

Relevance of allosteric conformations and homocarnosine concentration on carnosinase activity

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Abstract Activity of carnosinase (CN1), the only dipeptidase with substrate specificity for carnosine or homocarnosine, varies greatly between individuals but increases clearly and significantly with age. Surprisingly, the lower CN1 activity in children is not reflected by differences in CN1 protein concentrations. CN1 is present in different allosteric conformations in children and adults since all sera obtained from children but not from adults were positive in ELISA and addition of DTT to the latter sera increased OD450 values. There was no quantitative difference in the amount of monomeric CN1 between children and adults. Further, CN1 activity was dose dependently inhibited by

homocarnosine. Addition of 80 μM homocarnosine lowered V_{max} for carnosine from 440 to 356 pmol/min/ μg and increased K_{m} from 175 to 210 μM . The estimated K_{i} for homocarnosine was higher (240 μM). Homocarnosine inhibits carnosine degradation and high homocarnosine concentrations in cerebrospinal fluid (CSF) may explain the lower carnosine degradation in CSF compared to serum. Because CN1 is implicated in the susceptibility for diabetic nephropathy (DN), our findings may have clinical implications for the treatment of diabetic patients with a high risk to develop DN. Homocarnosine treatment can be expected to reduce CN1 activity toward carnosine, resulting in higher carnosine levels.

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Introduction

Carnosine is a β -alanyl-L-histidine dipeptide found in high concentrations in the muscle but also in the central nervous system (for review see Crush 1970) from where it is released into the serum and cerebrospinal fluid. The role of carnosine is not completely understood (Bauer 2005; Quinn et al. 1992; Boldyrev and Severin 1990); it appears to function as an antioxidant and free radical scavenger (Baran 2000; Trombley et al. 2000; Boldyrev 1993, Boldyrev et al. 1993), can delay senescence of cultured fibroblasts (McFarland and Holliday 1994), may serve as an anti-glycation agent (Hipkiss 1998) and has a positive effect on red blood cell deformability (Aydogan et al. 2008). In the central nervous system, carnosine meets many criteria for a neurotransmitter (Baslow 2009) modulating synaptic processes but also appears to be involved in neuroprotection

(Tabakman et al. 2002). Treatment with carnosine significantly reduced infarct volume and improved neurological function in a mouse model of ischaemic stroke (Min et al. 2008). Treatment with β -alanine increased muscle carnosine concentrations (Kendrick et al. 2008; Baguet et al. 2009). Whereas there seems to be no positive effect of muscle carnosine loading on muscle strength (Kendrick et al. 2008), we can conclude from the data reported by Baguet et al. that carnosine is a stable compound in human skeletal muscle. A comparison of carnosine content between a group of elderly Korean males and young elite Korean swimmers showed no difference in muscle carnosine concentration (Kim 2009). Recently, we reported evidence that carnosine acts as a protective factor for the development of nephropathy in diabetes (Janssen et al. 2005). Carnosine is found in a low concentration (usually below 100 nM) in blood and cerebrospinal fluid (CSF). Homocarnosine (γ -aminobutyric acid-L-histidine) also has a low concentration in blood (around 100 nM) but the concentration is much higher (up to 4 μ M) in brain and CSF where it may act as a precursor for the neurotransmitter GABA (Perry et al. 1974). Homocarnosine concentration in the brain is about 100-fold higher than carnosine concentration (Gjessing et al. 1990) and its concentration in children's CSF is higher than in adults (Perry et al. 1968).

Two dipeptidases are known to cleave carnosine, denoted CN1 and CN2 (Teufel et al. 2003) and classified as members of the M20 metalloprotease family. CN2 functions as a cytosolic non-specific dipeptidase (EC 3.4.13.18) and is not present in serum or CSF. CN2 does not degrade homocarnosine and hydrolyzes carnosine only at alkaline pH (Teufel et al. 2003). CN1 represents human serum carnosinase (EC 3.4.13.20), the only dipeptidase with substrate specificity for carnosine or homocarnosine. The CN1 gene is expressed in the central nervous system of adults and to a lesser extent in liver (Teufel et al. 2003). The concentrations of CN1, carnosine and homocarnosine have been reported to vary greatly between individuals. Both enzyme and dipeptides are found only in small amounts in newborns (Lenney et al. 1982). CN1 activity increases with age; it is higher in females than males (Bando et al. 1984).

Reduced carnosinase activity (Willi et al. 1997) has been reported in patients with certain muscle disorders (Bando et al. 1990, Duane and Peters 1988) as well as in patients with Parkinson disease, multiple sclerosis or cerebrovascular accidents. In contrast, normal serum carnosinase activity was reported in patients with idiopathic epilepsy and motor neuron disease (Wassif et al. 1994). Recently, Balion et al. (2007) showed that patients with dementia had significant lower carnosinase activities in serum compared to controls. It remains unclear whether the

reduced activities in some patients are disease-related or have an independent cause, for example reduced liver function.

Although serum carnosinase activity has been well characterized (Lenney et al. 1985; Pegova et al. 2000; Dunnett et al. 2002; Teufel et al. 2003) using carnosine as a substrate, homocarnosine metabolism and the differential effects of the two primary substrates on CN1 activity have not been studied in detail. Furthermore, little is known concerning the relationship between carnosine and homocarnosine concentrations and CN1 activity in CSF. Here we report that homocarnosine has a strong, competitive inhibitory effect on carnosine turnover mediated by CN1. Our results extend the understanding of brain carnosine and homocarnosine metabolism, as well as carnosine regulation in other tissues, and may have implications for the treatment of diabetic nephropathy.

Materials and methods

Study cohort

Blood samples were obtained from healthy controls for measuring CN1 activity and protein concentration as described above. Healthy controls consisted of 108 adults (range 19–62 years, 36 male and 72 female) and 52 infants and young children (range 6 days–18 years). There was no significant age difference between male and female individuals. All participants or their parents gave informed consent. Further, cerebrospinal fluid was obtained from anonymised spare material in 27 children in whom a lumbar puncture was performed in as a diagnostic measure but in whom other CSF tests showed normal results. The study was approved by the ethical committee (Second Ethics Committee of the Heidelberg University; amendment no. 2 and 3 to ethical approval no. 0193/2001).

Dipeptide concentrations and CN1 activity

Carnosine and homocarnosine concentrations were measured fluorometrically by high-performance liquid chromatography as previously described by Jansen et al. (2006) and Schönherr (2002). CN1 activity was assayed according to a method described by Teufel et al. (2003). Briefly, the reaction was initiated by addition of carnosine and/or homocarnosine to serum or recombinant enzyme. The reaction was stopped after defined periods by adding 1% trichloroacetic acid. Liberated histidine was derivatized by adding *o*-phthaldialdehyde (OPA) and fluorescence was read using a MicroTek plate reader (λ_{Exc} 360 nm, λ_{Em} 460 nm). Recombinant human CN1 was obtained from R&D Systems (Minneapolis, USA).

CN1 ELISA

To assess the amount of CN1 protein we developed a capture ELISA for the detection of human CN1. To this end, we raised monoclonal antibodies against recombinant human CN1. Balb/c mice were immunized with the recombinant protein and boosted after 2 and 4 weeks, respectively. Serum was collected to assess the antibody titre. Hereafter, the mice were sacrificed and splenocytes were fused with SP2/0 myeloma cells according to standard procedures. After fusion, the cells were plated at a density of 0.5 cells/well. Wells containing proliferating cells were tested for the presence of anti-carnosinase antibody by indirect immune fluorescence (IIF). Positive cultures were subsequently seeded at a density of 0.5 cells/well and retested by IIF. Two stable anti-carnosinase clones, i.e. RKYS1 and RYSK173 (both IgG1), were selected for further analysis. The antibodies recognize an epitope in, or near, the dimerization domain of the CN1 protein.

A human CN1 ELISA was developed by coating high absorbant microtitre plates (Greiner BioChemia, Fracht, Germany) overnight with 100 μ l of goat polyclonal anti-human CN1 (10 μ g/ml) (R&D, Wiesbaden Germany). The plates were extensively washed and incubated with 5% W/V of dry milk powder to avoid nonspecific binding. For each sample and standard serial dilutions were carried out. The plates were placed on a shaker for 1 h and subsequently extensively washed with PBS/Tween. Hereafter purified anti-human carnosinase monoclonal IgG (clone RKYS1 or RYSK173, both gave identical results) was added for 1 h followed by extensively washing. Biotinylated goat anti-mouse IgG was added for 1 h followed by avidin-HRP. The plates were washed after each incubation. After addition of deep-blue peroxidase (POD) substrate (Roche diagnostics, Mannheim, Germany) the reaction was stopped after 15 min by addition of 50 μ l of 1 M H_2SO_4 and read in an ELISA reader at 450 nm. CN1 protein concentrations were assessed in the linear part of the dilution curve. Sensitivity of the ELISA was approximately 20 ng/ml.

Western blot analysis

Fifteen microliter of human serum was depleted for albumin and immunoglobulin using a commercially available kit (Amersham, Freiburg, Germany). After depletion, the sample was approximately 40 \times diluted. Ten microliter of the diluted sample was denatured by boiling for 10 min in β -mercaptoethanol containing Laemmli sample buffer (Bio-Rad, München, Germany) prior to separation by 10% SDS-PAGE. In some experiments N-deglycosylation was performed by PNGase F treatment. Thereafter proteins

were precipitated from the diluted sample by addition of 5 volumes of acetone and subsequently centrifuged for 10 min at 15.000 RPM. The pellet was dissolved in denaturation buffer (5% SDS, 20 mM Tris pH 7.5, 0.4 M DTT) and process as recommended by the manufacturer (New England Biolabs, Frankfurt, Germany). After electrophoresis proteins were transferred to a PVDF membrane (Roche, Mannheim, Germany) using semi-dry blotting. Membranes were blocked for 1 h at room temperature with 5% non-fat dry milk powder dissolved in Tris-buffered saline (TBS) containing 0.1% Tween 20 (Sigma, Steinheim, Germany). The blots were incubated overnight at 4°C with a mouse monoclonal anti-CN1 antibody (Atlas Antibodies, Stockholm, Sweden). After washing with TBS/Tween, the membranes were incubated with a horseradish peroxidase—conjugated secondary antibody (Santa Cruz Biotechnology, Heidelberg, Germany) for 1 h at room temperature. Immunoreactive bands were visualized using chemiluminescence (PerkinElmer, Boston, USA).

Results

Carnosine and homocarnosine degradation in serum and CSF

CN1 activity for carnosine as a substrate shows great variability but clearly increases with age for both serum (Fig. 1) and CSF (data not shown). CN1 activity was considerably higher in serum than in CSF (Table 1), and serum activities were non-significantly higher in females (3.4 ± 1.1 μ mol/ml/h, $n = 62$) than in males (2.9 ± 0.8 μ mol/ml/h, $n = 32$).

Homocarnosinase activity was below the detection limit in young children in both CSF and sera. For older controls, activities ranged from 0.12 to 0.14 μ mol/ml/h in serum and

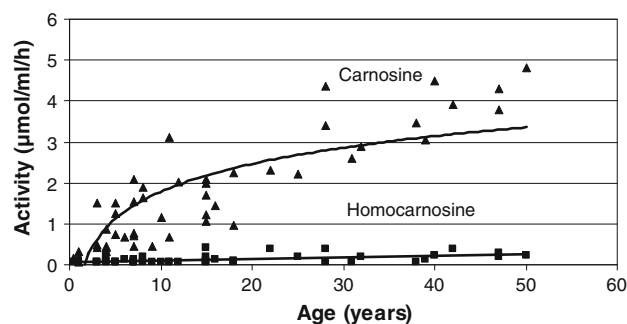


Fig. 1 CN1 activity in serum comparing both substrates (carnosine and homocarnosine) in healthy controls. CN1 activity varies between individuals and increases with carnosine as substrate (triangle) as a function of age, whereas CN1 activity with homocarnosine as substrate (square) is generally lower and remains stable with increasing age

Table 1 CN1 activity in serum and CSF for carnosine and homocarnosine as substrate

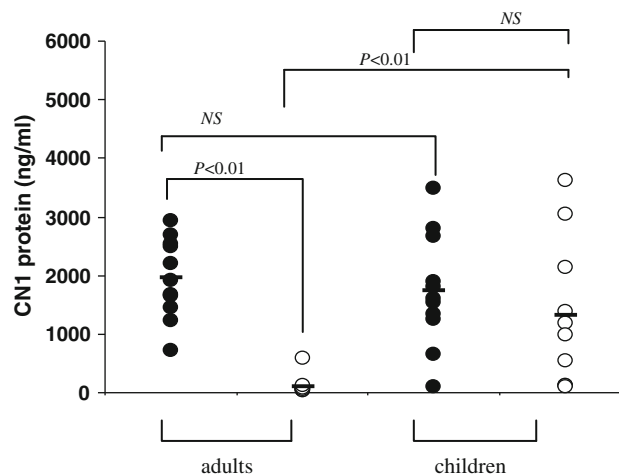
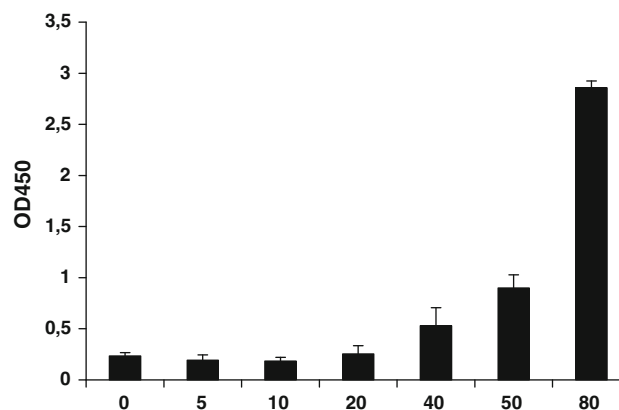
Material	Age (years)	CN1 activity with carnosine as substrate [$\mu\text{mol/ml/h}$]	N	CN1 activity with homocarnosine as substrate [$\mu\text{mol/ml/h}$]	n
Serum	0–1	0.34 ± 0.24	10	<0.05	5
	2–3	0.63 ± 0.4	12	<0.05	6
	4–10	1.3 ± 0.9	30	0.12 ± 0.06	14
	11–20	2.3 ± 1.2	20	0.14 ± 0.09	25
	Above 20	3.2 ± 0.9	94	0.12 ± 0.04	16
CSF	0–1	0.07 ± 0.006	5	<0.05	3
	2–3	0.17 ± 0.08	10	0.08 ± 0.02	5
	4–10	0.27 ± 0.15	14	0.09 ± 0.05	9
	11–20	0.35 ± 0.2	10	0.07 ± 0.03	9
	Above 20	0.8 ± 0.22	15	0.11 ± 0.09	11

from 0.07 to 0.11 $\mu\text{mol/ml/h}$ in CSF. The increase of CN1 activity with age was much less pronounced for homocarnosine than for carnosine (Fig. 1), and activity for homocarnosine was not significantly different in serum and CSF (Table 1). Furthermore the gender difference was less pronounced for homocarnosine than for carnosine (females = 0.113 ± 0.05 , males = 0.109 ± 0.06). Carnosine turnover increased with increasing carnosine concentrations, while increasing homocarnosine concentrations did not lead to a significant increase in homocarnosine turnover, suggesting that enzyme saturation is already achieved at homocarnosine concentrations of 40 $\mu\text{mol/l}$.

Because CN1 activity for carnosine as substrate increased in an age-dependent fashion, we next assessed if the amount of CN1 protein was different in children and adults. In adults CN1 protein was almost undetectable in serum, while in EDTA-plasma significant amounts were detected. In contrast, CN1 protein was easily detected in both serum and EDTA plasma from children (Fig. 2). Although CN1 activity was significantly lower in children than in adults, similar amounts of CN1 protein were detected in EDTA plasma.

Since CN1 protein was always detected in plasma but, with the exception of one individual, not in serum of adults, our data might suggested that serum CN1 is present in different conformations. Accordingly, we added DTT to sera of adults to potentially stabilize CN1 and assessed if detection of CN1 by ELISA occurred under this condition. DTT dose-dependently increased OD450 values, suggesting that detection of CN1 in serum by ELISA depends on the conformation the protein (Fig. 3).

Inasmuch as the amount of CN1 protein in serum was significantly higher in children compared to adults and addition of DTT increased the detection of CN1 in ELISA,

**Fig. 2** CN1 was measured by ELISA in serial dilutions of serum (open circles) or EDTA plasma (closed circles) obtained from children ($n = 11$) and adults ($n = 10$) as described in materials and methods. CN1 concentrations were determined in the linear part of the dilution curve. The results of all individual samples are depicted; the mean of each group is depicted as a line. Statistical significance was determined by Student's *t* test, a *P* value <0.05 was considered as significant**Fig. 3** Influence of DTT on the detection of CN1 protein in serum of adults. Undiluted serum was added in the presence of different concentration of DTT to the ELISA plates. Thereafter, the ELISA was performed as described. The result of a representative serum obtained from an adult is depicted. At least three different sera were tested; they gave essentially the same results

we used Westernblot analysis to determine whether monomeric CN1 was more prevalent in children than adults. Under non-reducing conditions two immune-reactive bands with apparent molecular weight of approximately 150 and 65 kDa were observed. The molecular weight of the latter band is in concordance with that of monomeric CN1. There was no consistent difference in the amount of monomeric CN1 in serum of adults and that of children. In two out of three children, a prominent 61 kDa band was also detected, which was markedly less abundant

in serum of adults (Fig. 4a). Under reducing conditions the upper band was shifted to approximately 130 kDa while the 65 kDa band did not change in molecular weight. Although addition of DTT clearly changed the conformation of CN1, as revealed by the change in mobility during electrophoreses, CN1 was not completely reduced by DTT to the monomeric form. To assess if the 150 kDa band was a differentially N-glycosylated CN1 isoform, the samples were treated with PNGase. The 150 kDa band was partially resistant to PNGase as revealed by the appearance of a 120 and 130 kDa band. The latter was likely due to the presence of DTT in the denaturation buffer which was required for PNGase treatment. The 65 kDa band was shifted to 61 kDa after PNGase treatment, suggesting that the 61 kDa band present in serum of children is most likely a monomeric CN1 isoform completely or partially devoid of N-glycosylation (Fig. 4b).

We next assessed in a different cohort of children if there was a difference in the amount of CN1 protein in serum, EDTA plasma and CSF within the same individual. We also assessed serum CN1 activity with carnosine as substrate in this cohort. In children below 1 year of age CN1 activity was not detectable. Consistent with this finding, CN1 protein was either not detectable by ELISA or was present in very small amounts when EDTA plasma was used. In general, the amount of CN1 protein was higher in EDTA plasma compared to serum. In CSF CN1 protein concentrations were lower than in serum. There was no clear correlation between CN1 activity and the amount of CN1, either when measured in EDTA plasma or when measured in serum (Table 2).

Competition experiment with both substrates in serum

To assess the ability of CN1 to utilize carnosine or homocarnosine, we measured CN1 activity with different combinations of carnosine and homocarnosine in serum samples obtained from healthy adults. The addition of increasing amounts of homocarnosine reduced CN1 activity with carnosine significantly in both serum (Fig. 5) and CSF (data not shown) compared to carnosine as sole substrate. The addition of 80 $\mu\text{mol/l}$ homocarnosine decreased the carnosinase activity for carnosine from 2.6 ± 0.15 to 1.8 ± 0.1 $\mu\text{mol/ml/h}$ significantly ($n = 5$; $P < 0.01$; Student's t test) and 800 $\mu\text{mol/l}$ homocarnosine decreased the activity to 0.6 ± 0.09 $\mu\text{mol/ml/h}$ ($n = 5$; $P < 0.01$; Student's t test). In contrast, another dipeptide (Leu-His) which is not degraded by carnosinase did not show any effect on CN1 activity (Fig. 5). Inhibition was immediate but reversible and enzyme activity recovered within 5–10 min. Hence, our results indicate that CN1 activity toward carnosine is inhibited by homocarnosine. Inhibition was dependent upon the amounts and ratio of both dipeptides in

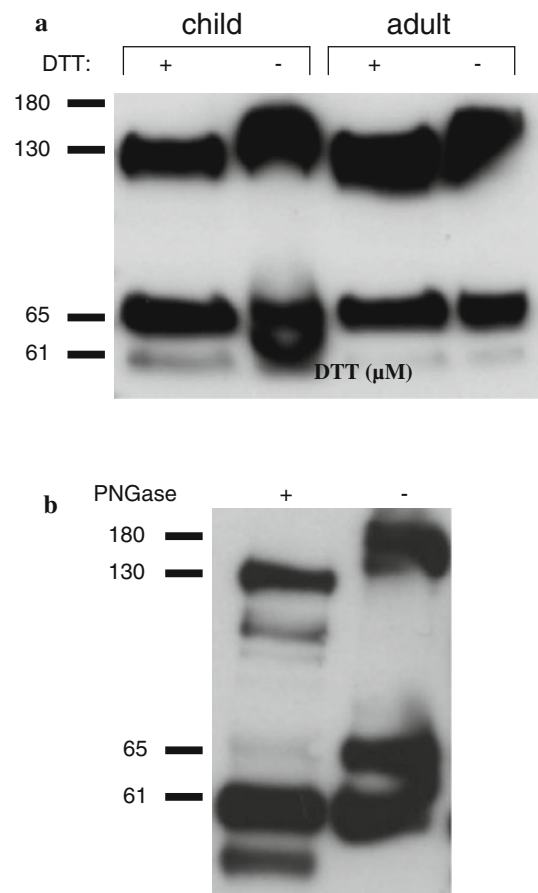


Fig. 4 Expression of CN1 in serum of adults and children. Albumin and IgG depleted serum was subjected to SDS-PAGE and Western blotting. **a** The samples were either treated with DTT (reducing conditions) or were left untreated. Under reducing conditions two bands with apparent molecular weight of 130 and 65 kDa respectively were detected. The latter band is in concordance to the molecular weight of monomeric CN1. Under non-reducing conditions immunoreactive band of approximately 150 and 65 kDa were detected. Note the presence of an additional 61 kDa band in serum of children. This was observed in two out of three children, while in adults ($n = 3$) this band was either not detected or weakly expressed. **b** Serum of a child was either treated with PNGase to remove N-glycosylation, or left untreated. SDS-PAGE was performed under non-reducing conditions for the untreated sample. Since DTT was present in denaturation buffer for PNGase, SDS-PAGE for the treated sample was performed under reducing conditions. The 150 kDa band was partly resistant to PNGase as demonstrated by the appearance of a 120 kDa band. The 130 kDa band occurred due to the presence of DTT in the denaturation buffer. The 65 kDa band was shifted to 61 kDa, corresponding to the lower band in the untreated serum. The lower band in the treated sample may represent a completely N-deglycosylated CN1 monomer or degradation product. The result of a representative blot ($n = 3$) is depicted in **a**, **b**

serum (Table 3). For all samples, carnosine turnover at a substrate concentration of 160 μM carnosine was between 1.3 and 1.8 $\mu\text{mol/ml/h}$ and addition of homocarnosine lowered the degradation significantly ($P < 0.01$, Student's t test). Activity decreased to 0.8–1.2 $\mu\text{mol/ml/h}$ when homocarnosine concentration was added (320 μM), and to

Table 2 Serum CN1 activity and protein concentration in serum, EDTA plasma and CSF of children

Age ^a	CN1 activity ^b	CN1 protein Plasma ^c	CN1 protein Serum ^c	CN1 protein CSF ^c
6 days	0	Not detectable	Not detectable	Not detectable
2 months	0	24	Not detectable	Not detectable
6 months	0	34 ± 5	Not detectable	Not detectable
9	0.3 ± 0.04	15,018 ± 1,852	788 ± 392	262 ± 35
1	0.01 ± 0.001	199 ± 21	Not detectable	Not detectable
1.5	0.1 ± 0.02	170 ± 78	39 ± 1	50 ± 14
2	0.1 ± 0.02	107 ± 12	90 ± 20	42 ± 23
4	2.6 ± 0.2	520 ± 277	304 ± 84	37 ± 18
6	1.8 ± 0.2	2,384 ± 821	1,617 ± 721	190 ± 11
9	0.4 ± 0.05	2,216 ± 647	1,704 ± 856	288 ± 7
12	2.2 ± 0.2	1,307 ± 551	450 ± 20	28
13	1.4 ± 0.1	11,650 ± 403	7,980 ± 2,458	181 ± 48

^a Age is provided in years as otherwise stated in months or days

^b CN1 activity was measured three times and is expressed in $\mu\text{mol/ml/h}$

^c CN1 concentrations are expressed as ng/ml

0.4–0.8 $\mu\text{mol/ml/h}$ with homocarnosine concentrations of 800 μM . Similar results were obtained for lower carnosine concentrations (data not shown).

We analyzed homocarnosine and carnosine degradation and histidine formation in samples with either carnosine as sole substrate or with both homocarnosine and carnosine together. When both substrates were available, both were degraded simultaneously (Fig. 6), but carnosine degradation and histidine formation were reduced in the presence of homocarnosine. We tested this effect for carnosine concentrations of 40, 80, 160, 320 and 960 μM in combination with homocarnosine concentrations of 160, 80 and 0 μM . Independent of the ratio of carnosine to homocarnosine, we observed significantly lower CN1 activities ($P < 0.01$; Student's t test.) in the presence of homocarnosine (data not shown), even when carnosine concentration was 12-fold higher than homocarnosine concentration.

Activity of recombinant human CN1

To confirm that carnosine and homocarnosine turnover in serum reflected CN1 activity and that no other serum component influenced the enzymatic reaction, we repeated the results with the recombinant human CN1. Without

homocarnosine the apparent K_m for the conversion of carnosine to β -alanine and L-histidine was 175 μM carnosine and V_{\max} about 440 $\text{pmol/min}/\mu\text{g}$. The addition of 80 μM homocarnosine lowered V_{\max} to 356 $\text{pmol/min}/\mu\text{g}$ and increased K_m to a concentration of 210 μM carnosine. Enzyme activity in the presence of 160 μM carnosine and 80 μM homocarnosine was 264 $\text{pmol/min}/\mu\text{g}$ (± 22.4 ; $n = 5$) whilst increasing homocarnosine to 160 μM lowered the maximal activity significantly ($P < 0.01$; Student's t test) to 122 $\text{pmol/min}/\mu\text{g}$ (± 16.2 ; $n = 5$). An activity of 40 $\text{pmol/min}/\mu\text{g}$ (± 5.3 ; $n = 5$) was observed when homocarnosine was present in a concentration of 480 μM . The estimated K_i for homocarnosine was 240 μM which shows a reduced affinity of CN1 toward carnosine in the presence of homocarnosine.

Carnosine turnover and homocarnosine concentrations in vivo

The correlation between carnosine turnover and homocarnosine concentrations in serum of control individuals is depicted in Fig. 7. Higher homocarnosine concentrations were associated with lower CN1 activity, in line with an inhibitory effect of homocarnosine on CN1 activity.

Fig. 5 CN1 activity with carnosine and an additional dipeptide. Carnosine degradation in serum can be lowered by the addition of homocarnosine (*striped*) whereas the addition of another dipeptide Leu-His (*gray*) has no effect on carnosine degradation ($n = 5$). With increasing amount of homocarnosine, CN1 activity for carnosine degradation decreases

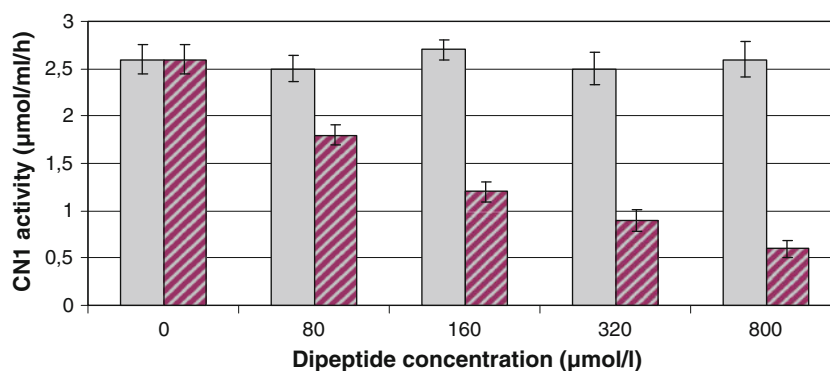
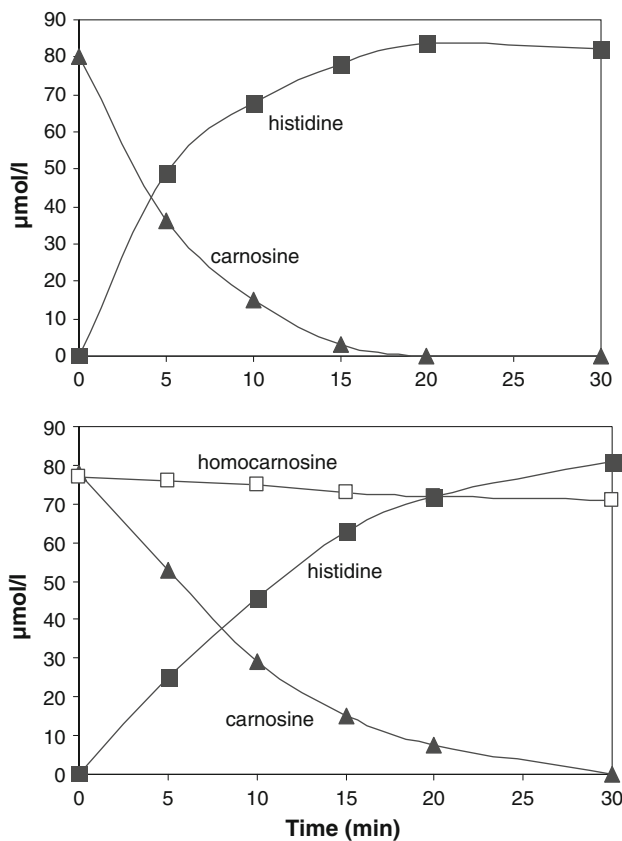


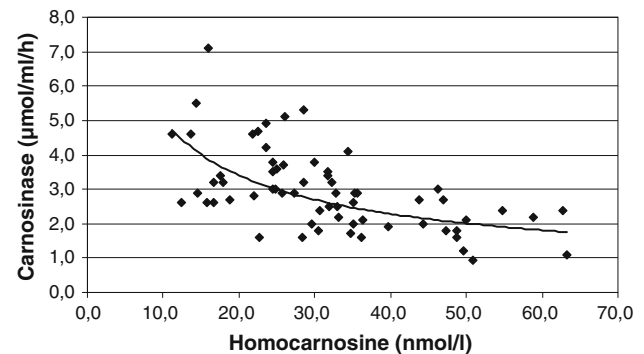
Table 3 CN1 activity in serum with a substrate combination of carnosine and homocarnosine

Ratio carnosine–homocarnosine	Substrate concentration		Activity [$\mu\text{mol/ml/h}$]				
	Carnosine (μM)	Homocarnosine (μM)	Control 1	Control 2	Control 3	Control 4	Average
	80	–	1.2 ± 0.1	0.9 ± 0.11	1.2 ± 0.1	1.3 ± 0.12	1.2
	160	–	1.8 ± 0.12	1.8 ± 0.14	1.5 ± 0.14	1.3 ± 0.4	1.6
	480	–	2.6 ± 0.1	2.8 ± 0.12	2.1 ± 0.2	3.1 ± 0.25	2.7
1:2	160	320	0.9 ± 0.01	1.2 ± 0.1	1.1 ± 0.26	0.8 ± 0.1	1.0
1:2	80	160	0.7 ± 0.14	0.7 ± 0.1	1.1 ± 0.2	0.9 ± 0.17	0.9
1:5	160	800	0.7 ± 0.12	0.8 ± 0.21	0.4 ± 0.06	0.6 ± 0.08	0.6
1:5	80	400	0.7 ± 0.14	0.7 ± 0.2	0.6 ± 0.06	0.6 ± 0.1	1.0

**Fig. 6** Carnosine degradation (triangle) and histidine formation (square) with and without the addition of homocarnosine (open square). Carnosine degradation is slower in the addition of homocarnosine compared to carnosine as the only substrate

Discussion

In the present study we provide further data on CN1 activity and protein concentrations in serum and cerebrospinal fluid (CSF) of children and adults. The main findings of this study are the following. First, in children CN1 activity in serum is lower than adults but this is not reflected by differences in CN1 protein concentrations.

**Fig. 7** CN1 activity with carnosine as substrate in serum and homocarnosine concentration in plasma was measured in healthy adults. Carnosinase degradation is correlated with homocarnosine concentration

Second, CN1 is present in different allosteric conformations in children and adults. This is suggested by the observation that CN1 is always detected in sera of children by ELISA, while 90% of the adults were negative. In contrast, both children and adults were positive in CN1 ELISA when EDTA-plasma was used. Third, in all sera homocarnosine dose-dependently inhibited CN1 activity toward carnosine. The amount of homocarnosine in serum and CSF correlated inversely with CN1 activity.

Whereas CN1 activity for carnosine as substrate is well characterized (Teufel et al. 2003), little is known about its role in homocarnosine metabolism. CN1 is the only known enzyme with relevant activity toward both dipeptides, but the biological basis of the divergent concentrations of carnosine and homocarnosine in blood (low amounts of both dipeptides) and CNS (low amounts of carnosine but higher amounts of homocarnosine) has not been well characterized. Enzyme analyses carried out in the present study showed that homocarnosine turnover in blood is much lower than carnosine. CN1 activity in serum and CSF differ for carnosine and homocarnosine. In all age groups, CN1 activity for carnosine is four to six times higher in serum than in CSF, while this effect is not observed for

homocarnosine as substrate. Although ELISA demonstrated higher amounts of CN1 protein in serum than in CSF, this does not sufficiently explain the biochemical patterns observed *in vivo*.

Importantly, our study demonstrates that CN1 seems to be present in a different allosteric conformation in children as compared to adults. Several arguments are in favor of this assumption. First, CN1 activity was significantly lower in children compared to adults, yet CN1 protein was seldomly detected in serum of the latter, while in all sera obtained from children CN1 protein was measured by ELISA. Because EDTA plasma of adults was always positive for CN1, we propose that in sera of adults the CN1 protein is either masked or not in the appropriate conformation for detection by ELISA. Second, addition of DTT to the sera significantly increased the OD450 values, suggesting that conformational changes of CN1 render the sera to become positive in ELISA.

It might be argued that addition of DTT confers CN1 dimers into monomers, and that the CN1 ELISA is preferentially measuring monomeric CN1. Differences between children and adults in the serum CN1 ELISA therefore could be due to differences in mono versus dimeric CN1. Westernblot analysis, however, demonstrated that there was no consistent difference in monomeric CN1 between sera from children and adults. We did, however, observe the presence of a monomeric CN1 isoform that is devoid of N-glycosylation. This isoform was abundantly present in two out of three sera from children but was weakly present or absent in sera from adults. It should also be emphasized that if the CN1 ELISA is only recognizing monomeric CN1, a positive ELISA in EDTA plasma would not be expected.

To our knowledge, this is the first report suggesting that *in vivo* CN1 is present in different allosteric conformations. Although our study does not provide a structural explanation for the different allosteric conformations, recently a study by Vistoli et al. (2006) has postulated that citrate ions can alter the conformation and activity of CN1. Given the fact that in the active center of CN1 different metal ions may be bound, it is conceivable that this might result in different allosteric conformations. Qualitative differences in, or the lack of, metal ion binding might explain a different CN1 conformation and low activity in children. The influence of metal ion binding, e.g. Mn or Cd, on CN1 activity has already been demonstrated (Lenney et al. 1982).

The results of the present study also indicate that dipeptide and dipeptidase patterns observed in blood and CSF are partially explained by a competitive inhibitory effect of homocarnosine on CN1. In control subjects there was a negative correlation between carnosine turnover and homocarnosine concentrations. Competition experiments in serum and with recombinant human CN1 showed that

the presence of homocarnosine reduced CN1 activity for carnosine even at low homocarnosine concentrations whereas CN1 activity was unaffected by the addition of histidine or Leucine–Histidine (Leu–His) dipeptide. Hence, the correlation observed in controls was not explained by a cofactor or confounding variables. Rather, reduced carnosine degradation in the presence of homocarnosine is most likely due to competition of both substrates binding at the same active site of the CN1 enzyme. Since the turnover of homocarnosine is lower and carnosine cannot enter the active site while homocarnosine binds, the degradation of carnosine is also lowered. Even a 12-fold higher carnosine concentration compared to homocarnosine did not out-compete homocarnosine in binding to the enzyme. The inhibitory effect is reversible since after cleavage or removal of homocarnosine, carnosine metabolism is comparable to those samples in the absence of homocarnosine. Decreased carnosinase activity was not found for the dipeptide Leu–His showing that the inhibition is due only to homocarnosine. Our findings are consistent with those of Margolis et al. (1979) who demonstrated an inhibitory effect of homocarnosine on carnosinase.

The physiological role of carnosine is still uncertain. Carnosine is mainly found in skeletal muscle and the concentration is closely related to muscle activity. Carnosine presumably exerts antioxidative effects and we recently demonstrated that a leucine repeat polymorphism in the CN1 protein, affecting CN1 secretion across the cell membrane, is associated with susceptibility to developing diabetic nephropathy Janssen et al. (2005). Homocarnosine is thought to be a precursor for the neurotransmitter GABA, and this may explain the relatively high concentrations of homocarnosine in the brain and consequently the reduced dipeptidase activity toward carnosine.

In conclusion, homocarnosine appears to be an effective regulator of CN1 activity, and therefore its concentration might be an important metabolic parameter affecting the carnosine pathway. Moreover, a possible therapeutic option of homocarnosine for patients at risk for diabetic nephropathy has to be considered with caution. On the one hand, we would expect that homocarnosine treatment could reduce the serum degradation of carnosine. On the other hand, high concentrations of homocarnosine might lead to high concentrations of its degradation products γ -aminobutyric acid (GABA) and histidine. Histidine can be easily converted into histamine and the impact on autonomic nerve activity would be unpredictable and could result in further progression of renal insufficiency, since progressive renal disease has been shown to be associated with increased nerve activity.

However, the regulatory mechanisms of carnosine synthesis and degradation, its physiological role and a possible relevance of reduced activity require further investigation.

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