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ORIGINAL ARTICLE

Carnosine metabolism in diabetes is altered by reactive metabolites

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Abstract Carnosinase 1 (CN1) contributes to diabetic nephropathy by cleaving histidine-dipeptides which scavenge reactive oxygen and carbonyl species and increase nitric oxide (NO) production. In diabetic mice renal CN1 activity is increased, the regulatory mechanisms are unknown. We therefore analysed the in vitro and in vivo regulation of CN1 activity using recombinant and human CN1, and the db/db mouse model of diabetes. Glucose, leptin and insulin did not modify recombinant and human CN1 activity in vitro, glucose did not alter renal CN1 activity of WT or db/db mice ex vivo. Reactive metabolite methylglyoxal and Fenton reagent carbonylated recombinant CN1 and doubled CN1 efficiency. NO *S*-nitrosylated CN1 and decreased CN1 efficiency for carnosine by 70 % ($p < 0.01$), but not for anserine. Both CN1 cysteine residues were nitrosylated, the cysteine at position 102 but not at position 229 regulated CN1 activities. In db/db mice, renal CN1 mRNA and protein levels were similar as in non-diabetic controls, CN1 efficiency 1.9 and 1.6 fold higher for carnosine and anserine. Renal carbonyl stress was strongly increased and NO production halved, CN1 highly carbonylated and less *S*-nitrosylated compared to WT mice. GSH and $NO_2/_3$ concentrations were reduced and inversely related with carnosine degradation rate ($r = -0.82/-0.85$). Thus, reactive metabolites of diabetes upregulate CN1 activity by post-translational modifications, and thus decrease the availability of reactive metabolite-scavenging histidine dipeptides in the kidney in a positive feedback loop. Interference with this vicious circle may represent a new therapeutic target for mitigation of DN.

Keywords Diabetic nephropathy · Carnosinase · Carnosine · Anserine · *N*-carbonylation · *S*-nitrosylation

Abbreviations

CN1	Carnosinase 1
ROS	Reactive oxygen species
RCS	Reactive carbonyl species
NO	Nitric oxide
HDP	Histidine-dipeptides
H_2O_2	Hydrogen peroxide
MG	Methylglyoxal
DN	Diabetic nephropathy

Introduction

Carnosinase (CN1, EC 3.4.13.20) plays an important role in the development of nephropathy in diabetic patients. Susceptibility to diabetic nephropathy (DN) is strongly associated with a leucine repeat polymorphism in the *CNDP1* gene encoding the CN1 protein. The shortest allelic form is associated with lower CN1 activities and

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a reduced probability of developing DN (Freedman et al. 2007; Janssen et al. 2005; Mooyaart et al. 2010). CN1 belongs to the M20 family of metalloproteases (MEROPS database; Rawlings ND 2004) and cleaves the histidine-dipeptides (HDP) carnosine (β-alanine-L-histidine) and anserine (β-alanyl-1-1-methylhistidine) (Boldyrev et al. 2013; Budzen and Rymaszewska 2013; Peters et al. 2010, 2011; Teufel et al. 2003). Both peptides have cytoprotective properties. Carnosine scavenges carbonyls (Barski et al. 2013; Negre-Salvayre et al. 2008; Vistoli et al. 2009), inhibits glycation (Alhamdani et al. 2007) and acts as ACE inhibitor (Hou et al. 2003; Nakagawa et al. 2006). Its function as antioxidant is debated (Babizhayev et al. 2013; Decker et al. 2000; Hipkiss 2011; Mozdan et al. 2005; Velez et al. 2008). Anserine has been shown to scavenge carbonyls (Aldini et al. 2005), to act as antioxidant (Kohen et al. 1988), and to reduce renal sympathetic nerve activity and blood pressure (Tanida et al. 2010). Anserine and carnosine have distinct actions; carnosine, but not anserine, reduces ischemic infarct size and preserves neurological function in a mouse model of permanent focal cerebral ischemia (Min et al. 2008). In diabetic mice, carnosine supplementation mitigates DN, reduces renal vasculopathy, normalizes vascular permeability (Peters et al. 2012), and improves wound-healing (Ansurudeen et al. 2012). In streptozotocin-induced diabetic rats, carnosine treatment prevents apoptosis of glomerular cells, podocyte loss (Peters et al. 2014; Riedl et al. 2011), and vascular damage (Pfister et al. 2011).

Diabetes mellitus is characterized by increased blood glucose, insulin and leptin levels (Somineni et al. 2014) and by impaired nitric oxide (NO) homeostasis with reduced availability of NO (Baylis 2012; Masha et al. 2011; Pollock and Pollock 2011), resulting in endothelial dysfunction, a critical mediator of diabetic vasculopathy (Sena et al. 2013). NO is required for *S*-nitrosylation, i.e. the covalent binding of NO to sulphur containing cysteines. This post-translational modification controls protein function (Foster et al. 2009; Hartmanova et al. 2013). Excessive production of reactive carbonyl species (RCS) and oxygen species (ROS) is another important mechanism underlying the pathogenesis of diabetic complication (Voziyan et al. 2014) such as cardiovascular and renal sequelae (Cheang et al. 2011; Forstermann and Sessa 2012). ROS such as hydrogen peroxide (H_2O_2) are known to lead to post-translational modifications by oxidizing amino acids in proteins. All amino acids can be modified by ROS but cysteine residues are most susceptible to oxidative changes due to the highly reactive thiol group (Gould et al. 2013). RCS react preferentially with arginine residues. The current paradigm of RCS-derived protein damage places the focus on methylglyoxal (MG), a spontaneous decomposition product of dihydroxyacetone phosphate and gylderaldehyde-3-phosphate, which contributes to extracellular matrix damage in diabetes (Voziyan et al. 2014).

ROS and RCS accumulate in diabetes mellitus, NO concentrations are low. We now tested whether reactive metabolites and NO modify CN1 activity by post-translational modification and whether this modification results in increased CN1 activity. This reduces the availability of reactive metabolite-scavenging histidine dipeptides anserine and carnosine in the kidney, which in turn should increase post-translational modification of CN1 in a positive feedback loop.

Materials and methods

CN1 activity

CN1 activity was assayed according to the method described by Teufel et al. (2003). Briefly, the reaction was initiated by addition of carnosine to renal tissue homogenate at a pH of 7. The reaction was stopped after defined periods by adding 1 % trichloracetic acid (final concentration in the test 0.3 %). Liberated histidine was derivatized by adding o-pthaldialdehyde (OPA) and fluorescence was read using a MicroTek plate reader (λ_{Exc} 360 nm; λ_{Em} 460 nm). V_{max} values were obtained from Dixon plots using a linear regression program from at least three different assays. The effect of the *S*-nitrosylation on CN1 activity was measured after incubation with either 3-morpholinosydnonimine (SIN-1) or $NaNO_3$ in different concentrations (between 0.2 and 2 mM) for 1 h at 30 °C. The effect of carbonylation was measured after incubation with H_2O_2 (with concentrations between 0.1 and 2 mM) and $FeSO_4$ (between 50 and 500 µM) and following incubation with MG (between 0.1 and 2 mM) for 1 h at 30 °C. The kinetic parameters were determined by using various concentrations of substrates, and data fitting was performed according to the Michaelis–Menten equation.

Site-directed mutagenesis and overexpression of recombinant CN1 enzyme

We substituted cysteine with serine in CN1 ($Mut1^{C102S}$ and $Mut2^{C229S}$). Recombinant FLAG-tagged proteins have been purified from CHO supernatant. The CDS of WT CN1 was cloned into the p3XFLAG-CM-13 Expression vector (Sigma-Aldrich). After sequence confirmation, site-directed mutagenesis (Site directed mutagenesis kit, Agilent technologies) was used to substitute the cysteine residues, respectively. Primer sequences were Mut1 sense CAC GAAAGGCACCGTGTCCTTCTACGGCCACTTGG

and Mut1 antisense CCAAGTGGCCGTAGAAGGA CACGGTGCCTTTCGTG, Mut2 sense CATGGTGGAG GTGAAATCCAGAGACCAGGATTTTC and Mut2 antisense GAAAATCCTGGTCTCTGGATTTCACCTCCAC CATG (sense TGC → TCC, antisense GCA → GGA, respectively). CHO cells were transfected with the expression vectors for CN1 wild type, Mut1 and Mut2. Cells were grown in the presence of geneticin (500 µg/ml, G418, Biochrom) in order to get a stable expression of CN1 protein and mutants. CHO cells were grown in protein- and serum-free media supplemented with 10 % L-glutamate in an incubator with 8 % CO_2 at 37 °C. FLAG-tagged CN1 was secreted into the supernatant. After 72 h supernatant was collected and an aliquot was used for Anti-FLAG Western Blot (Monoclonal mouse Anti-FLAG M2 antibody, Sigma-Aldrich) for verification of CN1 expression. CHO supernatant was concentrated to 5 ml using Vivaspin 20 concentrator (Sartorius Stedim). The samples were loaded onto Anti-FLAG M2 affinity gel (Sigma-Aldrich) and washed with TBS. 3xFLAG peptide (100 µg/ml, Sigma-Aldrich) was used for competitive elution. Purity of recombinant proteins was checked by silver staining.

Diabetic mice (db/db mice)

Male C57BL/KsJm/Leptdb (db/db) mice (Stock 000662) and their normoglycemic heterozygous littermates were obtained from Charles River (Sulzfeld, Germany). The animals were housed in a 12-h light/dark cycle at 22 °C. Standard laboratory food and water was provided ad libidum. The experimental procedure was approved by the North Stockholm Ethical Committee for Care and Use of Laboratory Animals (for details see Peters et al. 2012).

Preparation of total protein extracts

25-week-old animals were euthanized by carbon-dioxide. The kidneys were removed, immediately homogenized in cold buffer containing 20 mM HEPES, 1 mM ethylene glycol-tetraacetic acid (EGTA), 210 mM mannitol and 70 mM sucrose per gram tissue, pH 7.2. The homogenate was centrifuged at 1500*g* for 5 min at 4 °C, and the supernatant was kept at −80 °C until analysis (see also Peters et al. 2012). Protein concentration was determined by Bradford assay.

mRNA quantification PCR

RT-PCR was performed according to a previously described protocol (Janssen et al. 2005). Equal loading was confirmed by RT-PCR for β-actin.

Plasma assays

Blood was collected from all animals from the tail tip and glucose was determined using an OneTouch Ultra Blood Glucose meter (LifeScan, Milpitas, CA, USA). Insulin was determined by using an Ultra-Sensitive Mouse Insulin ELISA kit (Crystal Chem, 130 Downers Grove, IL). Leptin was determined by using a Mouse Leptin ELISA Kit (Crystal Chem, 131 Downers Grove, IL).

GSH concentrations

GSH concentrations were measured using a fluorescence assay from BioAssay Systems, Hayward, USA.

NO production

NO_2 and NO_3 renal tissue concentrations, which reflect total local NO production, were measured with Cayman's Nitrate/Nitrite colorimetric assay kit according to the manufacturer's protocol (Cayman Chemical Company, MI, USA).

Detection of *N*-glycosylation

For detection of carnosinase in renal tissue, gel electrophoreses and subsequent Western blotting was performed according to the method described by Riedl et al. (2010). Samples were deglycosylated by PNGase F (New England Biolabs, Frankfurt, Germany) treatment according to the manufacturer's recommendations.

Detection of *S*-nitrosylation

S-nitrosylation was detected by the Biotin Switch Technique (Forrester et al. 2009) from Cayman's (*S*-nitrosylated Detection Assay). Briefly, free SH groups are blocked by *S*-methylthiolation. Subsequent cleavage of the S–NO bonds in the sample and biotinylation of the newly formed SH groups provides the basis for visualization using streptavidin-based colorimetric visualization of *S*-nitrosylated proteins.

CN1 carbonylation

For determination of protein carbonylation the reaction of carbonyl groups with 2,4-dinitrophenylhydrazine (DNPH) to form protein-bound 2,4-dinitrophenylhydrazones was used. Hydrazones were detected immunochemically with anti-dinitrophenyl antibodies using the OxyBlot™ protein detection kit (Chemicon/Millipore, Germany). In brief, 1 µg recombinant human CNDP1 (2489-ZN; R&D Systems, Germany) was incubated with Fenton reagent (70 µM

$FeSO_4$ plus 1 mM H_2O_2) and MG (1 mM), respectively, for 30 min at 37 °C followed by derivatization with 2,4-dinitrophenylhydrazine or a derivatization-control solution following manufacturer's recommendations. SDS-PAGE and Western immunoblotting analysis of the carbonylated CN1 was performed using a standard protocol as published previously (Wagner et al. 2011) using the following antibodies: rabbit anti-CN1 (1:1000; HPA008933, Sigma-Aldrich, Germany) and rabbit anti-dinitrophenyl (1:2500; A6430, Invitrogen- Life Technologies, Germany). Secondary antibodies were purchased from Dianova, Germany.

Immunoprecipitation

Protein was isolated from whole kidney tissue homogenates of diabetic db/db and wild-type mice as described above. Protein (50 µg) was precipitated with 2 µg of a rabbit polyclonal serum carnosinase antibody (sc-98739, Santa Cruz Biotechnology, Germany) or rabbit control IgG (#2729, Cell Signaling Technology, Germany) at 4 °C overnight. Samples were then incubated with Dynabeads Protein G (10004D, novex through life technologies, Darmstadt, Germany) for 30 min at room temperature, centrifuged, and washed. Precipitates were eluted in 2× standard protein sample buffer in 10 min at 70 °C and analysed for CN1 (sc-98739, Santa Cruz Biotechnology) and CN1 carbonylation using the anti-dinitrophenyl antibody (Invitrogen-Life Technologies, Germany).

Statistical analysis

A minimum of three independent experiments were performed in duplicates and more. Data are given as mean ± SD. For comparison of three or more groups a one-way analysis of variance was performed, followed by post hoc analyses using Tukey's test. Differences were considered significant at $p < 0.05$.

Results

Influence of glucose, insulin and leptin on recombinant CN1 activity

To analyse CN1 activity, recombinant CN1 (rCN1) was produced in CHO cells, purified and characterized for carnosine and anserine degradation. rCN1 was more effective in degrading carnosine than anserine (Table 1). Similar findings were obtained using human serum CN1. V_{max} for human carnosine was 4.9 ± 0.6 and for anserine 0.9 ± 0.1 µmol/mg/h (K_m for carnosine 0.9 mM, for anserine 1.2 mM). Incubation of recombinant and human CN1 with glucose (1–25 mM), insulin or leptin (each 0.01–1 mM) did not affect enzyme activity (Table 1). Ex vivo incubation of renal tissue slides of db/db and WT mice with glucose (1–25 mM) also did not increase CN1 activity.

Influence of recombinant metabolites on recombinant CN1 activity

Methylglyoxal (MG) dose-dependently increased carnosine cleavage activity of rCN1. Already at a concentration of 0.3 mM MG, rCN1 showed significantly increased cleavage of 1 mM carnosine (4.6 ± 0.4 vs. 3.8 ± 0.5 µmol/mg/h; $p < 0.05$, Fig. 1a) and of anserine (0.8 ± 0.1 vs. 0.6 ± 0.1 µmol/mg/h; $p < 0.05$). At 0.5 mM MG, cleavage activity of rCN1 was increased by 75 % for carnosine and by 30 % for anserine (Table 1).

Table 1 Kinetic parameters of rCN1

	Carnosine			Anserine		
	V_{max} (µmol/mg/h)	K_m (mM)	Efficiency (V_{max}/K_m)	V_{max} (µmol/mg/h)	K_m (mM)	Efficiency (V_{max}/K_m)
Control	5.5 ± 0.4	0.8 ± 0.09	6.9 ± 0.5	0.7 ± 0.05	1.04 ± 0.12	0.7 ± 0.06
Glucose	5.4 ± 0.8	0.8 ± 0.1	6.8 ± 0.9	0.7 ± 0.08	1.0 ± 0.11	0.70 ± 0.05
Leptin	5.7 ± 0.5	0.9 ± 0.1	6.3 ± 0.7	0.7 ± 0.1	0.9 ± 0.12	0.77 ± 0.08
Insulin	5.3 ± 0.7	0.8 ± 0.1	6.6 ± 0.8	0.7 ± 0.05	1.0 ± 0.15	0.70 ± 0.09
Methylglyoxal	6.0 ± 0.2*	0.5 ± 0.02***	12.0 ± 1.5***	0.7 ± 0.05	0.79 ± 0.08***	0.9 ± 0.06***
Fenton reagent ($H_2O_2 + FeSO_4$)	6.3 ± 0.4**	0.4 ± 0.01***	15.8 ± 1.9***	0.8 ± 0.06	0.67 ± 0.05***	1.2 ± 0.09***
3-Morpholinosydnonimine	3.2 ± 0.3***	1.8 ± 0.1***	2.0 ± 0.3***	0.7 ± 0.03	1.1 ± 0.09	0.6 ± 0.04*

V_{max} and K_m values for recombinant CN1 activity were calculated and efficiency was calculated by the ratio V_{max} and K_m. Glucose (25 mmol/l), leptin (0.5 mmol/l) and insulin (0.5 mmol/l) had no effect on CN1 activity, carbonylation by methylglyoxal (0.5 mmol/) or Fenton reagent (0.5 mmol/l) increased V_{max} and decreased K_m; efficiency increased. *S*-nitrosylation by 3-morpholinosydnonimine (0.5 mmol/l) decreased V_{max} and increased K_m compared to untreated rCN1; efficiency decreased

* $p < 0.05$; ** $p < 0.01$; *** $p < 0.005$; compared to control (untreated rCN1)

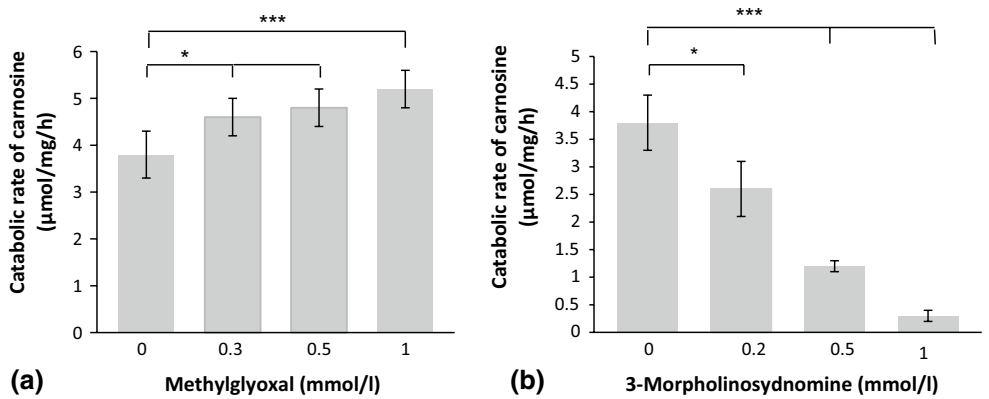

Fig. 1 Influence of recombinant metabolites on recombinant CN1 activity. **a** Methylgloxal dose-dependently increases rCN1 activity, **b** SIN-1 dose-dependently decreases rCN1 activity

Incubation with Fenton reagent also dose-dependently increased histidine dipeptide cleavage by rCN1. 0.25 mM Fenton reagent increased turnover of 1 mM carnosine to 4.8 ± 0.8 as compared to 3.8 ± 0.8 µmol/mg/h in Fenton free medium ($p < 0.05$). At a Fenton reagent concentration of 0.5 mmol/l, carnosine and anserine cleavage efficiency of rCN1 was increased by 130 and 70 %, respectively (Table 1). The addition of 3-morpholinosyndomine (SIN-1), an NO donor, caused a dose-dependent reduction of the rCN1 catalytic rate for carnosine (Fig. 1b). This was already significant at 0.2 mM SIN-1, (2.6 ± 0.5 vs. 3.8 ± 0.5 µmol carnosine/mg/h; $p < 0.05$). At a concentration of 1 mM SIN-1, carnosine cleavage rate was less than 10 % compared to the NO free control (0.3 ± 0.1 vs. 3.8 ± 0.5 µmol/mg/h; $p < 0.001$). 0.5 mM SIN-1 reduced CN1 cleavage activity for carnosine to 30 % and for anserine to 85 % of control ($p < 0.005/0.05$; Table 1). Coincubation of SIN-1 with Fenton reagent and MG, respectively, reduced the CN1 activity suppressing action of SIN-1 dose-dependently. Incubation of Mut2^{C229S} with 0.5 mM SIN-1 reduced efficiency for carnosine and anserine turnover to 42 and 85 % (2.9 ± 0.4 and 0.6 ± 0.05; $p < 0.005/0.05$ vs. untreated controls).

Post-translational modifications of rCN1

To determine whether the change in CN1 activity is caused by carbonylation and *S*-nitrosylation, rCN1 was incubated with methylglyoxal (MG), Fenton reagent (H_2O_2 + $FeSO_4$) and SIN-1. Introduction of carbonyl groups by oxidative reactions was demonstrated by OxyBlot analysis of rCN1 (Fig. 2a). Biotin-Switch assay showed that both rCN1 cysteines are *S*-nitrosylated by 3-morpholinosyndomine (SIN-1), an NO donor (Fig. 2b).

Role of cysteine residues for CN1 activity

To investigate the role of the two cysteine residues on rCN1 activity, the cysteines were substituted by serine (Mut1^{C102S} and Mut2^{C229S}). Mut1^{C102S} largely abolished enzyme activity for carnosine and anserine (V_{max}/K_m for carnosine 0.9 ± 0.7; for anserine 0.1 ± 0.05 µmol/mg/h/mM), whereas substitution of cysteine at position 229 (Mut2^{C229S}) did not significantly affect cleavage function compared to unsubstituted rCN1 (V_{max}/K_m for carnosine 6.5 ± 0.8, for anserine 0.6 ± 0.1 µmol/mg/h/mM). Incubation of Mut2^{C229S} with MG and with Fenton reagent doubled carnosine and anserine turnover compared to untreated Mut2^{C229S} (V_{max}/K_m for carnosine/anserine with MG: 11.5 ± 1.4/1.1 ± 0.09; with 0.5 mM Fenton reagent: 14.1 ± 2.1/1.2 ± 0.15; untreated Mut2^{C229S} 6.5 ± 0.8/0.6 ± 0.1 µmol/mg/h/mM; $p < 0.005$ vs. untreated Mut2^{C229S}). Activity of Mut1^{C102S} was low and unchanged when incubated with MG or Fenton reagent (data not shown).

Activity and post-translational modifications of CN1 diabetic mice

In db/db mice renal carnosine degrading activity was 175 % (0.51 ± 0.13) of WT controls (0.29 ± 0.17 µmol/mg/h; $p < 0.001$). V_{max} for carnosine and anserine was increased, substrate affinity higher as shown by a lowered K_m value, and thus efficiency (V_{max}/K_m) of the renal CN1 for carnosine and anserine cleavage 88 and 61 % higher than in WT (Table 2). Proteinuria, blood glucose, leptin and insulin were higher in db/db mice compared to WT controls (Fig. 3a–d; 99 ± 36 vs. 2.2 ± 1.4 µg albumin/mg creatinine, 23 ± 2 vs. 8 ± 1 mmol/l; 72 ± 8.7 vs. 5.3 ± 0.9 ng/ml; each $p < 0.005$ and 3.5 ± 1.6 vs. 1.1 ng/ml; $p < 0.01$). Carbonylation of renal proteins was increased, as demonstrated by oxyblot (Fig. 3e). Renal tissue GSH and

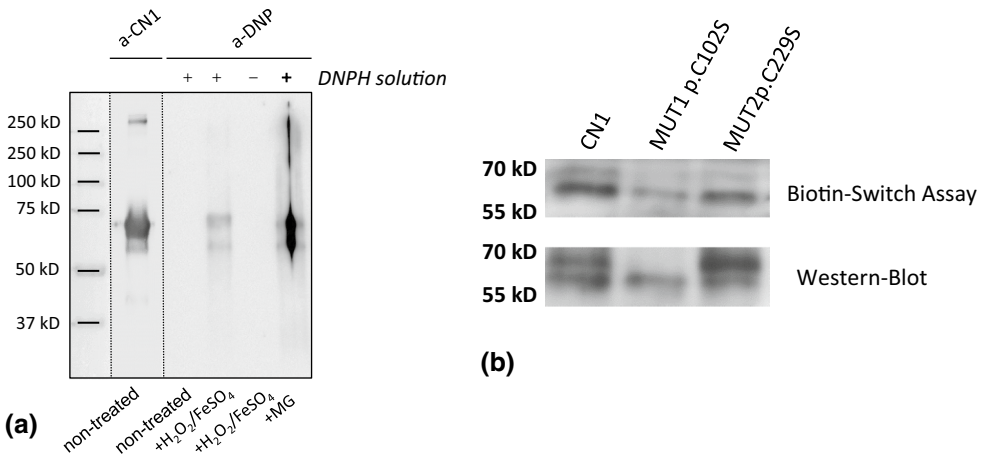

Fig. 2 Post-translational modifications of CN1. **a** Incubation of recombinant CN1 with Fenton reagent (70 µM $FeSO_4$ + 1 mM H_2O_2) or methylglyoxal (MG, 1 mM) causes substantial protein carbonylation as shown by OxyBlot™. Carbonylated CN1 was identified by SDS-PAGE and Western immunoblotting with anti-dinitrophenyl (a-DNP). **b** Recombinant CN1 *S*-nitrosylation after incubation with the NO donor 3-morpholinosydnonimine (SIN-1, 0.5 mM) was measured using the Biotin-Switch assay. Both cysteine residues at position 102 and 229 of CN1 were nitrosylated. Western blot served as loading control

Table 2 Kinetic of CN1 activity for carnosine and anserine degradation in renal tissue of WT and diabetic mice (db/db)

	Carnosine			Anserine		
	V_{max} (µmol/mg/h)	K_m (mM)	Efficiency (V_{max}/K_m)	V_{max} (µmol/mg/h)	K_m (mM)	Efficiency (V_{max}/K_m)
WT mice	3.9 ± 0.3	0.8 ± 0.06	4.9 ± 0.3	2.6 ± 0.2	0.8 ± 0.07	3.3 ± 0.2
Diabetic mice	5.9 ± 0.4***	0.6 ± 0.04***	9.2 ± 0.5***	3.2 ± 0.2**	0.6 ± 0.04***	5.3 ± 0.3***

V_{max} and K_m values for CN1 activity were determined and efficiency calculated (ratio of V_{max} and K_m). V_{max} was significantly higher for diabetic mice compared to WT mice for carnosine ($p < 0.005$) and for anserine ($p < 0.05$), and K_m decreased. Thus, efficiency of carnosine and anserine turnover by renal CN1 was significantly increased in db/db mice

* $p < 0.05$; ** $p < 0.01$; *** $p < 0.005$ compared to WT

NO_2/NO_3 concentrations were reduced (700 ± 99 vs. 932 ± 108 µM in WT, and 9.2 ± 6.6 vs. 18.1 ± 5.3 µM in WT, both $p < 0.01$) and inversely correlated with the carnosine degradation rate of CN1 ($r = -0.82$ and -0.85; both $p < 0.01$). Renal CN1 protein concentrations were similar in db/db mice as in WT controls. All three glycosylation sites of CN1 were glycosylated in db/db and in WT animals, demonstrating that in diabetic milieu CN1 activity is not increased by direct glycation. Western immunoblotting detected only one band corresponding to the fully *N*-glycosylated CN1 protein, deglycosylation by PNGase treatment reduced its molecular weight to 61 kDa (data not shown). Renal CN1 in db/db mice was more carbonylated (as demonstrated by immunoprecipitation) and less *S*-nitrosylated (as demonstrated by Biotin-Switch assay, Fig. 4a, b) than in WT controls. Renal tissue carnosine and anserine concentrations were lower in diabetic mice than in WT controls and correlated with renal tissue CN1 activity ($r = -0.7$ and -0.85 in db/db and -0.65 and -0.8 in WT mice).

Discussion

CN1 degrades histidine-dipeptides such as carnosine and anserine which have significant cytoprotective properties. In diabetic mice, the activity of CN1 is increased approximately twofold and histidine-containing dipeptide levels are reduced (Peters et al. 2012). The mechanisms leading to the up-regulation of CN1 activity in diabetes have been unknown. In the present study, we confirm increased CN1 activity in diabetic mice. This increase was not due to changes in the rate of transcription, protein synthesis or *N*-glycosylation required for protein secretion and enzyme activity (Riedl et al. 2010). Neither glucose, leptin nor insulin influenced CN1 activity in vitro; incubation of renal tissue with glucose did not modify CN1 activity of db/db and WT mice. In contrast, post-translational CN1 modifications substantially altered CN1 activity.

Carbonylation is a common form of oxidative protein modification leading to the semialdehyde derivatives of

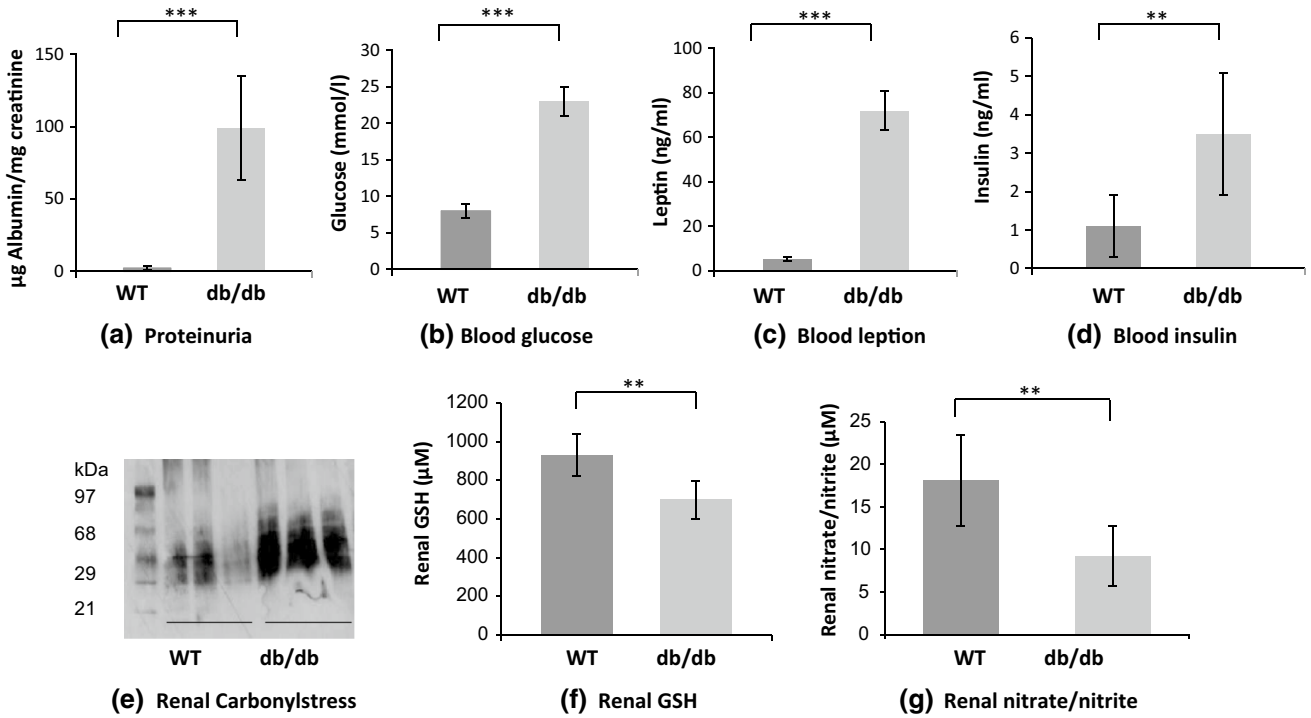

Fig. 3 Comparison of WT and db/db mice. **a** Proteinuria (µg Albumin/mg creatinine), **b** blood glucose (mmol), **c** blood Leptin (ng/ml), **d** blood Insulin (ng/ml) and **e** renal carbonyl stress, demonstrated by Oxblot, were increased, **f** renal GSH and **g** renal nitrate/nitrite levels were decreased in db/db mice compared to controls

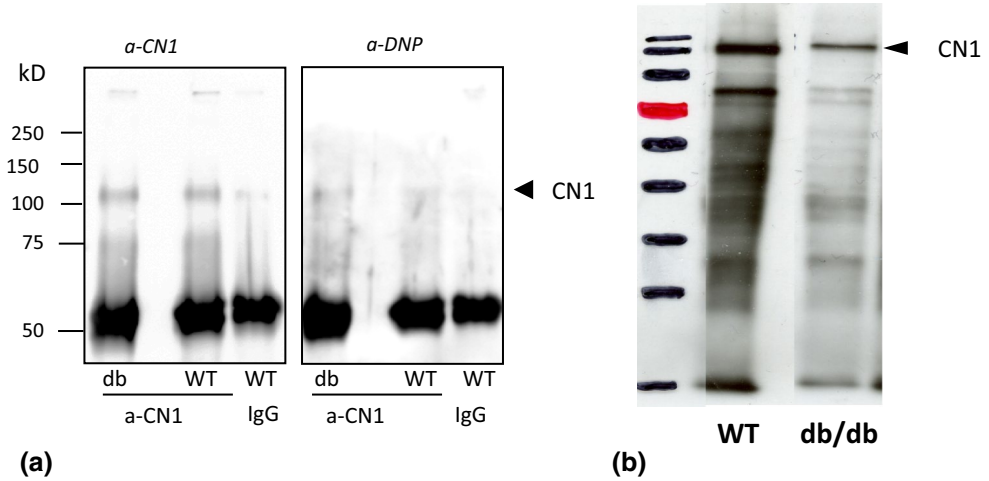

Fig. 4 CN1 carbonylation and *S*-nitrosylation in db/db mice compared to WT. **a** Immunoprecipitation of mice kidney tissue lysates. Protein was precipitated with CN1 antibody (a-CN1) or control (IgG). CN1 was demonstrated in db/db and wild-type mice by a-CN1 (*left panel*), CN1 carbonylation was detected by using the anti-dinitrophenyl antibody (a-DNP) (*right panel*). The presence of the high molecular band demonstrates that CN1 was present also in its dimeric form, as previously reported (Chang et al. 2010). **b** *S*-nitrosylation of renal tissue CN1 of control and db/db mice. *S*-nitrosylation was lower for diabetic mice compared to controls (Biotin Switch Assay)

lysine, arginine, proline, and threonine (Curtis et al. 2012; Dalle-Donne et al. 2006; Ruskovska and Bernlohr 2013). Carbonyl derivatives of lysine, cysteine, and histidine can be formed by secondary reactions, but the quantitatively most important product of carbonylation is glutamic semialdehyde from arginine (Nystrom 2005). In silico analysis (available online at http://www.lcb.cnrs-mrs.fr/CSPD/; Maisonneuve et al. 2009) predicted two sites in the CN1 amino acid sequence prone to carbonylation located at position 95–99 and at position 333–336. The putative

carbonylation site at position 95–99 is located in the active site, a highly conserved metal binding site of CN1 and seems to be responsible for the effects described. In view of the highly reactive nature of hydroxyl radicals, further reactions beyond carbonylation may occur, resulting in heterogeneous oxidative modification products such as methionine sulphoxide, dityrosine, as well cysteine oxidation products. These may also affect the conformation of the active site. As shown by the present experiments, the cysteine residues undergo *S*-nitrosylation which reduces CN1 activity, and carbonylation which increases CN1 activity. Modifications of cysteine at position 102 regulate CN1 activity while cysteine at position 229 does not appear to be relevant for enzyme function. The active site of CN1 contains no cysteine residues but Cys102 is located in the same β-strand as His106. It is possible that *S*-nitrosylation of Cys102 causes derangement of the local environment, impairs the coordination of zinc ions, and inhibits catalysis. Further analysis of the modified proteins should include high-resolution mass spectroscopy. Of note, the in vitro setting differs substantially from in vivo conditions. MG concentration used in the cell-free experiments to demonstrate post-translational modifications within 30 min were about 100-fold higher than that reported for intracellular MG concentrations (Dobler et al. 2006). On the other hand, higher MG concentrations have been observed in diabetic patients on dialysis (Lapolla et al. 2005), and our in vivo findings in mice support the impact of carbonylation on CN1 activity.

The regulation of CN1 activity by post-translational modification was not only shown in vitro, but also in the renal tissue of db/db mice. Under conditions of increased oxidative stress and reduced GSH concentrations, as observed under diabetic conditions, we demonstrated increased carbonylation of CN1 and increased CN1 activity. Surprisingly, we found no effect of high glucose levels on CN1 *N*-glycosylation and activity. Under conditions of lower NO_2/NO_3, and consequently decreased *S*-nitrosylation, as also seen under diabetic conditions, CN1 activity was higher. Reduced *S*-nitrosylation of CN1 under diabetic conditions could moreover result from increased generation of peroxynitrite.

Increased activity of CN1 would be an explanation for the increased turnover of histidine-dipeptides in diabetes. The shortest allelic form of the CN1 is associated with lower CN1 activity and protects from diabetic nephropathy in patients with type 2 diabetes, a correlation of serum CN1 activity with serum HDP concentrations, however, has not been established (Peters et al. 2011). In diabetic rodents a decrease in carnosine content was reported in retina, kidney and liver (Mong et al. 2011; Pfister et al. 2011; Riedl et al. 2011). In contrast, Stegen et al. (2015) demonstrated an increase in muscle carnosine concentrations with obesity and progressive glucose intolerance in rodents and in male humans with type 2 diabetes. We now demonstrate a decrease in renal carnosine concentration in the kidney of diabetic rodents in correlation with the tissue CN1 activity. CN1 activity is not the only mechanism by which renal turnover of histidine dipeptides is regulated. Two enzymes, carnosine synthase (Drozak et al. 2010) which regulates the ATP-dependent conjugation of histidine and alanine, and carnosine methyltransferase (Drozak et al. 2013) which methylates carnosine to anserine, have recently been identified as regulatory enzymes of carnosine metabolism. Likewise, the impact of histidine resulting from carnosine degradation needs further investigations. Histidine is known as a carbonyl scavenger (Orioli et al. 2007), but high concentrations may have toxic effects (Holliday and McFarland 1996; Letzien et al. 2014). Further studies are required to determine whether these enzymes are altered in diabetes and whether post-translational modifications play a role in their regulation.

Conclusions

We demonstrate that increased CN1 activity of diabetes is due to post-translational modifications of CN1 by reactive metabolites. Thus, increased HDP cleavage by CN1 under diabetic conditions, reducing the scavenging capacity for reactive metabolites by HDP to render them harmless, further increases CN1 activity in a positive feedback loop. Interference with this vicious circle, e.g. by inhibition of post-translational modification of CN1 or by suppression of CN1 activity represent intriguing therapeutic strategies for mitigation of diabetic complications such as DN.

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

Statement on the welfare of animals All procedures performed in studies involving animals were in accordance with the ethical standards of the institution or practice at which the studies were conducted.

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