On the relevance of carnosine and carnosinase for the development of diabetic nephropathy
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CHAPTER 6

SERUM CARNOSINASE IN A DIABETIC POPULATION:
A PRELIMINARY PILOT STUDY

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* Equally contributing authors

Work in Progress
ABSTRACT

Serum carnosinase (CN-1) is poorly secreted when healthy individuals are homozygous for the (CTG)$_5$ allele of the CNDP1 gene. Accordingly, low serum CN-1 activity is found in these individuals. We recently demonstrated that secretion of (CTG)$_5$ allele encoded CN-1 is increased when CNDP1 transfected Cos-7 cells are cultured in high glucose medium. This suggests that apart from the genotype, hyperglycemia might be an additional factor that influences CN-1 secretion. We therefore hypothesized in the present study that the correlation between the (CTG)$_n$ polymorphism and CN-1 in serum is less clear in a diabetic population. Moreover, we aimed to investigate the influence of blood glucose and lipid control on the amount of CN-1 secreted into the serum of diabetic patients. To this end, 70 type 2 diabetic patients and 34 healthy controls were genotyped and CN-1 concentration was determined in serum. Blood glucose, HbA$_{1c}$, triglycerides and cholesterol levels were measured as clinical parameters to estimate blood glucose and lipid control of the diabetic patients.

Similar as previously demonstrated, it was found that serum CN-1 concentrations was significantly lower in healthy individuals that are homozygous for the (CTG)$_5$ allele compared to that of individuals with other genotypes. The serum CN-1 concentration in all diabetic patients was approximately 5 times higher compared to healthy controls and in diabetic patients homozygosity for the (CTG)$_5$ allele was not associated with lower serum CN-1 concentration. In (CTG)$_5$ homozygous diabetic patients, CN-1 concentration correlated positively with blood glucose, but not with HbA$_{1c}$, cholesterol and triglycerides levels. In patients that carried an allele longer than (CTG)$_5$ no correlations were found between CN-1 concentration and the tested factors.

In conclusion, our data suggest that hyperglycemia is indeed an additional factor that influences secretion of (CTG)$_5$ allele encoded CN-1. This might explain why the association between homozygosity for the (CTG)$_5$ allele and CN-1 concentration is lost in diabetic patients. Poor blood glucose control in these patients might result in high serum CN-1 expression and consequently low carnosine levels. Hence, even in the presence of a protective genotype diabetic patients are at risk to develop DN, if blood glucose control is poor. Our data also underscore the importance of consequent glycemic control.
INTRODUCTION

Diabetic nephropathy (DN) is the most common cause for end stage renal disease (ESRD) and is recognized as an urgent medical problem of world wide dimensions (2). Approximately one third of the diabetic patients develop DN (118). A number of risk factors are associated with the development of DN, including prolonged duration of diabetes (188, 189), poor glycemic control, raised blood pressure and hypercholesterolemia (4, 5, 190-193). Yet, these risk factors alone are not sufficient for the development of DN, but most likely require a specific genetic background (33, 121).

In a linkage analysis performed on 18 Turkish families with type 2 diabetes and nephropathy, we have previously identified a susceptibility locus for diabetic nephropathy on chromosome 18q22.3-q23. Association between DN and this locus was subsequently confirmed in other, genetic heterogeneous groups (40, 41), suggesting that this locus harbours important genes with a major effect on DN. In 2005 the region was narrowed down to a single gene, i.e. the CNDP1 gene, encoding serum carnosinase or CN-1 (44). Based on the number of CTG repeats, 5 different genotypes of CNDP1 can be found that encode for a hydrophobic stretch of 4, 5, 6, 7 or 8 leucines (4L-8L) in the signal peptide of CN-1. Individuals homozygous for the (CTG)_5 allele are at a 2.56-fold reduced risk to develop DN compared to individuals with other genotypes. In line with the observation that CN-1 activity is low in (CTG)_5 homozygous healthy individuals (44), we have demonstrated that a low CN-1 secretion is observed when (CTG)_5 is present in the signal peptide of CN-1 (133). Since diabetic patients homozygous for the (CTG)_5 develop less frequently DN, a low CN-1 activity seem to be advantageous.

We have recently shown in CNDP1 transfected Cos-7 cells that secretion of CN-1 is increased when the cells are cultured in the presence of 25 mM of D-glucose. Moreover, we could show that on average serum CN-1 activity was higher in (CTG)_5 homozygous diabetic patients compared to genotype matched healthy controls (171). The latter observation was however made in a limited number of patients and thus needs to be confirmed in a larger well-characterized cohort of patients. There is experimental evidence that hyperglycemia increases N-glycosylation (24) and, as previously shown, proper N-glycosylation is required for CN-1 secretion (171). Based on these findings, it is conceivable that in diabetic patient serum CN-1 activity and concentrations are changed. The present study was conducted to test whether serum CN-1 concentrations are increased in diabetic patients and whether the association between homozygosity for the (CTG)_5 allele and low serum CN-1 is still true in these patients. Moreover, we investigated the relationship between blood glucose/lipid control and CN-1 concentration in the diabetic patients.
RESEARCH DESIGN AND METHODS

Study cohort
Patients with type 2 diabetes were recruited at the Isala Clinics (Zwolle, the Netherlands). Blood samples (serum and EDTA-plasma) were taken from 70 diabetic patients. For inclusion and exclusion criteria see Table 1. Blood samples were also taken from 34 adults that served as healthy controls (mean age 40 range 22-66 years, 19 females and 15 males. There was no significant age difference between male and female individuals). The healthy controls had no known history of diabetes or any kidney disease. All participants gave informed written consent. The trial was approved by the medical ethics committee. HbA1c, Blood glucose, cholesterol and triglyceride levels were determined by laboratory measurements according to standard procedures.

### TABLE 1

<table>
<thead>
<tr>
<th>Inclusion Criteria</th>
<th>Exclusion Criteria</th>
</tr>
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<tbody>
<tr>
<td>Age 50-75 years</td>
<td>Blood pressure &gt; 160/90 mmHg</td>
</tr>
<tr>
<td>Type 2 diabetes</td>
<td>Renal impairment due to other cause than DN</td>
</tr>
<tr>
<td>Diabetic nephropathy:</td>
<td>More severe stages of DN</td>
</tr>
<tr>
<td>-microalbuminuria 15-300 mg/24h</td>
<td>(macroalbuminuria, renal insufficiency)</td>
</tr>
<tr>
<td>-albumin to creatinine ratio</td>
<td></td>
</tr>
<tr>
<td>1.25-25 in males</td>
<td></td>
</tr>
<tr>
<td>1.75-35 in females</td>
<td></td>
</tr>
<tr>
<td>Use of RAAS-system blocking</td>
<td>Use of vitamine B-containing</td>
</tr>
<tr>
<td>agents (ACEi or AII antagonists)</td>
<td>supplements during the last 3 months</td>
</tr>
<tr>
<td>HbA1c&lt;8.5%</td>
<td>Use of NSAIDS</td>
</tr>
<tr>
<td>eGFR (MDRD) &gt; 30 ml/min</td>
<td>Tumors</td>
</tr>
</tbody>
</table>

*Table 1. Inclusion and exclusion criteria used to select the diabetic patients examined in this study.*

Genotyping
The CNDP1 exon 2 trinucleotide repeat polymorphism (D18S880) was genotyped as described previously (44). In brief, CNDP1 exon 2 was amplified using intronic primers and
subsequently subjected to fragment analysis on an ABI 310 capillary sequencer (Applied Biosystems, Darmstadt, Germany) according to standard operating procedures.

**CN1 ELISA**

CN-1 concentrations in EDTA-plasma samples were measured by ELISA. A human CN1 ELISA was developed by coating high absorbent microtitre plates (Greiner, Labortechnik, Frickenhausen, Germany) overnight with 100 µl of goat polyclonal anti-human CN1 (1 µg/ml) (R&D, Wiesbaden Germany). Unspecific binding was blocked with 0.05 % W/V of dry milk powder. Plates were incubated with the plasma sample for 1h under constant agitation. Rabbit polyclonal IgG (ATLAS, Abcam plc, Cambridge, United Kingdom) was added for 1 hr and consecutively HRP conjugated goat anti-rabbit IgG was added for an additional hour. In between the different steps, the plate was extensively washed with PBS/Tween. Deep-Blue peroxidase (POD) (Roche diagnostics, Mannheim, Germany) was used for colour development, which was generally stopped after 15 minutes by addition of 50 µl of 1 M H2SO4. The plates were directly read at 450 nm. Recombinant human CN1 (R&D Systems, Minneapolis, USA) was used as standard. CN1 protein concentrations were assessed in the linear part of the dilution curve. Sensitivity of the ELISA assays was approximately 20 ng/ml.

**Statistical analysis**

Variables with are presented as means ± SD. Data were analyzed by ANOVA measurements. Results were considered statistically significant with P < 0.05. Pearson correlation coefficients were calculated to test the strength of the linear relationship between two variables.
RESULTS

In the present study we measured CN-1 activity and concentration in serum of genotyped type 2 diabetic patients (n=70) and healthy controls (n=34). The CNDP1 genotype in the diabetic cohort was similar distributed as previously described (44). The majority of patients carried the 5L-5L or 5L-6L genotype (37 % and 36 %, respectively), while the other genotypes were less frequently found (6L-6L:16 %, 5L-7L: 9 %, 6L-7L: 0%, 7L-7L= 3 % ) (Fig 1).

The clinical parameters, i.e. blood glucose, HbA1c, triglycerides and cholesterol, were similar between the different genotypes (Tab. 2).

FIGURE 1

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Fig. 1. Genotype distribution in the type 2 diabetic study cohort. 70 diabetic patients were genotyped for the length of the leucine (L) stretch encoded by the CNDP1 gene. 26 patients were homozygous for the 5L allele (5L-5L), 25 patients had the 5L allele and the 6L allele (5L-6L), 6 patients had the 5L allele and the 7L allele (5L-7L), 11 patients were homozygous for the 6L allele (6L-6L), no patient had the 6L allele and the 7L allele, and 2 patients were homozygous for the 7L allele (7L-7L).
**Table 2.** Clinical parameters in the type 2 diabetic study cohort. Patients with different CNDP1 genotypes showed no differences in clinical parameters, such as age, gender, diabetes duration, BMI, body weight, blood glucose and cholesterol. Data given as mean ± SD.

Similar as previously reported, CN-1 concentration (Fig 2A) in healthy individuals that carry the 5L-5L genotype were lower compared to that of individuals with other genotypes (-35%, p<0.05, 5L-5L compared to other genotypes). The serum CN-1 concentration in all diabetic patients was approximately 5 times higher compared to healthy controls (diabetic patients: 114257.6 ± 32773.1; healthy controls: 24874.9 ng/ml ± 8909.5; p<0.05) and was approximately the same for both groups (Fig 2B).

**FIGURE 2**

![Fig. 2. CN-1 concentration in serum of healthy subjects and diabetic patients. A In the healthy population, the 5L homozygous (5L-5L) individuals show significantly lower CN-1 concentration compared to individuals with other genotypes. B In the diabetic population, the serum CN-1 concentration is approximately 5 times higher compared to healthy controls.](image-url)
SERUM CARNOSINASE IN A DIABETIC POPULATION

with other genotypes (mean ± SD, p<0.05). B: In diabetic patients, CN-1 concentration did not differ between 5L homozygous patients (5L-5L) and patients carrying other genotypes (mean ± SD).

We next tested if differences in clinical parameters were associated with CN-1 concentration in the serum of diabetic patients. A positive correlation was found between CN-1 concentration and blood glucose when the patients were 5L homozygous (r= 0.46, p<0.05, Fig. 3A). In contrast, no significant correlation was found between CN-1 concentration and blood glucose when the patients carried a longer allele of the CNDP1 gene (Fig. 3B). HbA1c levels did not correlate with CN-1 concentration for both groups (Fig 3C,D). Similarly, no correlation was observed between CN-1 concentrations and the levels of triglycerides (Fig. 3E,F) and cholesterol levels (Fig. 3G,H).

FIGURE 3

A

B

C

D
Fig. 3. Correlations between CN-1 concentration and parameters of lipid and blood glucose control in 5L-5L homozygous diabetic patients (A, C, E, G) and in patients that carry another allele of the CNDP1 gene (B, D, F, H). In 5L homozygous diabetic patients, CN-1 concentration significantly correlated with blood glucose ($p<0.05$, $r=0.46$) (A), whereas no correlation was observed between CN-1 concentration and blood glucose in patients that carry another allele (B). CN-1 concentration did not correlate with HbA1c (C;D), triglycerides (E,F) and cholesterol (G,H).
DISCUSSION

Diabetic patients homozygous for the (CTG)₅ allele of the CNDP1 gene have a 2.56-fold reduced risk for developing DN when compared to patients that carry other genotypes (42, 44). A short CTG repeat in the CNDP1 gene, i.e. (CTG)₅ or less, results in poor secretion of CN-1 (168). Hence, (CTG)₅ homozygous individuals have a low CN-1 activity in serum (44). Recently, we have shown that in (CTG)₅ CNDP1 transfected cells cultured under high glucose conditions, secretion of CN-1 is increased (171). This suggests that in serum of hyperglycemic patients homozygous for the (CTG)₅ allele, CN-1 concentration might be higher compared to genotype matched healthy controls. This assumption was supported in a study with a limited number of diabetic patients that demonstrated an elevated CN-1 activity in 5L homozygous patients (171). The present study was conducted to test whether serum CN-1 concentrations are increased in diabetic patients and whether the association between homozygosity for the (CTG)₅ allele and low serum CN-1 is still true in these patients. Moreover, we investigated the relationship between blood glucose/lipid control and CN-1 concentration in the diabetic patients.

Our study indeed suggests that when comparing healthy controls and diabetic patients CN-1 concentration is significantly higher in diabetics. Besides, a lower CN-1 concentration was only found in the (CTG)₅ homozygous healthy controls but not in the (CTG)₅ homozygous diabetics. In addition, it seems that CN-1 concentration is associated with blood glucose in the (CTG)₅ homozygous diabetic patients.

Our study demonstrates that only in healthy individuals, but not in diabetic patients, homozygosity for the (CTG)₅ allele is associated with a low serum CN-1 concentration. That finding might argue against the relevance of serum CN-1 as a risk factor for developing DN as it does not explain why this genotype is protecting diabetic patients for DN. Recently, it has been suggested that the association between the CNDP1 gene and diabetic nephropathy is sex specific and independent of susceptibility for type 2 diabetes (194). It might therefore be that homozygous (CTG)₅ males and females are quite distinct with respect to serum CN-1 concentrations and perhaps behave differently in this regard to hyperglycemia. The imbalanced male/female (17/9) ratio in the (CTG)₅ homozygous group in our study may have influenced our results and needs to be addressed in detail in further studies.

Similar to our in vitro observations (171), we found that in the seems that in diabetic patients more CN-1 is secreted into the serum. Of importance, CN-1 concentration did not correlate with HbA1c level as one would expect from the observed association between blood glucose
and CN-1 expression in the 5L homozygous patients. HbA1c levels do not represent current glycemic status but represents the average plasma glucose concentration of the last 120 days. High HbA1c levels are caused by fluctuating high and low blood glucose levels or reflect elevated blood glucose levels that do not vary throughout the day. If short term glucose elevation is an important stimulus for CN-1 secretion this might explain why the association between CN-1 activity or concentration and blood glucose levels were found, despite the fact that this association was not found for HbA1c levels. Elevation of blood glucose levels in a physiologic range, e.g. postprandial, does not affect CN-1 activity or concentration, as recently shown in healthy subjects by Peters et al. (195).

In conclusion, we have shown that serum CN-1 concentrations are increased in diabetic patients. In addition, it seems that the association between homozygosity for the (CTG)$_5$ allele and low CN-1 activity or concentration is lost in these patients. These findings challenge the hypothesis that diabetic patients homozygous for the (CTG)$_5$ allele have low CN-1 activity and hence are protected against DN. Nevertheless, if serum CN-1 is a significant factor for developing DN, our results also underscore the relevance of appropriate glycemic control. Yet, further studies are warranted to elucidate why the (CTG)$_n$ polymorphism in the CNDP1 gene is associated with susceptibility to develop DN.
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