ORIGINAL ARTICLE

Different conformational forms of serum carnosinase detected by a newly developed sandwich ELISA for the measurements of carnosinase concentrations

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Abstract Serum carnosinase (CN-1) measurements are at present mainly performed by assessing enzyme activity. This method is time-consuming, not well suited for large series of samples and can be discordant to measurements of CN-1 protein concentrations. To overcome these limitations, we developed sandwich ELISA assays using different anti-CN-1 antibodies, i.e., ATLAS (polyclonal IgG) and RYSK173 (monoclonal IgG1). With the ATLAS-based assay, similar amounts of CN-1 were detected in serum and both EDTA and heparin plasma. The RYSKS173-based assay detected CN-1 in serum in all individuals at significantly lower concentrations compared to the ATLAS-based assay (range: 0.1–1.8 vs. 1–50 µg/ml, RYSK- vs. ATLAS-based, $P < 0.01$). CN-1 detection with the RYSK-based assay was increased in EDTA plasma, albeit at significantly lower concentrations compared to ATLAS. In heparin plasma, CN-1 was also poorly detected with the RYSK-based assay. Addition of DTT to serum increased the detection of CN-1 in the RYSK-based assay almost to the levels found in the ATLAS-based assay. Both ELISA assays were highly reproducible (R: 0.99, $P < 0.01$ and R: 0.93, $P < 0.01$, for the RYSK- and ATLAS-based assays, respectively). Results of the ATLAS-based assay showed a positive correlation with CN-1 activity (R: 0.62, $P < 0.01$), while this was not the case for the RYSK-based assay. However, there was a negative correlation between CN-1 activity and the proportion of CN-1 detected in the RYSK-based assay, i.e., CN-1 detected with the RYSK-based assay/CN-1 detected with the ATLAS-based assay × 100% (Spearman–Rang correlation coefficient: −0.6, $P < 0.01$), suggesting that the RYSK-based assay most likely detects a CN-1 conformation with low CN-1 activity. RYSK173 and ATLAS antibodies reacted similarly in Western blot, irrespective of PNGase treatment. Binding of RYSK173 in serum was not due to differential N-glycosylation as demonstrated by mutant CN-1 cDNA constructs. In conclusion, our study demonstrates a good correlation between enzyme activity and CN-1 protein concentration in ELISA and suggests the presence of different CN-1 conformations in serum. The relevance of these different conformations is still elusive and needs to be addressed in further studies.

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Introduction

Carnosine-like peptides, i.e., L-carnosine (β-alanyl-L-histidine), homocarnosine (γ-aminobutyryl-L-histidine) and anserine (β-alanyl-l-1-methylhistidine), are a family of histidine derivatives that are present in a wide variety of vertebrate tissues, e.g., skeletal muscles and brain (Bonfanti et al. 1999). Elevated carnosine concentrations in human skeletal muscle lead to improved muscle performance in high-intensity exercise in both untrained and trained individuals (Harris et al. 2006; Hill et al. 2007). This suggests an important role of the dipeptide in the homeostasis of contracting muscle cells during anaerobic energy delivery, most likely by acting as a physiological pH buffer (Derave et al. 2007; Stout et al. 2007). Apart from muscle performance improving properties (Harris et al. 2006; Hill et al. 2007; Derave et al. 2007; Stout et al. 2007), L-carnosine seems to be implicated in neuroprotection (Tabakman et al. 2002) and to play a role in the development of the olfactory system and organization of hypothalamic neuronal networks (De Marchis et al. 2000).

Plasma carnosine concentrations vary significantly over a 24-h period, depending on intestinal absorption of intact carnosine (Gardner et al. 1991; Park et al. 2005) muscle exercise (Baguet et al. 2009) and activity of the carnosine synthetase (Drozak et al. 2010). In addition, carnosine concentrations in plasma and tissue depend on the activities of intra- and extracellular dipeptidases known as carnosinases.

Carnosinases, first isolated from the porcine kidney (Hanson and Smith 1949), are widely distributed in tissues of rodents and higher mammals (Drozak et al. 2010; Hanson and Smith 1949; Margolis et al. 1983; Wood 1957). Two types of carnosinases have been identified in humans, encoded by the *CNDP1* and *CNDP2* genes (Teufel et al. 2003). The gene products are also known as serum carnosinase (CN-1) and non-specific carnosine dipeptidase (CN-2), respectively. While CN-1 was identified as a dipeptidase that specifically hydrolyzes carnosine and homocarnosine, CN-2 has a broader specificity than CN-1 and is not able to hydrolyze homocarnosine and is sensitive to inhibition by bestatin (Teufel et al. 2003).

We have recently demonstrated that susceptibility to diabetic nephropathy (DN) is strongly associated with a $(CTG)_n$ polymorphism in the *CNDP1* gene (Janssen et al. 2005) affecting serum carnosinase secretion (Riedl et al. 2007). Diabetic patients homozygous for $(CTG)_5$ have a lower risk of developing diabetic nephropathy and have a lower plasma CN-1 activity (Janssen et al. 2005). Interestingly, overexpression of *CNDP1* in the Db/Db model results in early onset of diabetes, while in Db/Db mice fed with carnosine the onset of diabetes is retarded (Sauerhofer et al. 2007).

Currently, carnosinase measurements are mostly performed by measuring enzyme activity (Teufel et al. 2003;

Janssen et al. 2005; Lenney et al. 1982; Wassif et al. 1994). Yet, CN-1 activity can be modulated by the presence of homocarnosine (Peters et al. 2010), binding of metal ions in the active center (Lenney et al. 1982) and N-glycosylation of CN-1 (Riedl et al. 1984). Hence, serum CN-1 activities can be discordant to CN-1 protein concentrations. In order to assess whether a low serum CN-1 activity is due to a low CN-1 concentration, estimation of the amount of CN-1 in serum is required. Although this could be performed by Western blotting in a semi-quantitative fashion, this method is time-consuming and therefore is not well suited for routine testing of numerous samples. The present study was therefore conducted to set up ELISA assays that would allow the measurement of CN-1 in large series of individuals.

Research design and methods

Cell culture and transfection

Cos-7 cells (Invitrogen, Karlsruhe, Germany) were cultured in Dulbecco's Modified Eagle Medium (DMEM, PAA The Cell Culture Company, Pasching, Austria) enriched with 10% FCS and 1% penicillin/streptomycin at 37°C and 5% CO_2. The cells were co-transfected with the various CNDP1 constructs and pCruz-GFPTM (Santa Cruz, Heidelberg, Germany) by Nanofectin according to the manufacturer's instructions (PAA, The Cell Culture Company, Pasching, Austria). Four hours later, the medium was replaced by DMEM medium containing 1% penicillin/ streptomycin. Cells and supernatants were analyzed 48 h later. Transfection efficiency was assessed on an aliquot of the cell suspension by FACS analysis using green fluorescent protein (GFP) as readout. In general, transfection efficiency was above 70%. In all experiments, supernatants were concentrated using a Centricon Centrifugal Filter device of 30.000 MW (Millipore, Schwalbach, Germany). Cells were lysed on ice by the addition of 1% Triton $\times 100$ containing lysis buffer, supplemented with 1 mM 1,4-dithiol-DL-threitol (Fluka Chemie GmbH, Buchs, Germany), phosphatase-inhibitor (Sigma, Steinheim, Germany) and protease-inhibitor (Roche, Mannheim, Germany). Cell lysates were centrifuged for 10 min (14,000g at 4°C) to remove insoluble debris.

Generation of RYSK173 CN-1 monoclonal antibody

Recombinant human CN-1 was produced by using a cell-free rapid translation system (Roche, Mannheim, Germany) according to the manufacture's recommendations. Purified adjuvant-free recombinant human CN-1 was injected intraperitoneally into Balb/c mice to generate the RYSK173 monoclonal antibody. The mice were boosted 2

and 4 weeks after immunization. Serum was collected 3 days after the last boost to assess CN-1 antibody titer by indirect immune-fluorescence (IIF) on CN-1 transfected Cos-7 cells. Mice were killed and splenocytes were fused with SP2/0 myeloma cells according to standard procedures. After fusion, the cells were plated at a density of 1×10^3 cells/well. Wells containing proliferating cells were tested for the presence of anti-carnosinase antibody by IIF. Positive cultures were subsequently seeded at a density of 70–80 cells/well in a 96-well plate and retested by IIF. Clone RYSK173 (IgG1) was selected for further analysis.

CN-1 ELISA

A human CN-1 ELISA was developed by coating high-absorbent microtiter plates (Greiner, Labortechnik, Frickenhausen, Germany) overnight with 100 µl of goat polyclonal anti-human CN-1 (10 µg/ml) (R&D, Wiesbaden Germany). The plates were extensively washed and incubated with 0.05% W/V of dry milk powder to avoid unspecific binding. For each sample, standard serial dilution was carried out. The plates were placed on a shaker for 1 h and subsequently extensively washed with PBS/Tween. Thereafter, purified anti-human carnosinase monoclonal IgG (clone RYSK173) or rabbit polyclonal IgG (ATLAS, Abcam plc, Cambridge, United Kingdom) was added for 1 h followed by extensive washing. Biotinylated goat anti-mouse or goat anti-rabbit IgG was added for 1 h, extensively washed, followed by the addition of avidin-HRP. deep-blue peroxidase (POD) (Roche diagnostics, Mannheim, Germany) was used for color development, which was generally stopped after 15 min by the addition of 50 µl of 1 M H_2SO_4. The plates were directly read at 450 nm. Recombinant human CN-1 (R&D Systems, Minneapolis, USA) was used as standard. CN-1 protein concentrations were assessed in the linear part of the dilution curve. Sensitivity of the ELISA assays was approximately 20 ng/ml.

Construction of CNDP1 mutants

A cDNA clone of the CNDP1 gene containing 6 CTG-repeats (RZPD Library 983, entry No BX094414), kindly provided by Dr. M. Moeller (Department of Nephrology, RWTH, Aachen, Germany), was used as template to construct different CNDP1 variants by PCR. Glycosylation sites of CN-1 were stepwise deleted using the Quik-Change® II Site-Directed Mutagenesis Kit (Stratagene Europe, Amsterdam, Netherlands). In brief, single amino acid exchanges, i.e., asparagine for glycine, were induced in the wild-type CNDP1 cDNA by mutagenic primers to delete N322, N382 or N402 as described by Riedl et al. (1984). PCR reactions were performed according to standard procedures. All CNDP1 constructs and mutants were cloned into pCSII + mt vector. For the N-glycosylation lacking mutants, stop codons were removed to generate an myc-tagged CN-1 protein.

Western blot analysis

For detection of carnosinase in cell lysates or serum, gel electrophoreses and subsequent Western blotting were performed. In some experiments, samples were deglycosylated by PNGase F (New England Biolabs, Frankfurt, Germany) treatment according to the manufacturer's recommendations. All samples were boiled for 10 min in Laemmli sample buffer (Bio-Rad, München, Germany) prior to loading on an 8% SDS-PAGE. Proteins were transferred electrophoretically to a PVDF membrane (Roche, Mannheim, Germany) by semi-dry blotting. Thereafter, the membranes were blocked for 1 h at room temperature in TBS-Tween 20 (0.3%, Sigma, Steinheim, Germany) containing 10% milk powder. For detection of CN-1 in transfected cells or in human serum samples, mouse monoclonal anti-CN-1 antibody (Clone RYSK-173) was utilized. After incubation with appropriate horseradish peroxidase conjugated secondary antibodies (Santa Cruz Biotechnology, Heidelberg, Germany), antibody binding was visualized by enhanced chemiluminescence (PerkinElmer, Boston, USA).

Isoelectric focusing

Isoelectric focusing (IEF) was performed on precast 18-cm IPG strips with linear pH ranges using an IPGphor unit (Amersham Pharmacia, Freiburg, Germany). The strips were placed in ceramic strip holders and rehydrated in 350 µl of rehydration buffer (8 M urea, 2% CHAPS, 0.5% IPG buffer pH 3–10) containing 10 µg of serum proteins or 0.1 µg of recombinant CN-1, protease inhibitors and a few grains of bromophenol blue. The strips were covered with 2 ml dry strip cover fluid (Amersham Pharmacia, Freiburg, Germany) and rehydrated overnight by applying 30 V at constant temperature (20°C) on the IPGphor unit. The following IEF protocol was used: 30 min gradient up to 500 V, 30 min 500 V; 30 min gradient up to 2,000 V, 30 min 2,000 V; 1 h gradient up to 8,000 V, 4 h 8,000 V. After focusing, the strips were washed with water and immediately used for the second dimension. Before loading on SDS-polyacrylamide gels (PAGE), the strips were equilibrated for 20 min in equilibration buffer containing iodoacetamide. Thereafter, the strips were carefully washed with water and transferred to SDS-PAGE. The gels were sealed on top with agarose sealing solution (0.5% low melting agarose in 25 mM Tris, 192 mM glycine and 0.1% SDS). Electrophoresis was carried out overnight in 25 mM Tris, 192 mM glycine and 0.1% SDS at 15°C, applying a constant voltage of 100 V.

Measurement of carnosinase activity

Serum carnosinase activity (CN-1) was assayed according to a method described by Teufel et al. (2003). Shortly, the reaction was initiated by addition of carnosine and/or homocarnosine and was stopped by adding 1% trichloracetic acid. Liberated histidine was derivatized by adding o-phthaldialdehyd (OPA) and fluorescence was read using a MicroTek plate reader (λExc: 360 nm; λEm: 460 nm).

CNDP1 genotyping

Genomic DNA and CNDP1 genotyping was performed as described previously Janssen et al. (2005).

Statistical analysis

Quantitative data are given as mean $\pm$ SD. Student's t test was calculated to compare groups. Values of $P < 0.05$ were considered as statistically significant.

Results

Mouse splenocytes were fused with SP2/0 myeloma cells and plated at a density of 1×10^3 cells/well. A total of 740 supernatants obtained from the cells 2–3 weeks after fusion were screened for CN-1 binding by means of indirect immune-fluorescence (IIF) on cytospins made from CN-1 transfected Cos-7 cells. Out of the initial screen, 12 supernatants were found to be moderately positive in the IIF. These cells were further expanded and sub-cloned by limiting dilution in 96-well plates at a concentration of 70–80 cells/well. Based on the strong positive staining in the IIF, one clone (RYSK173) was selected after sub-cloning for further analysis (Fig. 1).

In addition to RYSK173, a rabbit polyclonal IgG (ATLAS) was used to set up ELISA assays for detection of CN-1 in serum and EDTA or heparin plasma. CN-1

detection by RYSK173 clearly differed, both qualitatively and quantitatively from CN-1 detection by ATLAS. In the latter, no differences were found for CN-1 detection in serum and EDTA or heparin plasma, while in the former detection of CN-1 was low in serum or heparin plasma, but significantly increased when EDTA plasma was used (Fig. 2a). Although addition of EDTA to serum dose dependently increased the detection of CN-1 with the RYSK-based assay (Fig. 2b), it was quantitatively lower significantly compared with the ATLAS-based assay (RYSK173 vs. ATLAS, 2.43 $\pm$ 0.52 vs. 27.6 $\pm$ 3.8 μg/ml; $P < 0.01$).

Apart from the addition of EDTA, detection of CN-1 with the RYSK-based assay could also be increased by protein denaturation. This was done either by incubating serum for 30 min at 60°C or by addition of the reducing agent DTT (Fig. 3). Detection of CN-1 with the RYSK-based assay dramatically increased when serum was incubated at 60°C, and remained high when the heated serum was placed at 4°C for an additional 20 min (Fig. 3a). Similarly, addition of DTT dose dependently increased the detection of CN-1 with the RYSK-based assay to the level as observed for the ATLAS-based assay (Fig. 3b).

Reproducibility of both ELISA assays was assessed by consecutive measurements of 40 serum samples on different days. As indicated in Fig. 4, both ELISAs were highly reproducible with a correlation coefficient (R) of 0.93 ($P < 0.01$) and 0.99 ($P < 0.01$) for the ATLAS- and RYSK-based assays, respectively (Fig. 4a, b). When the relative contribution of CN-1 detected with the RYSK-based assay to total CN-1 detected with the ATLAS-based assay was calculated, there was also a good reproducibility observed. CN-1 detected by the RYSK-based assay was in most samples below 5% of total CN-1. However in 7 out of 40 samples, this was clearly higher (Fig. 4c).

To investigate whether RYSK173 was detecting a different molecular weight variant of CN-1, we performed Western blot analyses of serum samples with all anti-CN-1 antibodies that were used in ELISA, including the catching

Fig. 1 Expression of CN-1 in transfected Cos-7 cells. Three days after transfection, coverslips of CN-1 transfected Cos-7 cells were incubated with hybridoma supernatants. The detection of CN-1 by RYSK173 is shown
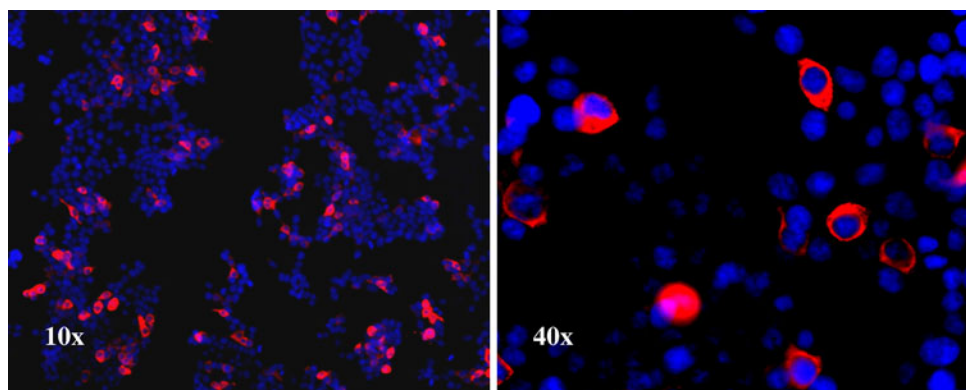
Fig. 2 Performance of CN-1 ELISA assays in serum and plasma samples. **a** Serum, EDTA- and heparin plasma obtained from five different individuals were tested in ELISA for CN-1 using ATLAS or RYSK173 as detecting antibody. In each assay, a standard curve with recombinant CN-1 was included to assess the concentration of CN-1 in the samples. The results for each individual are depicted by *different symbols* and expressed as mean CN-1 concentration (μg/ml). The mean of each group is depicted by a *horizontal line*. **b** Different concentrations of EDTA were added to a serum sample and CN-1 was subsequently assessed in ELISA by RYSK173. The results are obtained from one individual and expressed as mean concentration (μg/ml) ±SD of triplicate measurements
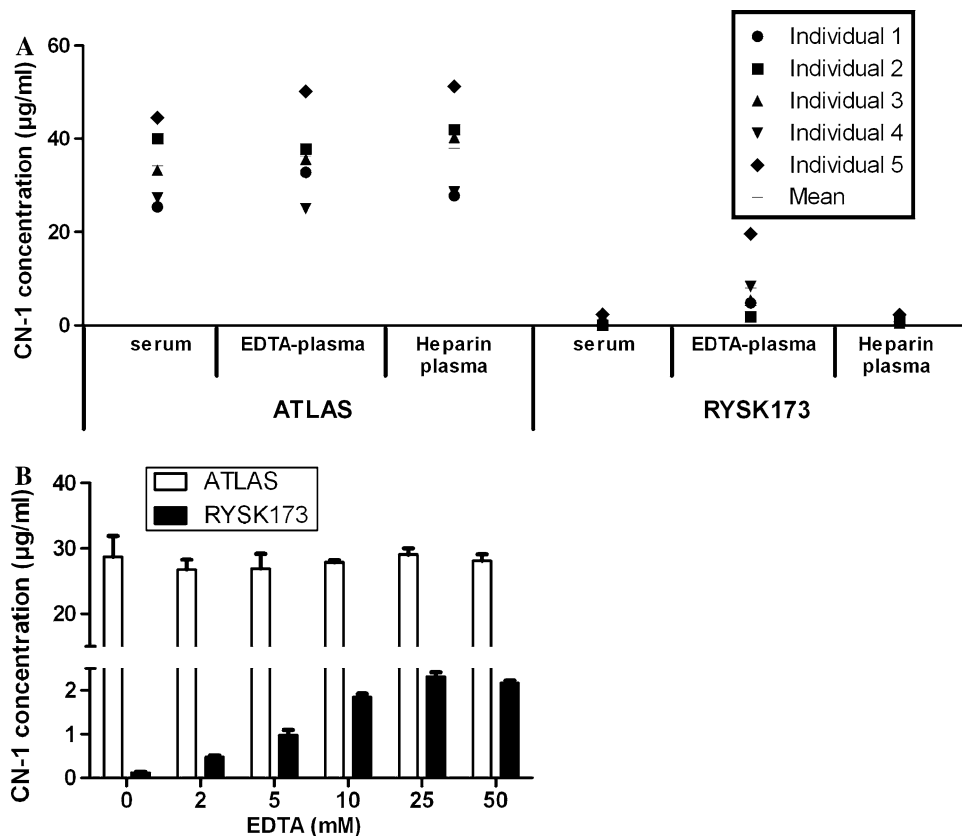
goat anti-human CN-1 polyclonal IgG. As depicted in Fig. 5a, all anti-CN-1 antibodies recognized a 65-kDa band under reducing and non-reducing conditions. While ATLAS, and to a lesser extent RYSK173, also reacted with a high molecular weight band of approximately 150 kDa, this was not observed with the goat anti-human CN-1 polyclonal IgG from R&D. To assess whether the upper band was a differentially N-glycosylated CN-1 isoform, aliquots of the samples were treated with PNGase. PNGase treatment resulted in a shift of both bands as shown in Fig. 5b, demonstrating that both bands were N-glycosylated. Isoelectric focusing of human serum and recombinant CN-1 followed by PAGE and Western blotting with RYSK173 showed that RYSK173 recognized a major protein with the expected pI of 4.4 for serum carnosinase (Fig. 5c). Because not all putative N-glycosylation sites within CN-1 are necessarily used for glycosylation, we also tested if RYSK173 recognized differentially N-glycosylated CN-1 forms. To this end, we transfected Cos-7 cells with myc-tagged CN-1 variants that lack 1, 2 or all 3 putative N-glycosylation sites by exchanging asparagine residues at N322, N382 and N402 for glycine. Although the expression of the differentially glycosylated variants largely differ, all variants were recognized by RYSK173 and ATLAS to a similar extent (Fig. 5d).

We next assessed in a total of 33 serum and corresponding EDTA samples, CN-1 concentrations using both ELISA assays and addressed whether enzyme activity correlated with serum CN-1 concentrations. As already shown in Fig. 2, the amount of CN-1 detected with the ATLAS-based assay did not significantly differ between serum and EDTA plasma (for serum: mean ± SD 24.8 ± 8.9 [range 11.7–40.8] μg/ml, for plasma 17.6 ± 5.3 [range 9.3–27.2]). While in most sera, the concentration of CN-1 detected with the RYSK-based assay was low (mean ± SD 0.2 ± 0.8 [range 0–4,8] μg/ml), for all individuals CN-1 concentrations were significantly higher in EDTA plasma when tested in the RYSK-based assay. These concentrations where however significantly lower when compared with ATLAS-based assay (mean ± SD 2 ± 2.1 [range 0.4–11.6] μg/ml, $P < 0.01$). CN-1 concentrations as assessed by the ATLAS-based assay correlated with CN-1 activity (Fig. 6a), but this was not found for the RYSK-based assay (data not shown). However, there was a negative correlation between CN-1 activity and the proportion of CN-1 detected with the RYSK-based assay (Spearman-Rang-correlation coefficient: −0.6, $P < 0.01$), suggesting that the RYSK-based assay most likely detects a CN-1 conformation with low activity (Fig. 6b). Similar to that reported previously for CN-1 activity, it was found that in $(CTG)_5$ homozygous individuals CN-1 concentrations were significantly lower compared to other $(CTG)_n$ genotypes (Fig. 6c). Differences between the groups were not attributed to gender or age
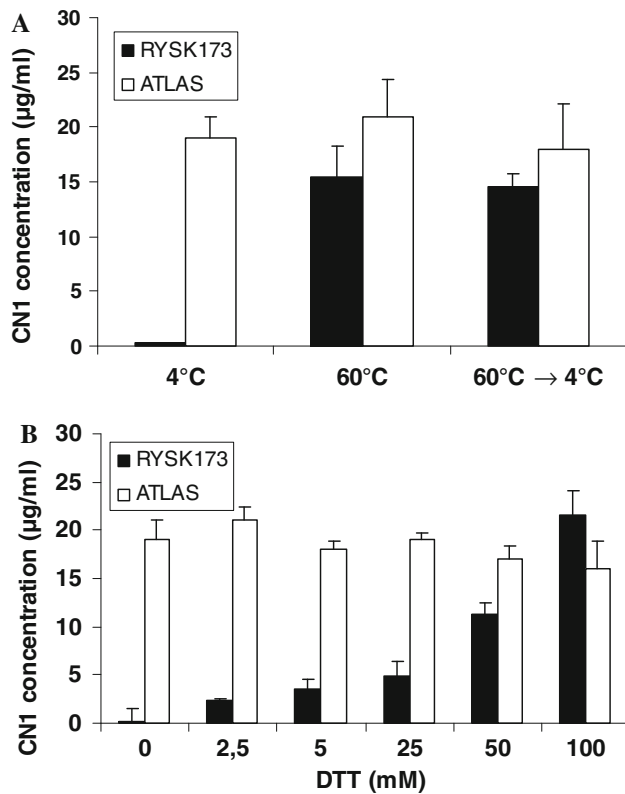
Fig. 3 CN-1 detection by RYSK173 is increased upon denaturation of serum. **a** Serum was either incubated at 4 or 60°C for 30 min. In addition, a serum sample was first incubated at 60°C for 30 min and subsequently put on ice for 20 min before assessment of CN-1. In all samples, CN-1 was detected by ELISA using ATLAS or RYK173 as detecting antibody. **b** Different concentrations of DTT were added to serum. Thereafter, CN-1 was detected by ELISA using ATLAS or RYK173 as detecting antibody. **a**, **b** The results of a representative experiment are depicted and expressed as mean concentration (μg/ml) ± SD of triplicate measurements. A total of four different individuals were tested, with essentially similar results

Fig. 4 Reproducibility of CN-1 detection in the ATLAS-based (**a**) and the RYSK-based (**b**) assay. A total of 40 serum samples were measured on two different days. **a**, **b** The results are expressed as μg/ml. **c** The relative contribution of CN-1 detected by the RYSK-based assay to total CN-1 was calculated as follows: (CN-1 measured with the RYSK-based assay/CN-1 measured with the ATLAS-based assay) × 100%. The results are expressed as percentage of total CN-1

differences, as no significant distribution was found between the groups [$(CTG)_5$ homozygous: $n = 14$, 7 males and 7 females, other genotypes: $n = 19$, 9 males and 10 females].

Discussion

To date, most available methods for measuring carnosinase in serum are based on measuring enzyme activity (Lenney et al. 1982; Wassif et al. 1994; Murphey et al. 1972). Carnosinase activity is however modulated by binding of metal ions (Lenney et al. 1982), the presence of homo-carnosine (Peters et al. 2010) and N-glycosylation of the enzyme (Riedl et al. 1984). Hence, carnosinase activity might be discordant to carnosinase protein concentrations. To assess whether low carnosinase activity is due to low enzyme concentrations, it is therefore crucial to estimate
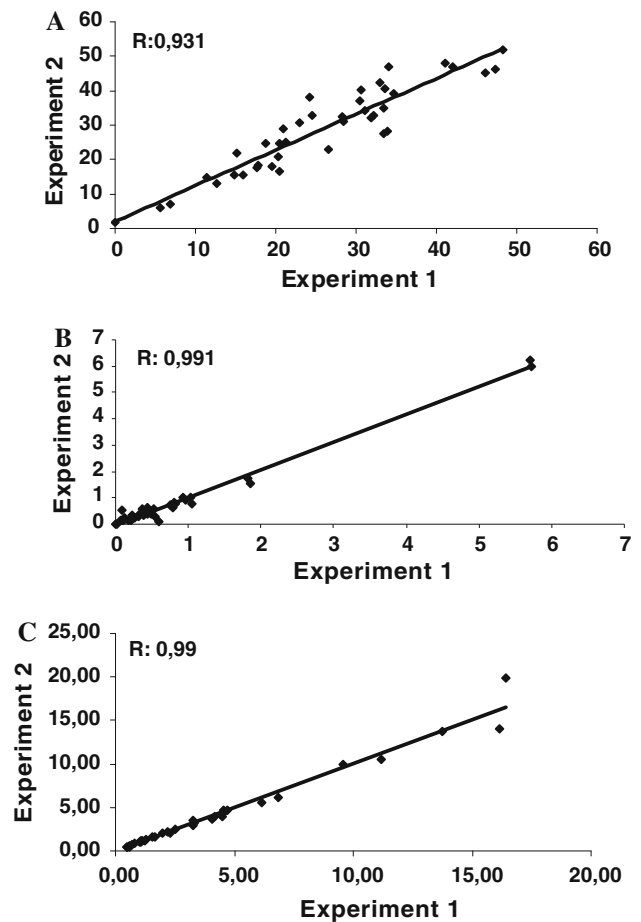
the concentration of CN-1. In the present study, we describe ELISA assays for assessment of CN-1 in serum or plasma samples. The main findings of this study are the following. Firstly, CN-1 concentrations can be assessed by ELISA in a highly reproducible manner. While the ATLAS-based assay correlates with CN-1 activity, this was not found for the RYSK-based assay. Secondly, CN-1 seems to be present in different conformations in serum or plasma, as only a fraction of CN-1 detected with the ATLAS-based assay is also detected with the RYSK-based assay. Thirdly, detection of CN-1 with the RYSK-based assay is increased by the addition of EDTA to serum or by denaturation of serum proteins.

Because ATLAS and RYSK173 showed marked differences in ELISA, it could be argued that these antibodies recognize structurally different CN-1 variants. This is however unlikely since in Western blot analysis similar bands were recognized by both antibodies. Moreover, in
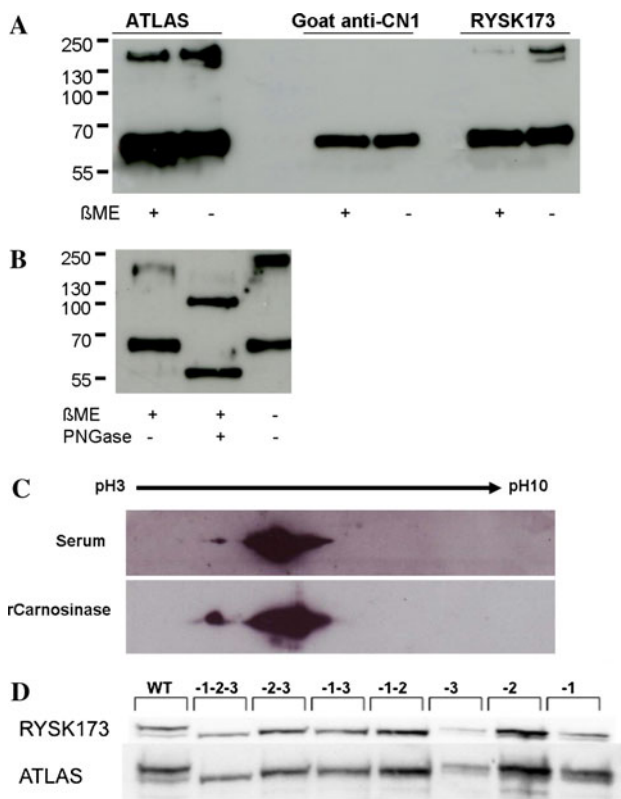
Fig. 5 Detection of CN-1 in serum by Western blot analysis. **a** Serum was subjected to PAGE under reducing (+) or non-reducing (−) conditions. Reducing conditions were achieved by the addition of ß-mercaptoethanol (ßME) to the sample buffer. Western blotting was performed using ATLAS (rabbit polyclonal anti-CN-1 IgG), goat polyclonal IgG and RYSK173 (mouse monoclonal anti-CN-1 IgG1). **b** To assess if the different bands at 150 and 65 kDa, respectively, were different, N-glycosylated CN-1 variants serum samples were treated with PNGase (+) or not (−). Similarly to that in **a**, PAGE was performed under reducing or non-reducing conditions by adding ßME (+) or not (−). **c** Serum and recombinant CN-1 were subjected to 2D-gel electrophoresis as described in "Materials and methods". After the second dimension, the gel was blotted and incubated with RYSK173 for detection of CN-1. **d** Cos-7 cells were transfected with different CN-1 cDNAs lacking one, two or all three N-glycosylation sites. Mutations in N-glycosylation sites at positions 1 (N322), 2 (N382) and 3 (N402) or combinations thereof are depicted by (−) followed by the position number. Cell extracts of transfected cells were subjected to PAGE and Western blotting using RYSK173. Thereafter, the blot was stripped and re-probed with ATLAS

2D-PAGE and subsequent Western blotting, RYSK173 recognized one major spot with comparable pI of 4.4 as reported for CN-1 (Teufel et al. 2003). CN-1 was recognized by RYSK173 both in its N-glycosylated and deglycosylated forms as demonstrated by PNGase treatment. Preferential recognition of a differentially N-glycosylated form of CN-1 by RYSK173 is also unlikely, since both RYSK173 and ATLAS gave comparable results when all possible combinations of N-glycosylation CN-1 mutants were tested in Western blotting.

It is more likely that recognition of CN-1 by RYSK173 in ELSA is dependent on the CN-1 conformation. Recently, a study by Vistoli et al. (2006) has postulated that citrate ions can alter the conformation and activity of CN-1. Given the fact that in the active center of CN-1 different metal ions may be bound, it is conceivable that this might result in different CN-1 conformations. Qualitative differences in—or the lack of—metal ion binding might explain why CN-1 concentration detected in serum with the RYSK-based assay is generally low and why this varies between individuals who have comparable amounts of CN-1 detected with the ATLAS-based assay. This notion is supported by the fact that addition of EDTA increased detection of CN-1, albeit the amount of CN-1 that was detected was still significantly lower to that detected by ATLAS. Based on a differential affinity of metal ions for EDTA, it is conceivable that not all metal ions within CN-1 can be chelated by EDTA. Consequently, only metal ions with a high affinity for EDTA will be chelated. That metal ion binding masks the RYSK173 epitope explains why only a proportion of CN-1 can be detected when EDTA is added to serum samples. Since RYSK173 also recognizes CN-1 under denaturing conditions, our data strongly suggest that RYSK173 does not recognize a conformational epitope, but more likely a linear epitope that is masked in a varying number of CN-1 molecules. We cannot exclude, however, that detection of CN-1 in serum with the RYSK-based assay is caused by damage or denaturation of the native CN-1 protein.

Previously, we have shown that the polymorphic $(CTG)_5$ repeat in the *CNDP1* gene is a susceptibility locus for developing DN (Janssen et al. 2005). We have also shown that in individuals homozygous for the $(CTG)_5$ allele, CN-1 activity is low (Janssen et al. 2005) and that this might be due to a poor secretion of CN-1 in individuals carrying this genotype (Riedl et al. 2007). We now not only show that CN-1 activity correlates with CN-1 protein concentrations when assessed by ATLAS, but also that indeed $(CTG)_5$ homozygous individuals have significant lower CN-1 concentrations compared to individuals with other genotypes. Although CN-1 concentrations assessed with the RYSK-based assay did not correlate with CN-1 activity, CN-1 activity inversely correlated with the proportion of CN-1 that was recognized by RYSK173. This therefore suggests that RYSK173 might recognize a CN-1 conformation with low enzyme activity. Both explanations for detection of CN-1 in serum with the RYSK-based assay, i.e., differential metal ion binding (Lenney et al. 1982; Vistoli et al. 2006) or denaturation of CN-1, fit with the assumption that RYSK173 recognizes a CN-1 conformation with low enzyme activity.

In conclusion, we have shown that CN-1 concentrations can be detected by ELISA in a highly reproducible manner.

Fig. 6 Detection of CN-1 in a cohort of different individuals ($n = 33$). **a** Correlation between CN-1 protein concentrations and CN-1 activity in serum. CN-1 protein concentrations were assessed by ELISA using ATLAS. **b** Correlation between CN-1 activity and the proportion of CN-1 detected in the RYSK-based assay. The proportion of CN-1 detected by the RYSK-based assay to total CN-1 was calculated as follows: (CN-1 measured with the RYSK-based assay/CN-1 measured with the ATLAS-based assay) $\times 100\%$. **c** CN-1 protein concentrations in serum of homozygous $(CTG)_5$ individuals (5–5, $n = 14$, 7 males and 7 females) and in individuals carrying a different genotype (others, $n = 19$, 9 males and 10 females)
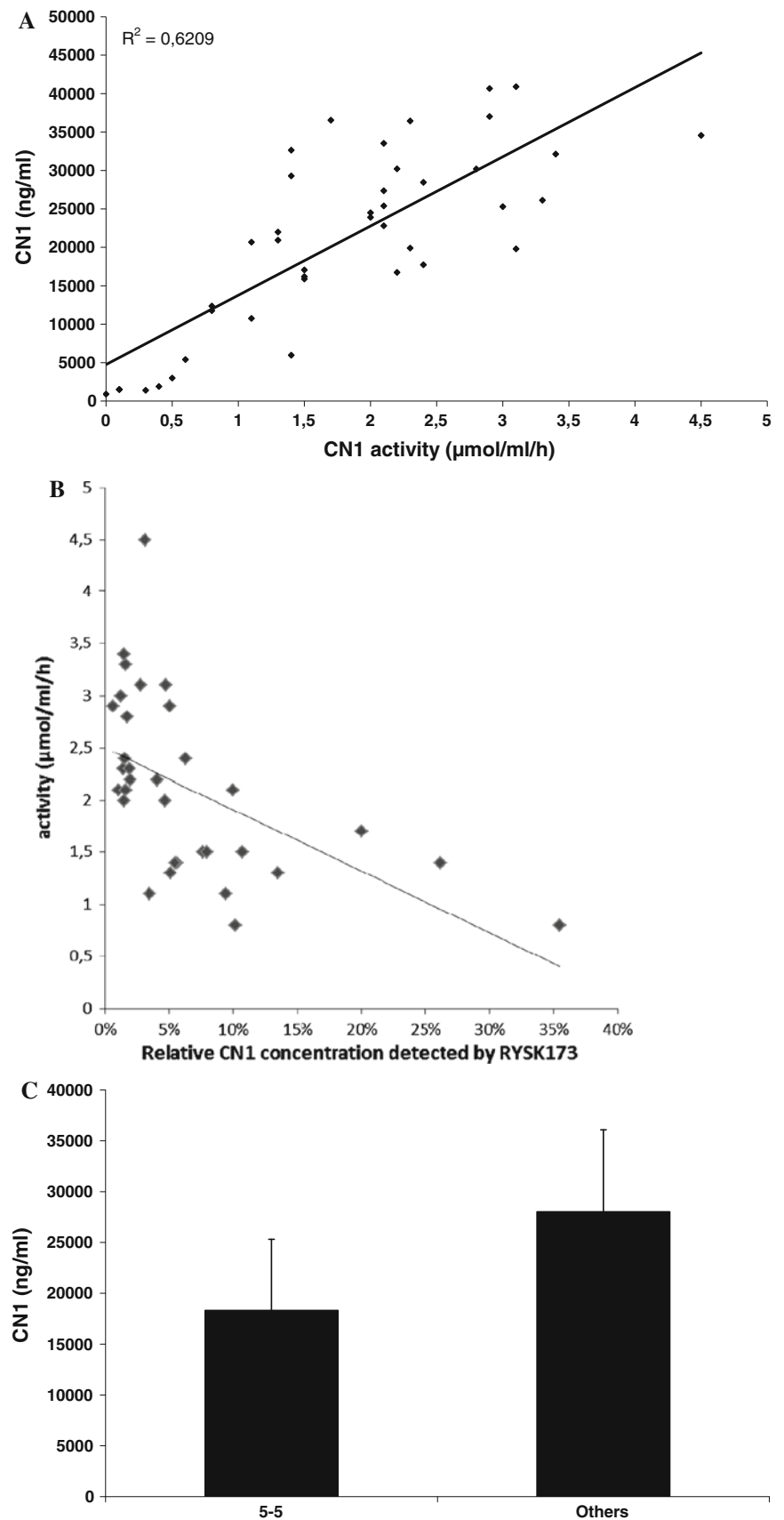
We have developed two ELISA assays, which can be used for quantitative and qualitative CN-1 measurements. While the ATLAS-based assay demonstrates a good correlation between enzyme activity and CN-1 protein concentration, the RYSK-based assay seems to detect a specific CN-1 conformation. Although we cannot discriminate whether this conformation is related to differential metal ion binding or damage of the native CN-1 protein, it seems that RYSK173 recognizes a CN-1 variant with low enzyme activity. The relevance of these different CN-1 conformations is still elusive and needs to be addressed in further studies.

Conflict of interest The authors declare that they have no conflict of interest.

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