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ORIGINAL ARTICLE

Profiling histidine dipeptides in plasma and urine after ingesting beef, chicken or chicken broth in humans

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Abstract The in vitro metabolic stability of histidine-dipeptides (HD), carnosine (CAR) and anserine (ANS), in human serum, and their absorption kinetics after ingesting pure carnosine or HD rich foods in humans have been investigated. Healthy women ($n = 4$) went through four phases of taking one dose of either 450 mg of pure carnosine, 150 g beef (B), 150 g chicken (C), or chicken broth (CB) from 150 g chicken with a >2-week washout period between each phase. Blood samples were collected at 0, 30, 60, 100, 180, 240, and 300 min, and urine samples before and after (up to 7 h) ingesting pure carnosine or food. Both plasma and urine samples were analyzed for HD concentrations using a sensitive and selective LC–ESI-MS/MS method. CAR was undetectable in plasma after ingesting pure carnosine, B, C or CB. By contrast, plasma ANS concentration was significantly increased ($P < 0.05$) after ingesting C or CB, respectively. Urinary concentrations of both CAR and ANS were 13- to 14-fold increased after ingesting B, and 14.8- and 243-fold after CB ingestion, respectively. Thus, dietary HD, which are rapidly hydrolyzed by carnosinase in plasma, and excreted in urine, may act as reactive carbonyl species sequestering agents.

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Introduction

Carnosine (β-alanyl-L-histidine, CAR) is the archetype of a class of aminoacyl histidine dipeptides (HD), which includes homocarnosine (γ-amino-butyryl-histidine, HCAR), anserine (*N*-β-alanyl-3-methyl-l-histidine, ANS), and balenine (*N*-β-alanyl-1-methyl-histidine) (Fig. 1). In humans, CAR is synthesized from the two constitutive amino acids by carnosine synthase (EC 6.3.2.11) (Horinishi et al. 1978), and is degraded by carnosinase. Two different carnosinase iso-forms have been identified up to now: a cytosolic form (tissue carnosinase, CN2, EC 3.4.13.18), acting as a non-specific dipeptidase, and a highly specific metal-ion dependent homodimeric dipeptidase, named serum carnosinase (CN1, EC 3.4.13.20), which is located in both serum and brain (Teufel et al. 2003). The activities of CAR synthase and carnosinases, as well as their tissue localization, regulate the tissue content of CAR, which is very abundant in skeletal muscles (mM concentration range), and in some regions of the central nervous system (CNS), such as the olfactory bulb (Bonfanti et al. 1999; Crush 1970; Stuerenburg 2000). In humans, the diet represents the main source of CAR and derivatives, which are contained in significant amounts in red and white meats (beef, chicken, pork) and fish (Gil-Agusti et al. 2008).

Although CAR (and other HD) was discovered at the beginning of the last century, their biological function(s) still remain enigmatic, although several hypotheses have been proposed (Aldini et al. 2005; Guiotto et al. 2005). In the nervous system, HCAR was suggested to act as a GABA reservoir through the control of one or several

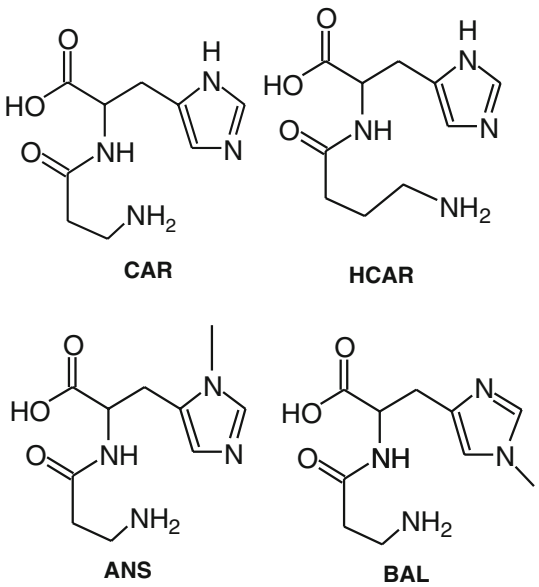

Fig. 1 Structures of Histidine dipeptides. *CAR* carnosine (*β*-alanyl-L-histidine), *HCAR* homocarnosine (*N*-4-aminobytyryl-L-histidine), *ANS* anserine (*N*-*β*-alanyl-3-methyl-L-hitidine), *BAL* balenine (*N*-*β*-alanyl-1-methyl-histidine)

carnosinases (Petroff et al. 2001), since CAR meets many criteria as a neurotransmitter and/or neuromodulator, in particular in the olfactory pathway (Margolis 1974). In skeletal muscles, since it is present in mM concentration range, CAR may play an important role as cytosolic buffer to neutralize lactic acid (Abe 2000).

More recently, several pharmacological properties have been reported for CAR, such as anti-ischemic, anti-aging and hypoglycemic activities as well as effects on the CNS, in particular on behavior. Also, it may have preventive/protective efficacy toward diseases related to the complications of diabetes and metabolic syndrome, such as nephropathy and cardiovascular disease (Alhamdani et al. 2007; Kurata et al. 2006; Min et al. 2008; Rajanikant et al. 2007; Rashid et al. 2007; Sauerhofer et al. 2007; Tang et al. 2007). The exact mechanism by which CAR might exert these reported effects has not yet been clarified, although several hypotheses have been proposed such as: (1) CAR, through the imidazolic ring, could act both as a direct antioxidant by scavenging peroxyl radicals and singlet oxygen, and as an indirect antioxidant by deactivating the pro-oxidant effect of transition metal ions through a chelating mechanism (Egorov et al. 1997; Kang et al. 2002; Kohen et al. 1988); (2) CAR could act as a histamine precursor due to its enzymatic hydrolysis to L-histidine and subsequent conversion to L-histamine; (3) CAR could act as an efficient scavenger of reactive carbonyl species generated by glucose and lipid oxidation such as methylglyoxal (Brownson and Hipkiss 2000), acrolein (Carini et al. 2003) and hydroxynonenal (Aldini et al. 2002).

Dietary CAR could be considered as a promising bioactive agent considering the lack of acute and chronic toxicity, as well as its richness in the diet (e.g. 300 mg of CAR can be obtained by ingesting 200 g of beef). Even though the above reported observations have potentially large relevance for human health, knowledge of absorption and bioavailability of HD in humans is scanty. Only few and controversial studies regarding the absorption, distribution, metabolism and excretion (ADME) profiles of CAR and other HD have been addressed (Gardner et al. 1991; Park et al. 2005; Harris et al. 2006). From rodent studies, CAR is actively transported across the brush border membrane via the peptide transporter PEPT1 (Son et al. 2004). However, studies conducted in rodents cannot be extrapolated to humans, since this animal species lacks serum carnosinases, which are supposed to be primarily involved in the metabolic fate of CAR in humans.

Hence, the current study was conducted to determine systemic delivery of CAR and other HD (ANS) in humans, by monitoring their plasma and urine concentrations following ingestion of pure CAR or foods known to be rich in HD. A highly sensitive and specific HPLC–ESI-MS/MS method to determine HD in biological fluids was set up, validated and applied for the current study.

Materials and methods

Chemicals

All chemicals and reagents were of analytical grade and purchased from Sigma-Fluka Aldrich Chemical Co (Milan, Italy). HPLC- and analytical-grade organic solvents were also purchased from Sigma-Aldrich (Milan, Italy). HPLC-grade water was prepared with a Milli-Q water purification system. Carnosine (*β*-alanyl-l-histidine, L-CAR), its isomer *β*-alanyl-D-histidine (D-CAR), the internal standard (IS) H-Tyr-His-OH were obtained from Flamma S.p.A (Chignolo d'Isola, Bergamo, Italy). Carnosine was encapsulated in a gelatin gel capsule for studying absorption kinetics. Anserine (*N*-*β*-alanyl-3-methyl-l-histidine, ANS), *N*-acetylcarnosine (*N*-AcCAR), *β*-alanine, phthaldialdehyde (OPA) were purchased from Sigma (Milan, Italy); heptafluorobutyric acid (HFBA) was purchased from Aldrich (Milan, Italy).

Histidine dipeptides analysis by LC–ESI-MS/MS

Analyses were performed using a ThermoFinnigan Surveyor LC system equipped with a quaternary pump, a Surveyor autosampler, a vacuum degasser, and connected to a TSQ Quantum Triple Quadrupole Mass Spectrometer (ThermoFinnigan Italia, Milan, Italy). Chromatographic

separations were done by reverse phase elution with a Phenomenex Sinergy polar-RP column (150 mm × 2 mm i.d.; particle size 4 μm) (Chemtek Analytica, Anzola Emilia, Italy) protected by a polar-RP guard column (4 mm × 2 mm i.d.; 4 μm) kept at 25°C. The mass spectrometer was equipped with an electrospray interface (ESI), which was operated in the positive-ion mode, and controlled by Xcalibur software (version 1.4).

For validation of the method, two fresh stock solutions of each analyte (1 mM CAR, ANS, *N*-AcCAR and the internal standard) were independently prepared in PBS (10 mM) and stored at −20°C. One solution was used to spike urine or plasma blanks for calibration samples, and the other to prepare the quality control (QC) samples. Stock solutions were diluted further with PBS and the solutions of each analyte were mixed together to obtain working solutions. Working solutions (20 μM) were analyzed by LC/MS/MS to ensure that the concentrations of the original solutions were within the limits of the maximum established error (±3%). Calibration samples were prepared by spiking urine or plasma blanks with each working solution to provide the following final concentrations: 0.5, 1.0, 5.0, 10.0, 20.0, 40.0, 60.0 μM (IS concentration was maintained at 20 μM). QC samples at four concentrations [LOQ (0.5), 1, 10, 60 μM] were prepared in the same way. Each calibration sample was processed as described below in assay validation for histidine dipeptides using LC–ESI-MS/MS.

Aliquots of 100 μl of serum or urine samples were spiked with the IS (20 μM final concentration), deproteinized by perchloric acid (PCA, 700 mM final concentration) and centrifuged at 18,000 rpm for 10 min. The supernatants were then diluted 1:1 with mobile phase A (CH_3CN/H_2O/HFBA 90/10/0.1 v:v:v), filtered through 0.2 μm filters and then injected into the LC–MS system.

Separations were done by gradient elution from 100% phase A to 80% phase B (CH_3CN) in 12 min at a flow rate of 0.2 ml min^{-1} (injection volume 10 μl); the composition of the eluent was then restored to 100% A within 1 min, and the system was re-equilibrated for 6 min. The samples rack was maintained at 4°C. ESI interface parameters (positive-ion mode) were set as follows: middle position; capillary temperature 270°C; spray voltage 4.0 kV. Nitrogen was used as the nebulizing gas at the following pressure: sheath gas 30 psi; auxiliary gas 5 a.u. MS conditions and tuning were performed by mixing through a T-connection the water-diluted stock solutions of analytes (flow rate 10 μl min^{-1}), with the mobile phase maintained at a flow rate of 0.2 ml min^{-1}: the intensity of the $[M + H]^+$ ions were monitored and adjusted to the maximum by using the Quantum Tune Master® software. Quantitations were performed in multiple reaction monitoring (MRM) mode at 2.00 kV multiplier voltage, and the following MRM transitions of $[M + H]^+$ precursor ion → product ions were selected for each analyte and the relative collision energies optimized by the Quantum Tune Master® software:

- H-Tyr-His-OH (IS) *m/z* 319.2 → 156.5 + 301.6 (collision energy, 25 eV);
- CAR: *m/z* 227.0 → 110.6 + 156.5 (collision energy, 25 eV);
- *N*-AcCAR: *m/z* 269.1 → 110.3 + 156.1 (collision energy, 20 eV);
- ANS: *m/z* 241.2 → 109.3 + 170.1 (collision energy, 40 eV).

The parameters influencing these transitions were optimized as follows: argon gas pressure in the collision Q2: 1.5 mbar; peak full width at half maximum (FWMH): 0.70 *m/z* at Q1 and Q3; scan width for all MRM channels: 1 *m/z*; scan rate (dwell time): 0.2 s/scan.

Validation of histidine dipeptides analysis using LC–ESI-MS/MS

Due to the endogenous content of the analytes in urine, a full validation procedure [linearity, limit of detection (LOD), limit of quantitation (LOQ), intra- and inter-day precision and accuracy, recovery and stability] has been executed in blank urine after 60 min incubation with a 5% (v/v) of human serum in order to obtain the complete hydrolytic consumption of endogenous dipeptides (Table 1). Calibration standards were prepared and analyzed in duplicate in three independent runs. The calibration curves were constructed by weighted ($1/x^2$) least-square linear regression analysis of the peak area ratios of CAR, ANS, and *N*-AcCAR to the IS against nominal analyte concentration. The lower limit of quantitation (LLOQ) was determined as the lowest concentration with values for precision and accuracy within ±20% and a signal-to-noise (S/N) ratio of the peak areas ≥10. Intra- and inter-day precisions and accuracies of the method were determined by assaying five replicates of each of the QC samples (four levels at LOQ and in the low, intermediate and high concentration range) in three separate analytical runs. Precision and accuracy were determined by calculating the coefficient of variation (CV%) and the relative error (RE%). The absolute recovery of the analytes after protein precipitation was determined by comparing the results for QC samples ($n = 3$) at the four concentrations with a corresponding set of deproteinized urine or plasma samples spiked with the same concentrations (containing 100% of the theoretical concentration). The overall absolute recovery was measured as the ratio of the slopes of the two calibration curves, and expressed as percentage. The stability of the analytes was investigated in the stock and

working solutions and in the final extract at 4°C (rack temperature) up to 24 h. The results were compared with those for freshly prepared QC samples and the percentage concentration deviation was calculated. The analytes were considered stable when 90–110% of the initial value was found.

β-Alanine analysis by HPLC with fluorimetric detection

β-Alanine plasma content was determined after derivatization with phthaldialdehyde/3-mercaptopropionic acid reagent according to the method previously reported by Harris et al. (2006). Derivatized β-alanine was determined by HPLC consisting of a Surveyor LC system (Thermoquest, Milan, Italy) equipped with a quaternary pump, a Surveyor autosampler (200-vial capacity) and connected to a fluorimetric detector (FL3000, Thermo Separation products). Data processing was performed by the ChromQuest 4.0 version software (Thermo Finnigan, Milan, Italy). Detector excitation and emission wavelengths were set at 340 and 445 nm, respectively. HPLC separations were carried out on a Waters Symmetry C18 column (5 µm, 4.6 × 250 mm) at a flow rate of 1 mL/min at room temperature under the conditions already described (Harris et al. 2006).

Analysis of HNE Michael adducts to histidine and carnosine by LC–ESI-MS/MS

Urinary content of the HNE Michael adduct with carnosine (CAR-HNE) and derivatives, namely His-1,4-dihydroxynonane (His-DHN), and protein bound His-HNE, were determined by LC–MS/MS in MRM mode, according to the methods previously described (Orioli et al. 2007).

Stabiltiy of histidine dipeptides in rat and human serum

Carnosine (50 µM final concentration) was incubated with human or rat serum as such or diluted 1:20 with PBS, and incubated at 37°C for 60 min. Aliquots of 100 µl were obtained after 5 min and then every 10 min until 60 min, spiked with the IS (50 µM final concentration), deproteinized, and the supernatants analyzed by LC–MS as above described.

Absorption kinetics of pure carnosine and dietary histidine dipeptides in humans

Four women aged 40–60 years were enrolled in the present study. Two study participants were in pre-menopause and the other two in post-menopause. All study subjects were in good health, as determined by a medial history questionnaire, physical examination, and normal results of clinical laboratory tests. All study subjects fulfilled the following eligibility criteria: (1) no history of cardiovascular, hepatic, gastrointestinal, or renal disease; (2) no alcoholism, no smoking, and no exogenous hormone use. The study protocol was approved by the Institutional review Board of Tufts Medical Center and Tufts University Health Sciences, and written informed consent was obtained from each study subject.

The subjects went through four experimental phases with at least a 2-week washout period between each phase. In the experimental phases subjects consumed either 450 mg of carnosine, beef, chicken breast or chicken broth prepared from chicken breast. After a 14-h fasting blood draw, subjects ingested either 450 mg of carnosine in gelatin gel capsule, 150 g beef, 150 g chicken breast or 280 ml of chicken broth prepared from 150 g of chicken breast with morning meal, which did not contain any source of carnosine. Blood samples were collected at 30,

Table 1 Intra- and inter-day accuracy and precision data for carnosine (CAR), anserine (ANS) and *N*-acetyl carnosine (*N*-AcCAR)

Analyte	Nominal concentration (µM)	Intra-day[a]		Inter-day[b]	
		CV (%)	RE (%)	CV (%)	RE (%)
CAR	0.5 (LOQ)	13.50	6.10	11.50	−10.98
	1	8.41	−1.54	8.99	5.24
	10	3.32	3.04	1.25	4.87
	60	2.80	3.25	5.54	−2.90
ANS	0.5 (LOQ)	11.21	−10.91	13.01	−12.91
	1	7.54	5.49	9.05	4.49
	10	3.14	2.58	3.14	3.06
	60	2.85	−0.98	3.44	−0.99
N-AcCAR	0.5 (LOQ)	11.54	−15.87	12.87	−16.17
	1	6.90	−8.97	4.91	−4.99
	10	4.27	2.22	5.89	6.04
	60	2.00	7.04	1.05	3.33

[a] Five replicates at each concentration

[b] Three runs, five replicates at each concentration over a period of 3 days ($n = 15$)

LOQ limit of quantitation, *CV* coefficient of variation, *RE* relative error

60, 100, 180, 240, and 300 min after consuming each meal. Blood samples were collected in evacuated containers containing EDTA. Plasma samples were separated immediately and stored at −80°C for subsequent analyses. Urine samples were collected up to 7 h in plastic bottle containing EDTA. Frozen plasma and urine samples were hand carried to the University of Milan, Italy in ice pack for LC–MS/MS analysis.

CAR profiling in rat lung tissue

Male Wistar rats (Charles River, Calco, Italy; body mass 250 ± 25 g) were maintained in compliance with the policy on animal care expressed in National Research Council guidelines (NRC 1985). Laboratory chow and drinking water were available ad libitum. Rats were treated with CAR (200 mg/kg, oral gavage) and after 0.5, 1, 1.5, 2, 3, and 4 hours the animals were killed by decapitation and the lungs were removed and stored in liquid nitrogen until processed. The 0 h data were obtained from our previous study showing no CAR in control animal (Aldini et al. 2004). Tissues were homogenized and analyzed by LC–ESI-MS/MS as previously reported (Aldini et al. 2004).

Statistical analysis

A repeated-measure analysis of variance with a Holm-Sidak multiple comparison test was used to measure the effect of beef, chicken or chicken broth ingestion on plasma and urine anserine and/or carnosine concentrations. When an equal variance test failed, Friedman repeated measures analysis of variance on ranks with a Tukey multiple comparison test was used. Data analysis was carried out with SigmaStat (Ver 3.1, Systat Software Inc, Point Richmond, CA).

Results

Analysis of histidine dipeptides by LC–ESI-MS/MS

The LC–MS/MS method, already developed by Aldini et al. (2004) for HD profiling in rat tissues, was re-validated on human plasma and urine. Standard curves for the analytes constructed on different working days showed good linearity over the entire calibration range for both matrices (0.5–60 μM), with correlation coefficients (r^2) > 0.998. The equations for the calibration lines were as follows:

$$\text{CAR } y = -0.00176493 + 0.0191128x$$
$$\text{ANS } y = 0.000528134 + 0.0170757x$$
$$\text{NAcCAR } y = -0.000480732 + 0.0571518x$$

The LOD was 0.2 μM for all the analytes, both in urine and plasma, and the LOQ was 0.5 μM. The intra- and inter-assay precision and accuracy of the method were determined on QC samples by analyzing five replicates at four concentrations, and the data are reported in Table 1. The intra-day precision (CV, %) was <14%, and accuracy ranged from −15.87 (at LOQ) to +7.04% of nominal concentrations; the inter-day CV values were <13.05% and accuracy was in the range −16.17 (at LOQ) to +6.04%. Recoveries at the two extremes of the calibration ranges were satisfactory, ranging from 96.2 to 101.3% for all the compounds (data not shown). The stability of CAR, ANS, in aqueous solutions and tissue extracts at room and higher temperatures (up to 105°C) has already been reported (Aldini et al. 2004). We therefore checked the stability of the analytes in QC samples, in the spiked plasma and urine only under the experimental conditions used. The stability was guaranteed for at least 24 h at 4°C and no significant differences (*t* test) were found between freshly prepared samples and samples prepared plasma or urine stored in liquid nitrogen (data not shown).

Histidine dipeptides stability in rat and human serum/plasma

Figure 2a shows the time-dependent stability of CAR in human and rat serum. CAR was found to be stable for at least 2 h when incubated with rat serum, while in human serum, it rapidly disappeared (since it was already undetectable after 5 min of incubation, the first time point considered). In order to measure the hydrolysis rate, serum was diluted 1:20. In this condition, the CAR consumption was significantly reduced (half-life of 3 min), and the calculated hydrolysis rate was of 170 nmoles ml^{-1} min^{-1}.

The stability of ANS and CAR was then studied in human serum (Fig. 2b). Similar to CAR, ANS also rapidly disappeared, although at a slower rate than CAR, since after 5 min of incubation a low, but well quantifiable amount of ANS was determined. In a 1:20 (v:v) diluted serum sample, the half life of ANS was 32 min, and the hydrolysis rate ≈16 nmoles ml^{-1} min^{-1}.

In order to ensure the stability of CAR during blood collection and plasma preparation for the human studies, stability studies were also performed in human plasma samples prepared using the metal ion chelating agent EDTA. The anticoagulant EDTA was chosen in view of its well known ability to chelate Zn^{2+} ions which are essential for the catalytic activity of carnosinase. The results indicate that unlike to that observed in serum, CAR does not undergo to hydrolysis in EDTA-plasma samples at least up to 1 h of incubation at room temperature (data not shown).

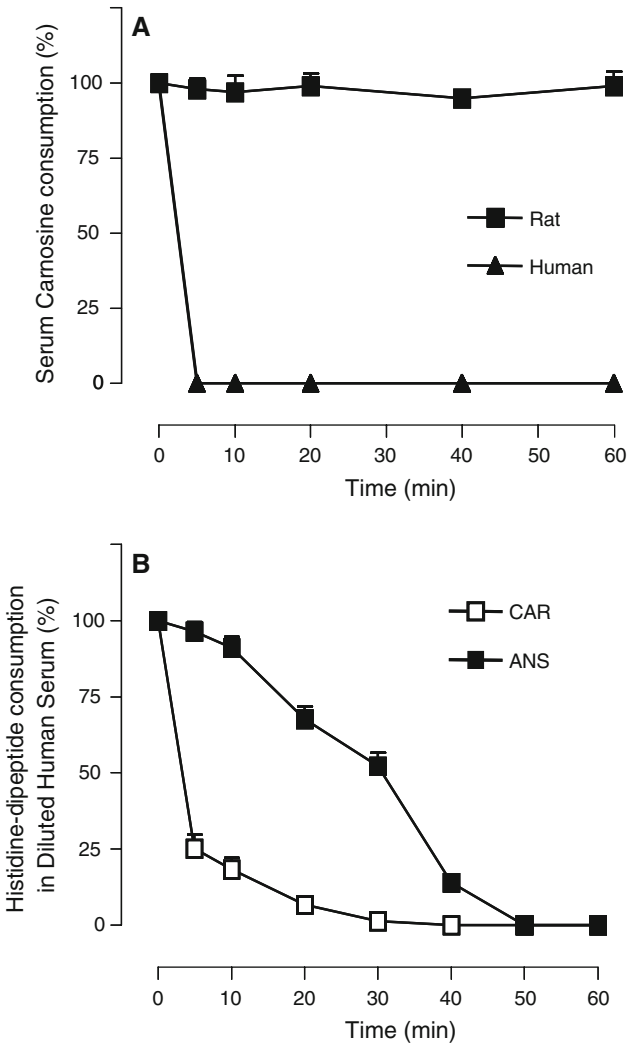

Fig. 2 **a** Time dependent stability of carnosine (20 μM) in human and rat serum samples and **b** depletion of histidine dipeptides, carnosine (20 μM) and anserine (20 μM) over time in diluted human serum with PBS (1:20, v:v). Values are mean ± SD ($n = 3$)

This confirms the effectiveness of EDTA in inactivating carnosinase activity, thus guaranteeing CAR stability during blood sample manipulation.

Profiling histidine dipeptides in plasma after ingesting pure carnosine and dietary histidine dipeptides in humans

None of the four volunteers receiving an oral dose (450 mg) of CAR showed detectable plasma levels of the dipeptide, which were always below the LOD of the validated LC–MS/MS methodology, at all the time points considered (data not shown).

Before measuring plasma levels of HD in humans following food consumption, the selected food (beef meat, chicken meat, and chicken broth) were analyzed for the contents of CAR and ANS.

Figure 3 shows a typical LC–ESI-MS/MS analysis of chicken broth, reporting the total ion current (TIC) (panel A), and the MRM ion traces for CAR (panel B), ANS (panel C) and the IS (panel D). The content of the both HD in the meat and broth samples used for the human study is shown in Table 2.

After either beef/chicken meat or chicken broth ingestion, CAR was undetectable in plasma (levels under the detection limit) of all volunteers, and at all the time points considered. After beef consumption, ANS was detectable but not quantifiable (levels between LOD and LOQ) in three of the four subjects, while it was determinable at concentrations greater than limit of quantitation in all the volunteers after chicken meat and chicken broth consumption. Figure 4 shows a typical LC–ESI-MS analysis of a plasma sample withdrawn after 60 min chicken meat consumption. The peak with a retention time of 19.14 min relative to ANS is well evident, while CAR is undetectable. Figure 5 (panel A) shows the plasma profile of ANS for each subject after chicken meat consumption, and panel B indicates the mean ± SEM showing a C_{max} of 2.72 ± 1.08 μM ($P < 0.05$), reached 100 min after meat ingestion. Similar results were obtained after chicken broth consumption, with maximal plasma levels of the compound being on average C_{max} of 0.78 ± 0.18 μM, which was reached after 60 min ($P < 0.01$). It is important to stress that both CAR and ANS were below the LOD determined by sensitive LC–MS/MS analysis before the meal. By contrast, *N*-AcCAR was well detectable in all the volunteers before ingestion of any type of meat (0.10 ± 0.010 μM), but its levels did not significantly change after beef or chicken consumption.

Profiling histidine dipeptides in urine after ingesting dietary histidine dipeptides in humans

Figure 6 shows the urinary levels of CAR, ANS and *N*-AcCAR in human subjects receiving 150 g of beef. All of the three analytes are well detectable, both before and after beef consumption. In particular, before beef ingestion, *N*-AcCAR was the most abundant histidine dipeptides (11.02 ± 4.0 μM), followed by CAR (3.50 ± 1.85 μM) and ANS (1.22 ± 0.74 μM). As was observed in plasma, the concentration of *N*-AcCAR in urine did not change significantly after beef consumption whereas significant increases over baseline levels of both CAR (42.35 ± 10.52 μM, $P < 0.01$) and ANS (16.55 ± 4.28 μM, $P < 0.01$) were observed. A similar trend was found for *N*-AcCAR (almost unchanged) even after chicken broth consumption as shown in Fig. 7. In view of the higher content of ANS in chicken compared to beef, the ANS urinary excretion after chicken broth ingestion was

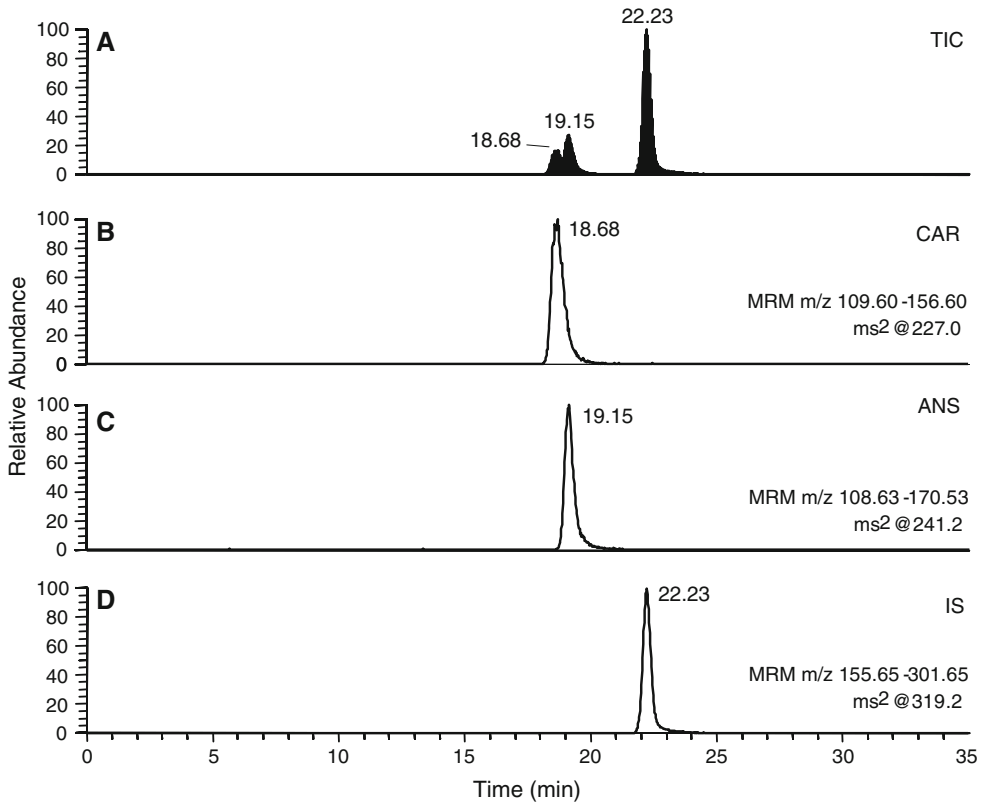

Fig. 3 LC–ESI-MS/MS profile of chicken broth. Total ion current (**a**) and multiple reaction monitoring (MRM) ion traces of carnosine (**b**), anserine (**c**), and the internal standard, Tyrosyl-Histidine (**d**)

Table 2 Carnosine and anserine contents in beef, chicken breast and chicken broth used for the in vivo studies

Meat	Carnosine	Anserine
Beef	2.29 mg g^{-1}	0.286 mg g^{-1}
	343.5 mg/150 g	42.9 mg/150 g
Chicken	2.15 mg g^{-1}	4.44 mg g^{-1}
	322.5 mg/150 g	660 mg/150 g
Chicken broth	1.13 mg ml^{-1}	1.70 mg ml^{-1}
	316.4 mg/280 ml	475 mg/280 ml

Peptides were determined by LC–ESI-MS/MS analysis

significantly higher than that of CAR (146.1 ± 35.88 vs. 37.07 ± 10.18 μM).

Profiling β-alanine in plasma after ingesting chicken broth

β-Alanine was not detected in the plasma of all the enrolled volunteers before the treatment (LOD = 0.3 μM). Figure 8 shows the plasma profile of β-alanine (mean ± SEM) after chicken broth ingestion: β-alanine was well detectable after 30 min, to reach the C_{max} between 60 and 90 min (64.14 ± 12.13 μM). After this time, the β-alanine plasma concentrations slowly began to decline (37.45 ± 6.54 μM at $t = 2$ h and 11.37 ± 3.76 μM at $t = 3$ h), to be undetectable at 240 min.

HNE Michael adducts to histidine and carnosine

Previously, we developed a quantitative LC–MS/MS method to profile two newly identified adducts originating from the conjugation of 4-hydroxy-*trans*-2-nonenal (HNE) to histidine (followed by reduction of the aldehyde His-1,4-dihydroxynonane; His-DHN), and to carnosine (CAR-HNE). Both adducts are recognized as new potential biomarkers of carbonyl stress (Orioli et al. 2007). The same method was applied in the current study to determine excretion of His-DHN and Car-HNE adducts following ingestion of CAR. No HNE Michael adducts to histidine and carnosine were detected in the urine of subjects receiving CAR (LOQ = 0.01 nmol/mL for both the analytes).

CAR profiling in rat lung tissue

As shown in Fig. 9, CAR was undetectable in lung tissue from control rats ($t = 0$ values). However, when the rat was supplemented with CAR by garbage, although CAR was not detectable at the first observation time ($t = 30$ min), significant increase occurred (4.2 ± 2.1 μmoles g^{-1} tissue) after 60 min of CAR oral intake, reached the peak levels at 90 min post-treatment (13.5 ± 2.8 μmoles g^{-1} tissue), and began to decline thereafter to be undetectable within 180 min.

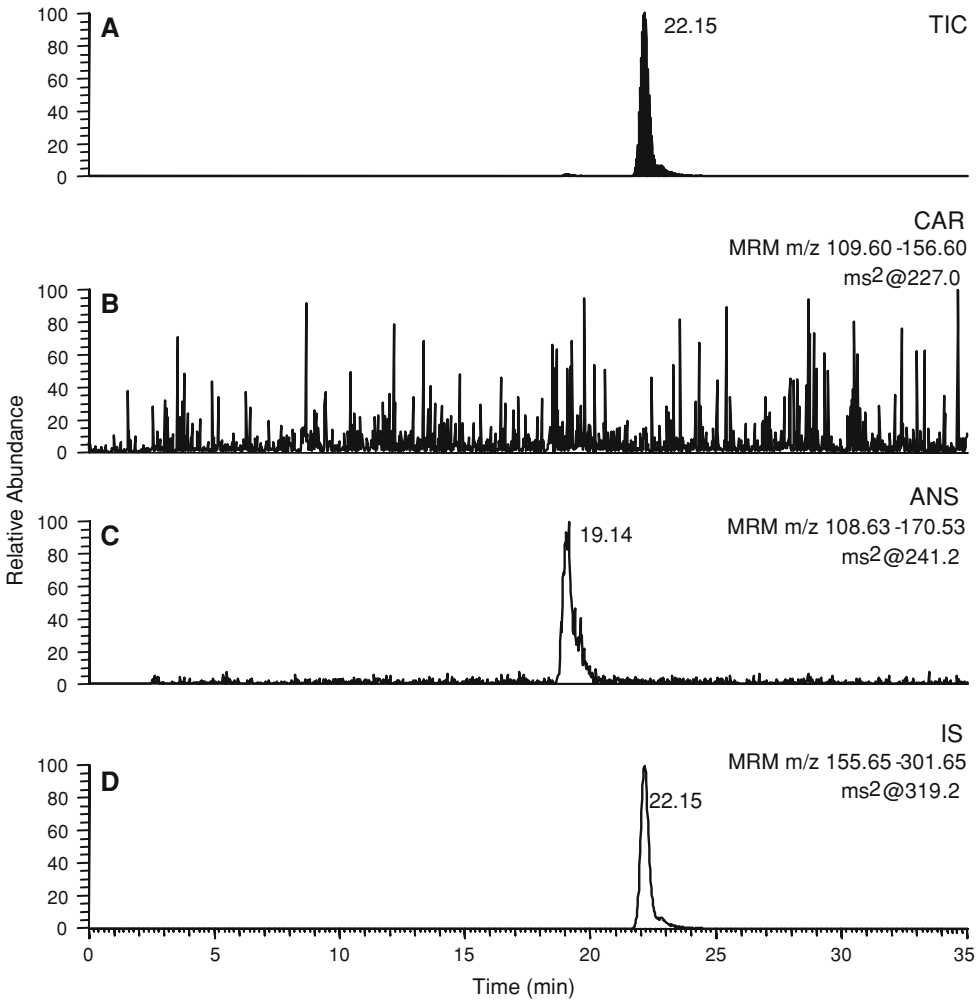

Fig. 4 LC–ESI-MS/MS profile of human plasma withdrawn 60 min after ingesting 150 g of chicken. Total ion current (**a**) and multiple reaction monitoring (MRM) ion traces of carnosine (**b**), anserine (**c**), and the internal standard, Tyrosyl-Histidine (**d**)

Discussion

The first step of the present study was to assess the stability of CAR and analogs in human and rat serum. In rat serum, CAR was found to be highly stable, and this is well explained by the lack of serum carnosinases. By contrast, CAR was hydrolyzed very efficiently in human serum, since 50 nmoles ml^{-1} were completely consumed within 5 min, the first time point considered. Experiments performed in diluted plasma permitted calculation of the rate of hydrolysis which was $\approx$170 nmoles ml^{-1} min^{-1}, a value close to that reported by Wassif et al. (1994) (161 nmoles ml^{-1} min^{-1}). ANS was found to be more stable than CAR, the hydrolysis rate being 10-fold lower ($\approx$16 nmoles ml^{-1} min^{-1}). These results correlate well with the docking scores previously reported (−40.25 for CAR and −30.75 for ANS) (Vistoli et al. 2006), showing the influence of the methyl residue on the imidazolic ring in reducing the substrate-enzyme interaction. To exclude the involvement of alternative mechanisms to the hydrolytic one in both CAR and ANS consumption, such as protein binding or metal ion chelation, D-carnosine was selected as a reference compound, because it is resistant to hydrolytic cleavage by peptidases. The D-isomer was found to be completely stable for at least 2 h incubation, thus confirming that the rapid disappearance of L-CAR in serum is only due to the cleavage of the β-alanine-histidine peptidic bond.

Since the absorption, distribution, metabolism, and excretion (ADME) profile of CAR in humans has not been fully elucidated and there are controversial data, the current study was conducted to monitor the plasma levels of CAR after ingesting 450 mg of pure CAR, a dose which can be easily achieved by the diet (the amount is contained in $\sim$300 g of beef). At all the observed times, and up to 5 h, CAR was undetectable in the plasma samples of all the subjects. These data seem in contrast with those reported by Gardner et al. (1991), since they found a plasma content of CAR ranging between 4.6 and 81.1 μM 1.5 h after CAR ingestion. This discrepancy might be explained by considering: (a) the higher dose used by Gardner (4 g vs. 0.45 g); (b) the analytical method employed for CAR

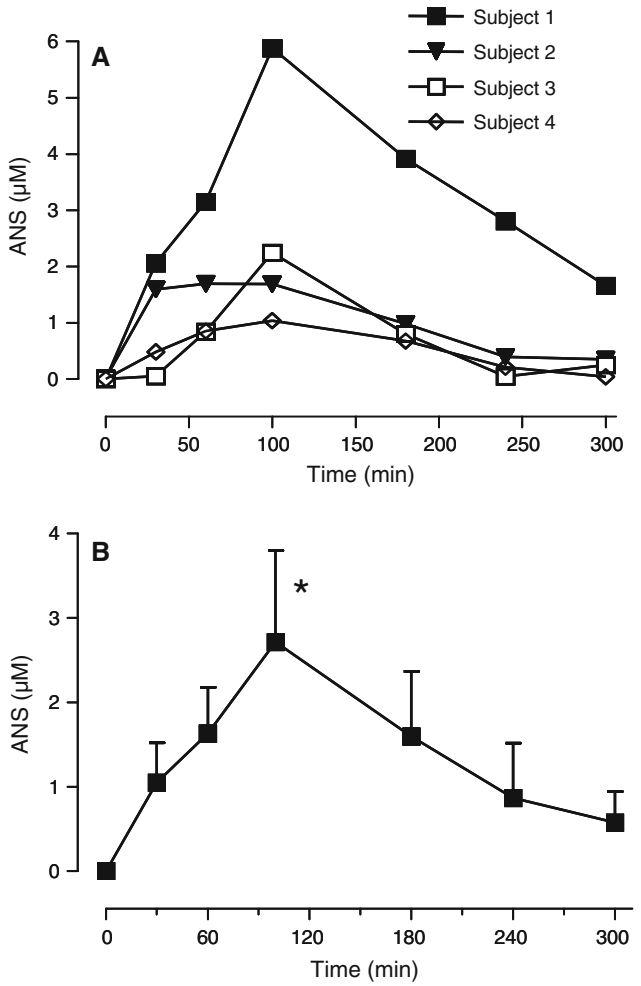

Fig. 5 Individual (**a**) and mean (**b**) absorption kinetics of plasma anserine in four subjects after ingesting 150 g of chicken breast determined by LC–ESI-MS/MS. $C_{max} = 2.72 \pm 1.08$ μM at 100 min. Repeated measure ANOVA with a Holm–Sidak multiple comparison test. (Friedman repeated measures analysis of variance on ranks with a Tukey multiple comparison test.) $^{*}P > 0.05$

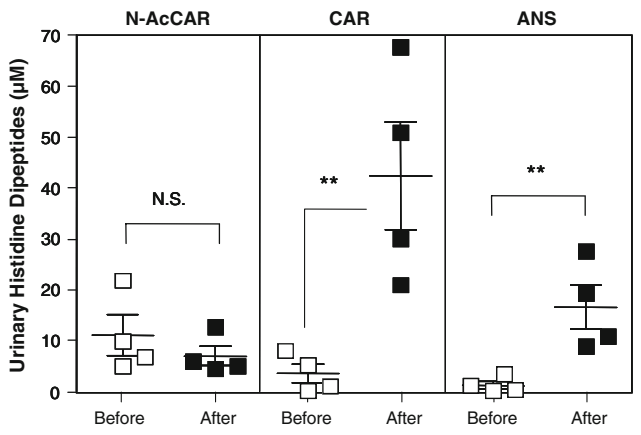

Fig. 6 Urinary excretion (up to 7 h) of histidine dipeptides after ingesting 150 g of beef. *N-AcCAR N*-acetyl carnosine, *CAR* carnosine, *ANS* anserine. Paired *t* test ($n = 4$), $^{**}P < 0.01$

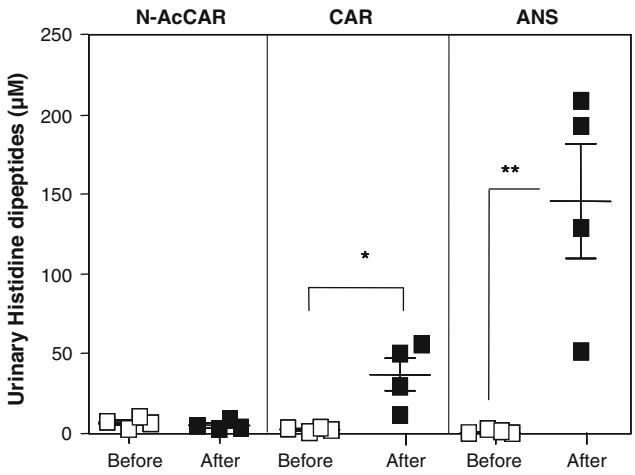

Fig. 7 Urinary excretion (up to 7 h) of histidine dipeptides after ingesting 280 ml of chicken broth prepared with 150 g of chicken breast. *N-AcCAR N*-acetyl carnosine, *CAR* carnosine, *ANS* anserine. Paired *t* test ($n = 4$), $^{*}P < 0.05$, $^{**}P < 0.01$

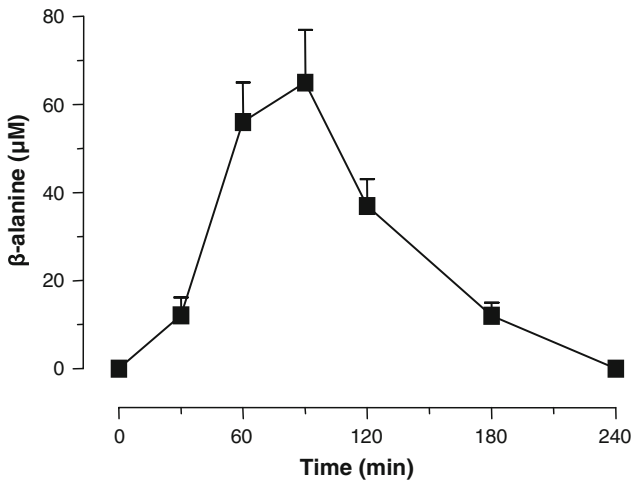

Fig. 8 Plasma concentrations of *β*-alanine after ingesting 280 ml of chicken broth prepared with 150 g of chicken breast. Values are mean ± SEM

quantification (an ion-exchange amino acid analyzer with ninydrin detection), which is less specific than the LC–ESI-MS/MS used in the present study; (c) possible genetic polymorphisms of plasma carnosinase which could greatly affect the CAR concentrations in blood.

More recently, Park et al. (2005) monitored the plasma concentrations of CAR in healthy volunteers after ingesting 200 g of ground beef (equivalent to a CAR dose of 267 mg) using an HPLC system. They reported that the plasma concentrations of CAR reached 40 μM immediately after ground beef consumption, with a further linear increase, to reach a C_{max} of 130 μM after 4 h. These data are in contrast with those reported by Harris et al. (2006), demonstrating that no CAR was detected in human serum

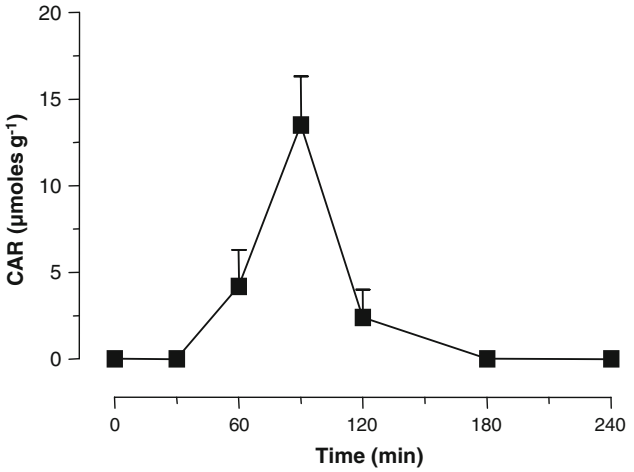

Fig. 9 CAR profiling in lung tissue of rat receiving an oral dose of CAR (200 mg kg^{-1}, oral gavage). Values are mean ± SEM

after ingestion of chicken broth (containing the equivalent of 40 mg kg^{-1} β-alanine in the form of anserine and carnosine). On the other hand, the current study using even a higher dosage of CAR (450 mg) did not observe any carnosine in blood after ingesting the pure carnosine. It is possible that the metabolic fate of CAR in humans can be substantially different depending on its sources. Beef may contain some compounds, which are able to stabilize CAR in serum, i.e. a carnosine inhibitor or its formation during meat digestion, or the formation of peptides which can compete with CAR in the hydrolytic process. Therefore, we repeated the experiment using meat (beef and chicken) as food source of CAR. In contrast to the results of Park et al. (2005), CAR was below the LOD at all the observation times in all of our study subjects. Assuming a complete absorption of meat CAR (1.18 mmoles), the plasma concentration would be of 470 nmoles ml^{-1}. Taking into account the hydrolysis rate (170 nmoles ml^{-1} min^{-1}), this amount would be hydrolyzed in less than 3 min. By contrast, ANS was well detected in serum, and this result well agrees with the in vitro and in silico studies, demonstrating that the methyl moiety significantly reduces the affinity of the histidine dipeptides for the enzyme, thus greatly enhancing its serum stability.

Conversely, both CAR and ANS were well detectable in the urine collected within the 0–7 h interval following meat consumption, reaching concentrations up to 42 μM (beef) and 146 μM (chicken), respectively. The higher urinary levels of ANS compared to CAR reflect the higher content ratio of ANS/CAR in the chicken meat. By considering the urinary volume (≈1.5 L collected in 7 h), and the amount of CAR and ANS in the ingested meat and chicken broth, as determined by LC–ESI-MS/MS analysis, their percentage of urinary excretion within 7 h accounts for approximately 5% of the ingested dose. These data agree well with those reported by Gardner et al. (1991), showing that CAR excreted in the urine ranges from 1.2 to 15% of the dose.

The data in our hands well agree with those recently reported by Harris et al. (2006), showing that CAR was not detected in the plasma of the volunteers after ingesting chicken broth (equivalent to 40 mg kg^{-1} of β-alanine), whereas ANS was found at trace levels. Moreover, the authors detected CAR and ANS in the urines.

There are at least three explanations regarding CAR disappearance in blood: (1) hydrolysis by carnosinase, (2) rapid uptake of the dipeptide into the tissues, (3) carnosine's reaction with electrophilic RCS such as HNE or with physiological aldehyde and ketons. The hydrolytic fate of CAR, suggested by the in vitro experiments, was firstly confirmed by monitoring the plasma formation of β-alanine, product of CAR hydrolysis, in the current study. The current study clearly indicates that the main metabolic fate of carnosine is the hydrolytic cleavage to the two constitutive amino acids catalyzed by carnosinase.

The uptake of carnosine in human tissues is not yet well established. However, it is well known that carnosine is transported by proton-coupled oligopeptide transportes (POTs) (Jappar et al. 2009), namely PEPT1, PEPT2, PHT1 and PHT2. Therefore, it is plausible that CAR can be taken up by tissues expressing these transporters. In particular, PEPT1 is highly expressed in apical membranes of small intestine and in the proximal tubule of kidney cortex. PEPT2 is primarily localized in the brush border in proximal tubule as well as in brain, choroid plexus, eye, lung and mammary gland. PHT1 is abundantly expressed in rat brain and eye, and PHT2 is abundant in lung, spleen thymus and immunocytes (Jappar et al. 2009). In order to partially support this hypothesis, the CAR content in the lung tissue of rats receiving a single dose of CAR (250 mg kg^{-1}) has been analyzed in the current study. Lung was chosen due to its expression of PHT2 and PEPT2 transporters. The results well show a significant uptake of CAR in the lung thus, indicating beside kidney other tissues expressing POTs, in fact, can take up exogenous carnosine. Further studies are needed to better understand the tissue uptake of CAR.

Finally, CAR may react with carbonyls such as HNE. We previously demonstrated that CAR acts as detoxifying agent of unsaturated aldehydes such as HNE and acrolein by forming the corresponding Michael adduct (Aldini et al. 2005). In particular, the carbonyl sequestering effect has been firstly demonstrated in in vitro conditions (Aldini et al. 2002) and then in biological matrices (Orioli et al. 2005) as well as in ex vivo experiments using Zucker rats as animal model of in vivo oxidative damage and protein carbonylation (Orioli et al. 2007). However, no detectable adduct of CAR with HNE was found in all the participants

in the current study. It can be explained by (1) healthy subjects enrolled in this study are not characterized by a significant lipid peroxidation cascade and hence only negligible amount of HNE, which can be detoxified by GSH, the first nucleophilic detoxifying agent, is formed and (2) the HNE-CAR metabolic pathways differ in humans with respect to those of rodents. Further analyses are in progress in healthy subjects and patients affected by oxidative stress associated diseases such as metabolic syndrome, nephropathy and atherosclerosis to better understand the RCS detoxifying role of CAR in humans. The CAR reaction with physiological unreactive aldehydes and ketons should be excluded since carnosine is a selective entrapping agent for electrophilic aldehydes and does not react with endogenous carbonyls such as pyridoxal as recently demonstrated using a mass spectrometry approach (Vistoli et al. 2009). The high selectivity of carnosine can be explained by considering the reaction mechanism of CAR toward electrophilic aldehydes (i.e. α,β-unsaturated aldehydes). In particular, it involves the Schiff base formation between the β-alanine amino group and the aldehydic function, which then catalyzes the Michael adduction between the C3 of the aldehyde and the Nτ of the histidine group by approaching the two reactive centers (Aldini et al. 2002). Hence, the high selectivity of CAR can be explained on the basis of this mechanism of reaction, which avoids cross reactivity with physiologically relevant carbonyl compounds, given the reversibility of the Schiff base in aqueous solutions.

The current study clearly indicates that CAR and ANS are mainly hydrolyzed by carnosinase (fast renal clearance well explains the lack of circulating CAR), and that a low but significant amount is excreted in the urine. Therefore, it is plausible that CAR reaches a steady state of very low concentration in serum, while ANS sustains higher concentrations than that of carnosine due to its greater stability vis-a-vis carnosinase after ingesting HD rich diet. CAR and ANS are continuously excreted in the urine where they accumulate in a micromolar concentration range. The current study results warrant further studies on biological functions of HD in humans.

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