Ltd. in this research.

Abbreviations

The following abbreviations are used in this manuscript:

Ans	L-anserine
b-Ala	β-alanine
Car	L-Carnosine
CN1	Serum carnosinase
IDPs	Imidazole-dipeptides
His	L-histidine
m-His	1-methyl-histidine (Nπ-methyl-L-histidine)

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