On the relevance of carnosine and carnosinase for the development of diabetic nephropathy
Riedl, Eva Maria Susanne

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CHAPTER 3

N-GLYCOSYLATION OF CARNOSINASE INFLUENCES PROTEIN SECRETION AND ENZYME ACTIVITY: IMPLICATIONS FOR HYPERGLYCEMIA

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* Equally contributing authors

OBJECTIVES: The (CTG)$_n$ polymorphism in the serum carnosinase (CN-1) gene affects CN-1 secretion. Since CN-1 is heavily glycosylated and glycosylation might influence protein secretion as well, we tested the role of N-glycosylation for CN-1 secretion and enzyme activity. We also tested if CN-1 secretion is changed under hyperglycemic conditions.

METHODS: N-glycosylation was either inhibited by Tunicamycin in pCSII-CN-1 transfected Cos-7 cells or by stepwise deletion of its 3 putative N-glycosylation sites of CN-1. CN-1 protein expression, N-glycosylation and enzyme activity were assessed in cell extracts and supernatants. The influence of hyperglycemia on CN-1 enzyme activity in human serum was tested in homozygous diabetic patients and healthy controls.

RESULTS: Tunicamycin completely inhibited CN-1 secretion. Deletion of all N-glycosylation sites was required to reduce CN-1 secretion efficiency. Enzyme activity was already diminished when 2 sites were deleted. In pCSII-CN-1 transfected Cos-7 cells cultured in medium containing 25mM D-glucose, the immature 61 kDa CN-1 immune reactive band was not detected. This was paralleled by an increased GlcNAc expression in cell lysates and CN-1 expression in the supernatants. Homozygous (CTG)$_5$ diabetic patients had significantly higher serum CN-1 activity compared to genotype matched healthy controls.

CONCLUSIONS: We conclude that apart from the (CTG)$_n$ polymorphism in the signal peptide of CN-1, N-glycosylation is essential for appropriate secretion and enzyme activity. Since hyperglycemia enhances CN-1 secretion and enzyme activity, our data suggest that poor blood glucose control in diabetic patients might result in an increased CN-1 secretion even in the presence of the (CTG)$_5$ allele.
INTRODUCTION

Diabetic nephropathy (DN) is the foremost cause for dialysis in the western world (131). Prime risk factors for developing DN are poor glycemic control and high blood pressure; yet, appropriate treatment of individual patients to minimize these risk factors can only delay the onset, but do not eliminate susceptibility to develop DN (119, 132). We have recently demonstrated that susceptibility to DN is strongly associated with a polymorphism in the CNDP1 gene (44). This gene encodes the serum carnosinase (CN-1) protein, which degrades carnosine into β-alanine and histidine. We have also demonstrated that the CNDP1 polymorphism affects carnosinase secretion, i.e. a low CN-1 secretion is observed when (CTG)₅ is present in the signal peptide of CN-1 (133). This is in line with the finding that CN-1 activity is low in (CTG)₅ homozygous individuals (44). Over-expression of CNDP1 in the Db/Db model results in early onset of diabetes, while in Db/Db mice that were fed with carnosine the onset of diabetes was delayed and glucose metabolism was improved (113). This unambiguously shows the relevance of the carnosine carnosinase system for diabetic complications, and can also explain why low serum CN-1 activity is protective. Several mechanisms for the protective role of carnosine have been postulated (44, 134-137), yet, definite proof for its beneficial effect on DN remains to be assessed.

Although CNDP1 has been confirmed as a susceptibility locus (41, 42), more recently this association was questioned by Wanic et al (138). The negative findings of Wanic et al may be explained by the fact that baseline renal function, proteinuria and mortality was not included in their Cox proportional hazards regression analyses (139). Alternatively, their data might indicate that apart from the (CTG)ₙ polymorphism, CN-1 secretion is influenced by other factors.

N-glycosylation is initiated in the endoplasmatic reticulum (ER) by the transfer of preassembled glucose₃-mannose₉-N-acetylglucosamine₂ (Glc)₃(Man)₉(GlcNAc)₂ core oligosaccharides from the dolichylpyrophosphate carrier to asparagine residues in nascent proteins. Three different nucleotide-activated sugar donors are required as substrates for the assembly of Dolichol linked core oligosaccharides: UDP-N-acetylglucosamine (UDP-GlcNAc), GDP-mannose and UDP-Glucose. N-glycosidic bonds anchor the core oligosaccharides to side chains of asparagines located within a consensus sequence of Asn-X-Thr/Ser. Not all consensus sequences within a given protein get occupied. This results in generation of differentially N-glycosylated proteins. Moreover, both the supply of oligosaccharide substrates as well as the activity of the oligosaccharide transferring enzyme determines N-glycosylation efficiency. N-glycosylation improves protein stability and
solubility and act as specific molecular recognition site for the recruitment of chaperones (140). Many proteins need proper N-glycosylation to be correctly folded. Inaccurately N-glycosylated proteins may therefore not be secreted and those that are still secreted may have altered substrate binding properties and modified enzyme activity.

The Hexosamine biosynthesis pathway is responsible for both O- and N-glycosylation, utilizing substrates (e.g. fructose-6P) that are key metabolites in carbon, nitrogen and energy homeostasis (141). Hence, the extent of GlcNAc branching is dependent on enzyme kinetics and metabolic flux through the hexosamine pathway to generate UDP-GlcNAc. Lau et al (142) have shown that the N-glycan branching pathway cooperates with the number of N-glycans on glycoproteins to differentially regulate their surface level in response to hexosamine flux. Similarly, Sasai et al (143) demonstrate that the level of UDP-GlcNAc is a critical factor in the production of β1,6 branched N-glycans. If high glucose concentrations lead to a higher flux through the hexosamine (19) pathway, it is conceivable that this might have potential consequences for N-glycans such as CNDP1. In fact, Bar-On et al have shown in experimental diabetes that a dolichol-mediated increase in protein N-glycosylation occurs (24). They suggest that the dolichol-N-glycosylation pathway may represent another detrimental aspect of hyperglycaemia and may operate by dolichol mass action rather than through glycosylating enzyme activity.

CN-1 is a heavily glycosylated protein with 3 putative N-glycosylation sites at asparagine N322, N382 and N402 (144). In the present study therefore we investigated, whether N-glycosylation affects CN-1 protein secretion and enzyme activity of CN-1. Because an increased glucose flux through the hexosamine pathway increases GlcNAc concentrations (19), we hypothesized that N-glycosylation of CN-1 is affected by hyperglycemia resulting in an increased CN-1 secretion and enzyme activity.
MATERIAL AND METHODS

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 (Dept. of Nephrology, RWTH, Aachen, Germany), was used as template to construct different CNDP1 variants by PCR. Glycosylation sites of CN-1 were step wise deleted using the QuikChange® 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. The sequence of the primers that were used is depicted in Table 1. PCR reactions were performed according to standard procedures. For mutants lacking one glycosylation site either one of the asparagines residues at N322, N382 or N402 were exchanged. For mutants lacking two glycosylation sites combinations of residues N322 and N382 or N322 and N402 or N382 and N402 were mutated. Finally mutants that lack all three N-glycosylation sites at position N322, N382 and N402 were constructed. All CNDP1 constructs and mutants were cloned into pCSII + mt vector. For the N-glycosylation lacking mutants, stopcodons were removed to generate a myc-tagged CN-1 protein.

**TABLE 1** Primers used for site directed mutagenesis.

<table>
<thead>
<tr>
<th>Forward primer</th>
<th>Reverse primer</th>
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<tbody>
<tr>
<td>N322G 5’gacctagaaagaataacgggttacagcgggttggaa3’</td>
<td>5’ttccaaccccggtctcttccttttctttc3’</td>
</tr>
<tr>
<td>N382G 5’ctctaggcctcaacagttggtcttttctttc3’</td>
<td>5’ttccaacccggtctcttccttttctttc3’</td>
</tr>
<tr>
<td>N402G 5’ctttgaaggtgatgctcacaagagttgcttcacaagatggtgttc3’</td>
<td>5’gaacaacacatttttggaactaacttttttctttt3’</td>
</tr>
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</table>

Table 1 N-Glycosylation sites were step-wise deleted using mutagenic primers. Mismatched base pairs containing the mutations are underlined.

Cell culture and transfection

Cos-7 cells (Invitrogen, Karlsruhe, Germany) were cultured in Dulbeccos Modified Eagle Medium (DMEM, PAA The Cell Culture Company, Pasching, Austria) enriched with 10% FCS and 1% Penicillin/ Streptomycin at 37°C and 5% CO₂. The cells were co-transfected with the various CNDP1 constructs and pCruz-GFP™ (Santa Cruz, Heidelberg, Germany) by Nanofectin according to the manufacturer’s instructions (PAA The Cell Culture Company, Pasching, Austria). Four hours later, medium was replaced by DMEM medium containing 1% Penicillin/ Streptomycin. To inhibit N-glycosylation 10µg/ml of Tunicamycin (Sigma-Aldrich Chemie, Steinheim, Germany) was added to the medium. Cells and supernatants were harvested 48 hours later. For high glucose experiments Cos-7 cells were transfected with the
CNDP1 (CTG) variant and cultured for 72 hours in DMEM containing 25mM D-(+)-Glucose (Sigma-Aldrich Chemie, Steinheim, Germany) and 1% Penicillin/ Streptomycin. To decrease GlcNAc production, hexosamine biosynthesis pathway was inhibited by supplementing 20 µM azaserine (Santa Cruz, Heidelberg, German) to the glucose conditioned DMEM medium during the incubation period. No cell toxicity was observed using this concentration as determined by trypan blue exclusion. Transfected cells cultured for the same time period in standard medium (DMEM, 5mM D-(+)-Glucose, 1% Penicillin/ Streptomycin) were used as control. Hereafter supernatants were harvested and transfection efficiency was assessed on an aliquot of the cell suspension by FACS analysis using green fluorescent protein (GFP) as read-out. In general, transfection efficiency was above 70%. In all experiments supernatants were concentrated using a Centricon Centrifugal Filter device 30,000MW (Millipore, Schwalbach, Germany). Cells were lysed on ice by addition of 1% Triton X 100 containing lysis buffer, supplemented with 1mM 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 minutes (14,000g at 4°C) to remove insoluble debris.

**Western blot analysis**

For detection of carnosinase in cell lysates and supernatants, gel electrophoreses and subsequent western blotting was 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 minutes 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. Hereafter the membranes were blocked for 1 hour at room temperature in TBS-Tween 20 (0.3%, Sigma, Steinheim, Germany) containing 10% milk powder. Anti-myc antibody (Abcam plc, Cambridge, United Kingdom) was used for the detection of CN-1 produced by transfected cells. For detection of CN-1 in human serum samples, mouse monoclonal anti-CN-1 antibody (Clone RYSK-173, raised against human recombinant CN-1) was utilized. To assess the amount of GlcNAc modified proteins, the membranes were incubated over night with anti-N-acetylglucosamine antibody (Abcam plc, Cambridge, United Kingdom). After incubation with appropriate horseradish peroxidase conjugated secondary antibodies (Santa Cruz Biotechnology, Heidelberg, Germany) antibody
binding was visualized by enhanced chemiluminescence (PerkinElmer, Boston, USA). Intensity of the bands was measured by densitometry using the ImageJ 1.36b software.

*Measurement of carnosinase activity:*

Serum carnosinase activity (CN1) was assayed according to a method described by Teufel et al (144). 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-phtaldialdehyde (OPA) and fluorescence was read using a MicroTek plate reader ($\lambda_{\text{Exc}}$: 360 nm; $\lambda_{\text{Em}}$: 460 nm). The recombinant human CN1 was obtained from R&D Systems, Minneapolis, USA. CN-1 activity in cell supernatants was normalized to transfection efficiency.

*Patients:*

Diabetic patients and healthy controls were recruited from our clinic. Blood samples were collected for measuring CN1 activity as described above and for CN-1 genotyping as described previously (44). Eleven (CTG)$_5$ homozygous diabetic patients were identified (6 female, 5 male, mean age 62 years (range 36 to 84 years)). There were 3 type 1 and 8 type 2 diabetic patients included. Healthy controls consisted of 15 individuals (7 male, 8 female, mean 41 years (range 36 to 84)). Demographic characteristics of patients and controls are shown in Table 2. All participants gave informed consent. Ethical approval was given by the 2nd ethics committee of the Heidelberg University (amendment no. 2 and 3 to ethical approval no. 0193/2001).

<table>
<thead>
<tr>
<th>TABLE 2 Demographic characteristics of patients and control subjects</th>
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<tr>
<td>-----------------------------</td>
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<tr>
<td>Diabetic patients</td>
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<tr>
<td>Control subjects</td>
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Table 2 All data are expressed as mean. Values in parentheses represent the range; DM = diabetes mellitus; DR = diabetic retinopathy; HbA1C = Glycated haemoglobin; n = number of patients; nd = not done; RR = hypertension (defined as repeatedly elevated blood pressure >140/90 mmHg).

*Statistical analysis:*

Quantitative data are given as mean ± SD. Student t-test was calculated to compare groups. Values of p< 0.05 were considered as statistically significant.
RESULTS

*N-Glycosylation modulates secretion and enzyme activity of CN-1*

Three CN-1 immunoreactive bands, ranging from 61 to 65 kDa, were found by western blotting in cell lysates of pCSII- *CNDP1* transfected Cos-7 cells. While the 65 kDa band was always weakly expressed in cell lysates, in supernatants it was the most prominent band. After PNGase F treatment only the 61 kDa band was found, suggesting that the lower CN-1 band represents the immature CN-1 protein devoid of N-glycosylation (Fig 1). In human serum only one CN-1 reactive band was detected corresponding to a molecular weight of 65 kDa. PNGase F treatment of serum reduced its molecular weight to 61 kDa (Fig 1).

**FIGURE 1**

![Image of CN-1 expression in CNDP1 transfected Cos-7 cells and human serum. After transfection cell extracts (C) and supernatants (S) were harvested as described. The samples were treated with PNGase (+) or were left untreated (-). Similarly, aliquots of human serum (Hu) were subjected to PNGase treatment. Note that in cell extracts two major immunoreactive bands for CN-1 were detected by western blot analysis with apparent molecular weight of 61 kDa and 63 kDa respectively. The 65 kDa band was prominent in serum and supernatants of transfected cells, while the expression hereof was weak in cell extracts. After PNGase treatment, the molecular weight of CN-1 in serum, supernatants and cell extracts shifted to 61 kDa.](image)

To study the role of N-glycosylation for CN-1 protein secretion we first used Tunicamycin to inhibit N-glycosylation. Tunicamycin is an antibiotic that blocks the reaction of UDP-GlcNAc and dolicholphosphate, as a consequence the synthesis of all N-linked glycoproteins is inhibited. Tunicamycin completely prevented secretion of CN-1 in pCSII- *CNDP1* transfected cells as demonstrated by western blotting. CN-1 expression in cell extracts was quantitatively and qualitatively changed by Tunicamycin. Only the immature 61 kDa CN-1 band was detected in significantly lower amounts (Fig 2A).
Because Tunicamycin may also affect protein secretion independent of its inhibitory effect on N-glycosylation, we constructed myc-tagged CN-1 variants that lack 1, 2 or all 3 putative N-glycosylation sites. To this end, asparagine residues at N322, N382 and N402 were exchanged for glycine. Similar to Tunicamycin treatment, the amount of total CN-1 produced by variants that were completely devoid of N-glycosylation sites was strongly decreased compared to wild type CNDP1 (Fig 2B). The low CN-1 expression in these variants was not due to a low transfection efficiency since the percentage of green fluorescent protein (GFP) expressing cells was not significantly different compared to wild type CNDP1 transfected cells (wild type 68%, CNDP1 -3 73%). In the supernatants of all CNDP1 variants that lack either 1, 2 or 3 N-glycosylation sites, CN-1 expression was lower compared to the wild type CNDP1 variant (Fig 2B).

CN-1 secretion efficiency, expressed as the proportion of CN-1 in supernatants to total CN-1 (cell extract + supernatant) was measured by densitometry. In comparison to wild type CNDP1, CN-1 secretion efficiency was not affected for CN-1 variants lacking one or two N-glycosylation sites. In contrast, secretion efficiency was severely impaired for variants that lack all 3 N-glycosylation sites (Fig 2C). In line with the western blot, CN-1 enzyme activity in the supernatants was decreasing when the number of deleted N-glycosylation sites increased. Enzyme activity of CN-1 variants lacking only one N-glycosylation site was not significantly different to that of wild type CN-1. However, enzyme activity of CN-1 variants lacking two N-glycosylation sites was significantly decreased (Fig 2D). The combination of N-glycosylation sites that were deleted was not important in this regard (data not shown). As expected, CN-1 activity was not detected in supernatants of CN-1 variants that were devoid of N-glycosylation sites.

**FIGURE 2**
Fig. 2. Influence of N-glycosylation on CN-1 secretion. A: CNDP1 transfected cells were treated with tunicamycin (10 µg/ml) (+) for 48 h or were left untreated (-). Hereafter cell extracts (C) and supernatant (S) were subjected to Western blot analysis. Note that in the presence of Tunicamycin qualitative and quantitative differences for CN-1 expression in cell extracts were observed, whereas in supernatants of these cells no CN-1 expression was found. B: N-glycosylation sites of wild type (wt) CNDP1 were deleted by site directed mutagenesis. Numbers below the blot represent the deletion of any one of three (-1), any two of three (-2) or all three (-3) glycosylation sites. The CNDP1 variants were transfected in Cos-7 cells and expressed as myc-tagged fusion protein as described. Cell extracts (C) and supernatants (S) were subjected to western blot analysis using anti-myc antibody. In A and B the results of a representative blot is depicted. A total of six different experiments were performed and with all essentially the same result. C: CN-1 secretion efficiency was measured by densitometry of the blots. Secretion efficiency was defined as the ratio of CN-1 in supernatant divided by the total amount of CN-1 (cell extracts + supernatant). The results are expressed as mean ratio ± SD of six individual experiments. D: CN-1 activity in supernatants of wild-type CNDP1-transfected cells and cells expressing CNDP1 variants lacking either one (-1), two (-2), or all three (-3) glycosylation sites. The results are expressed as mean CN-1 activity (µmol/ml/hr) ± SD. Again, the supernatants of six individual experiments were included.

Hyperglycemia influences CN-1 secretion and enzyme activity

Because hyperglycemia results in an increased glucose flux through the hexosamine pathway and thereby increases GlcNAc concentrations, we speculated that hyperglycemia might increase N-glycosylation and subsequently affect CN-1 secretion and activity. Since amongst the different genetic CNDP1 (CTG)_n variants CN-1 secretion is the lowest for the (CTG)_5, we chose this variant in the hyperglycemia experiments. CNDP1 (CTG)_5 transfected cells were cultured for three days in high glucose medium (25 mM D-glucose) (HG) or kept in normal medium containing 5 mM of D-glucose (NG). We first tested if substrate supply for N-glycosylation, i.e. GlcNAc, was increased in cells that were cultured in HG medium. As demonstrated in Fig 3A, the expression of GlcNAc modified proteins was significantly increased under hyperglycemic conditions. We subsequently tested if HG conditions would
affect N-glycosylation of CN-1 and to what extent this influenced CN-1 secretion and enzyme activity.

Similar as demonstrated in figure 1, in cell extracts obtained from cells that were cultured in NG medium the immature not N-glycosylated 61 kDa band was detected in western blot analysis. However, in cells that were cultured in HG medium, the 61 kDa band was not detected suggesting a relative increase in N-glycosylation under HG conditions. This was paralleled by a slight increase in CN-1 expression in the supernatants of these cells (Fig 3B). Densitometric quantification of immunoblots revealed, that N-glycosylation efficiency in cell extracts, expressed as the proportion of N-glycosylated CN-1 (upper band) to total CN-1 (upper band + lower band) was significantly increased in cells cultured in 25 mM D-glucose containing medium (Fig. 3C, NG vs. HG p<0.05). In addition, this analysis revealed that CN-1 secretion efficiency, expressed as the proportion of CN-1 in cell extract to total CN-1 (cell extract + supernatant) was significantly increased under hyperglycemic conditions (Fig. 3D, NG vs. HG p<0.05). To analyze whether limitation of oligosaccharide substrate supply would influence N-glycosylation and secretion efficiency under HG, we used azaserine an inhibitor of hexosamine biosynthesis pathway to impede auxiliary UDP-GlcNAc formation. Azaserine, as a glutamine analogue, inhibits amongst others the glutamine:fructose-6-phosphate amidotransferase (GFAT) enzyme. Restriction of UDP-GlcNAc caused a remarkable decrease in CN-1 expression in the supernatants. Nevertheless, increased N-glycosylation efficiency in cell extracts was not affected by azaserine conditioning (Fig. 3E).

FIGURE 3

![Figure 3](image-url)
Fig. 3. Influence of high glucose medium on GlcNAc expression, N-glycosylation of CN-1, and CN-1 secretion and impact of hexosamine synthesis pathway on CN-1 secretion. CNDP1 transfected Cos-7 cells were cultured for 72 hrs in the presence of 25 mmol/l (HG) ± 20 µmol/l azaserine or 5 mmol/l (NG) D-glucose. Hereafter cell extracts were prepared and supernatants were harvested. A: Western blot analysis of GlcNAc modified proteins in cell extracts. B: Western blot analysis of CN-1 expression in supernatants (S) and cell extracts (C). Note that the immature 61 kDa band is not present in cells that were cultured under HG conditions. In A and B the results of a representative experiment is depicted. Four different experiments were performed and all showed similar results. C: CN-1 glycosylation efficiency in cell extracts was measured by densitometry and defined as ratio of N-glycosylated CN-1 (upper band) to total CN-1 (upper band + lower band). D: CN-1 secretion efficiency was measured as described in Fig. 2. Four experiments were included. E: Result of a representative Western blot analysis of CN-1 expression in supernatants (S) and cell extracts (C) of cells cultured in the presence of HG with (+) or without (-) azaserine. Although the secretion of CN-1 is decreased by the limitation of GlcNAc, azaserine does not influence the relative increase in N-glycosylation in cell extracts under HG conditions. A total of four different experiments were performed and all showed similar results.

To test if our in vitro observations also have an in vivo relevance, we next addressed if serum CN-1 activity is different in homozygous (CTG)$_5$ diabetic patients and genotype matched
healthy controls. Compatible with the in vitro findings, serum CN-1 activity was significantly higher in diabetic patients (control vs. diabetic patients \( p<0.05 \)) (Fig 4).

**FIGURE 4**

![Graph showing CN-1 activity in 5L homozygous healthy controls (Co, n=15) vs. 5L homozygous diabetic patients (DM, n=11). Results are expressed for each individual patient and control. The line represents the mean of each group.]

*Fig. 4. CN-1 activity was measured in 5L homozygous healthy controls (Co, n=15) as well as in 5L homozygous diabetic patients (DM, n=11). Results are expressed for each individual patient and control. The line represents the mean of each group.*
SECRETION OF THE SERUM CARNOSINASE IS INFLUENCED BY THE LENGTH OF THE (CTG)\text{n} REPEAT, LOCATED IN THE SIGNAL PEPTIDE OF THIS ENZYME (10). BECAUSE CN-1 IS N-GLYOSYLATED AND GLYOSYLATION MIGHT INFLUENCE PROTEIN SECRETION, STABILITY AND ENZYME ACTIVITY, IT IS CONCEIVABLE THAT IN HYPERGLYCEMIC PATIENTS CN-1 SECRETION OR ACTIVITY IS NOT COMPLETELY DETERMINED BY THE (CTG)\text{n} GENOTYPE. IN THE PRESENT STUDY WE THEREFORE ASSESSED THE RELEVANCE OF N-GLYOSYLATION FOR CN-1 PROTEIN SECRETION AND ENZYME ACTIVITY AND ADDRESSED IF HYPERGLYCEMIA CAN INFLUENCE SERUM CN-1 SECRETION AND ACTIVITY. THE MAIN FINDINGS OF THIS STUDY ARE THE FOLLOWING. FIRST, ONLY DELETION OF ALL THREE N-GLYOSYLATION SITES IMPAIRS CN-1 SECRETION EFFICIENCY, WHILE CN-1 ACTIVITY IS ALREADY DIMINISHED WHEN TWO SITES ARE DELETED. SECOND, HYPERGLYCEMIA INCREASES N-GLYOSYLATION EFFICIENCY AND INCREASES CN-1 SECRETION. THIS MIGHT BE CAUSED BY AN INCREASED GLCNAC SUPPLY. THIRD, HOMOZYGOUS (CTG)\text{5} DIABETIC PATIENTS HAVE HIGHER SERUM CN-1 ACTIVITY COMPARED TO GENOTYPE MATCHED HEALTHY CONTROLS.

The human CN-1 glycoprotein contains three potential N-glycosylation sites at asparagine number N322, N382 and N402 (144). In pCSII-CNDP1 transfected Cos-7 cells three CN-1 immune reactive bands can be detected. The 61 and 63 kDa bands dominate in cell lysates while the 65 kDa band is almost not detectable in cell extracts. In contrast, the 65 kDa band is strongly expressed in supernatants of transfected cells. The different CN-1 bands detected in western blotting are likely due to differences in N-glycosylation since after PNGase F treatment only the 61 kDa band was detected. Hence, this band represents the immature not N-glycosylated CN-1 protein. Macroheterogeneity occurs for a number of glycosylated proteins and describes the differential use of glycosylation sites within a given protein (145-147). Neither in serum nor in supernatants of transfected cells the 61 or 63 kDa band was found. Although this might suggest that CN-1 secretion only occurs after complete N-glycosylation, our findings that CNDP-1 variants lacking 1 or 2 glycosylation sites also secrete CN-1 argues against this assumption. It is therefore more likely that secretion of the completely N-glycosylated CN-1 protein is favored, but that complete N-glycosylation is not a pre-requisite for CN-1 secretion. Nevertheless, when N-glycosylation is completely prevented, either by deletion of all three N-glycosylation sites or by Tunicamycin treatment, CN-1 secretion is severely impaired. N-glycosylation occurs co-translationally in the lumen of the endoplasmatic reticulum (ER) and facilitates the protein folding process by recruiting members of the calnexin chaperone system (148). Acquisition of the protein’s native structure may therefore fail or progress slowly when N-glycosylation is prevented. Folding-defective or
terminally misfolded proteins are disposed from the ER through cytosolic transport and subsequent proteasomal degradation (149). This might explain why CN-1 is significantly less expressed in cells transfected with CNDP1 variants that are completely devoid of N-glycosylation sites as opposed to cells expressing wild type CNDP1. The importance of N-glycosylation of CN-1 for protein secretion is not unique to CN-1, as it has also been reported for other proteins (147, 150, 151).

Our data also indicate that CN-1 enzyme activity was dependent on proper N-glycosylation since deletion of two N-glycosylation sites significantly decreased CN-1 activity. Enzyme activity as a function of N-glycosylation has also been reported by other groups (152, 153). Why CN-1 activity severely drops after deletion of two glycosylation sites remains to be elucidated. Because CN-1 was poorly secreted by CNDP1 variants that were devoid of N-glycosylation sites, no CN-1 activity was detected in supernatants of these cells as expected.

In diabetic patients the role of non-enzymatic advanced glycation end products for cell activation and/or damage has been well studied (154, 155). In contrast, the influence of enzymatic glycosylation and its possible pathogenic role in diabetic complications remains to be addressed. A few studies however, have reported that hyperglycemia increases N-glycosylation in rats (24, 156, 157). Our own data are in line with this assumption, as we could show that the immature not N-glycosylated CN-1 protein was not present in CNDP1 transfected cells that were grown under high glucose conditions. Yet, a possible increase in glycosylation was not reflected by a difference in molecular weight of the mature secreted CN-1 protein. Although under hyperglycemic conditions activation of the hexosamine pathway leads to synthesis of UDP-GlcNAc (19), an increase of substrates alone might not be sufficient to enhance N-glycosylation (158). These conclusions are in accordance with our observations, since in cells cultured under HG N-glycosylation efficiency was not disturbed by inhibition of hexosamine synthesis pathway. Nevertheless, restriction of UDP-GlcNAc led to decreased CN-1 expression in the cell supernatants. The assembly of the N-glycosylation core oligosaccharide is dependent on two UDP-GlcNAc molecules. Therefore, it is conceivable that the N-glycosylation and secretion process is slowed down under azaserine treatment. As expected, Azaserine did not completely block N-glycosylation of CN-1, because the oligosaccharide substrates can either be synthesized from glucose or be salvaged from glycoconjugates degraded within cells. The expression or activity of enzymes that attach sugars to growing proteins might equally have contributed to the increased N-glycosylation
efficiency under hyperglycemic conditions; these enzymes were not measured in this study. Hence, it must be emphasized that the increased CN-1 secretion observed under hyperglycemic conditions may be related to changes in the N-glycosylation machinery and substrate increment.

If hyperglycemia influences CN-1 secretion it is expected that diabetic patients would have more CN-1 activity compared to genotype matched healthy controls. Because healthy individuals homozygous for \textit{CNDP1 (CTG)}\textsubscript{5} have low CN-1 activity (44), we stratified for this genotype and compared CN-1 activity of diabetic patients with that of healthy controls. Indeed our data show that diabetic patients have a significantly higher CN-1 activity. We are aware that this study only included a relative small group of diabetic patients (n=11), and significant differences in the mean age between both groups were present. Because CN-1 activity increases with age until adulthood but not thereafter (54), it is unlikely, but not excluded, that age differences are a confounding factor in our analysis. Therefore these data should be confirmed in a larger cohort of age, sex and genotype matched patients, before firm conclusion can be drawn.

Recently, we have shown that \textit{CNDP1} activity towards carnosine is inhibited by homocarnosine (159). If carnosine is considered to be protective in terms of diabetic complications, than clearly other factors than \textit{CNDP1} genotype that also affect carnosine metabolism, e.g. serum homocarnosine concentrations, blood glucose control, should be taken into account for risk assessment to develop diabetic complications in this group of patients. Diabetic patients that do not have the protective \textit{CNDP1} genotype might be protected because of good glycemic control or by having sufficient homocarnosine levels. Vice versa, diabetic patients with the protective genotype may still develop diabetic nephropathy when glycemic control is poor or when low serum concentrations of homocarnosine is present.

Inasmuch as our study demonstrates that hyperglycemia increases CN-1 secretion, it was not our intention to investigate if a low CN-1 activity is associated with a diminished oxidative stress or AGE formation. CN-1 is only one of the parameters that influence the amount of serum carnosine. Activity of carnosine synthase and dietary carnosine intake are two additional factors. For future studies it would thus also be worthwhile to study if in diabetic patients CN-1 activity correlates with carnosine concentrations and to elucidate if this in turn correlates with parameters of oxidative stress and or AGE formation.
We conclude that apart from the (CTG)$_n$ polymorphism in the signal peptide of CN-1, N-glycosylation is essential for appropriate secretion and enzyme activity. Since hyperglycemia enhances CN-1 secretion and enzyme activity, our data suggest that poor blood glucose control in diabetic patients might result in an increased CN-1 secretion even in the presence of the (CTG)$_5$ allele.
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