Original Article

Carnosine as a Protective Factor in Diabetic Nephropathy

Association With a Leucine Repeat of the Carnosinase Gene *CNDP1*

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The risk of diabetic nephropathy is partially genetically determined. Diabetic nephropathy is linked to a gene locus on chromosome 18q22.3-q23. We aimed to identify the causative gene on chromosome 18 and to study the mechanism by which the product of this gene could be involved in the development of diabetic nephropathy. DNA polymorphisms were determined in 135 case (diabetic nephropathy) and 107 control (diabetes without nephropathy) subjects. The effect of carnosine on the production of extracellular matrix components and transforming growth factor-β (TGF-β) after exposure to 5 and 25 mmol/l D-glucose was studied in cultured human podocytes and mesangial cells, respectively. A trinucleotide repeat in exon 2 of the *CNDP1* gene, coding for a leucine repeat in the leader peptide of the carnosinase-1 precursor, was associated with nephropathy. The shortest allelic form (*CNDP1 Mannheim*) was more common in the absence of nephropathy (P = 0.0028, odds ratio 2.56 [95% CI 1.36–4.84]) and was associated with lower serum carnosinase levels. Carnosine inhibited the increased production of fibronectin and collagen type VI in podocytes and the increased production of TGF-β in mesangial cells induced by 25 mmol/l glucose. Diabetic patients with the *CNDP1* Mannheim variant are less susceptible for nephropathy. Carnosine protects against the adverse effects of high glucose levels on renal cells. *Diabetes* 54:2320–2327, 2005

Diabetic nephropathy is one of the most severe complications of type 1 and type 2 diabetes. Diabetic nephropathy has become the leading cause of end-stage renal failure in the western world. In the U.S., the number of cases of end-stage renal failure due to diabetic nephropathy continues to increase at almost 10% per year (1).

There is a large body of evidence (2,3) of familial aggregation of diabetic nephropathy, and the existence of at least one genetic susceptibility factor seems likely. The most intensively studied genetic variant is the "D/I" polymorphism in the ACE gene on chromosome 17 encoding the angiotensin converting enzyme. Meta-analyses (4–6) have suggested a weak association between the presence of the D-allele and diabetic nephropathy.

In 2002, our group presented results of a family-based linkage study on 18 Turkish families with type 2 diabetes showing that a major susceptibility locus maps to chromosome 18q22.3-q23 (7). The locus was mapped between the markers D18S43 and D18S50 and was supported by a highly significant logarithm of odds score of 6.1. Linkage of diabetic nephropathy to 18q was confirmed by an affected sibpair analysis performed on Pima Indians and, very recently, by a large sibpair analysis of African Americans (7,8). We postulated that diabetic nephropathy might be caused by a dominant-acting mutation at this gene locus. Consequently, a recessive allele at this locus could protect carriers from the development of this complication. ZNF236 is the only gene within the 18q candidate region

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AGE, advanced glycation end product; ROS, radical oxygen species; TGF-β, transforming growth factor-β.

that had previously been considered as a candidate gene for diabetic nephropathy (9). Since no mutations in the transcript of the gene were found, ZNF236 was subsequently excluded as a trait-causing gene (10). It therefore remained unknown which gene within the 18q region is responsible for the susceptibility of some diabetic subjects to develop diabetic nephropathy. We searched for genes related to the pathophysiology of microvascular disease and focused on the inhibition of oxidative stress and advanced glycation end products (AGEs). For example, AGEs have been found in glomeruli of diabetic animals and humans, the inhibition of AGEs is beneficial in experimental diabetic nephropathy, and AGEs have been shown to cause impaired proliferation of renal mesangial cells, a major feature of diabetic nephropathy development (11). The carnosinase genes *CNDP1* and *CNDP2* are such genes located in 18q22.3.

The enzyme carnosinase degrades the dipeptide carnosine (β-alanyl-L-histidine), while carnosine has been reported to inhibit the formation of AGE molecules (12). *CNDP1* encodes the secreted serum carnosinase, whereas *CNDP2* encodes tissue carnosinase, also known as cytosolic nonspecific dipeptidase (13). The genes are positioned adjacent to each other in a head-to-tail orientation. The aim of this study was to identify the causative gene on chromosome 18 and to gain insight in the mechanism by which the product of this gene could be involved in the development of diabetic nephropathy.

RESEARCH DESIGN AND METHODS

Sampling strategy for case-control study. We screened 5,500 patient records in different centers in Germany, The Netherlands, Qatar, and the Czech Republic. A complete list of participating centers can be found in the contributors section of the ACKNOWLEDGMENTS. To identify case and control subjects, screening of all patient records of a participating center was performed, and patients not fulfilling the inclusion criteria were excluded. The detailed sampling strategy, exclusion and inclusion criteria, and details on collected data are given in the addendum available from www.klinikum.uni-heidelberg.de/index.php?id = 6,791.

Power analysis. Single type 2 and type 1 diabetic patients (designated groups 2 and 3, respectively) were recruited for confirmatory testing of the sequence variant selected in the first phase (an explorative search using group 1). Together, groups 2 and 3 consisted of 99 patients with diabetes and 98 patients with diabetic nephropathy. Power estimations had shown that such a sample size would be sufficient to confirm association with >80% power if the relative risk exceeds 2.5 (allele frequency 40–50%, significance level 5%).

Sequence analysis and genotyping. CNDP1 exons were amplified using intronic primers (MWG-Biotech, Ebersberg, Germany) and sequenced using an ABI 3100 capillary sequencer (Applied Biosystems, Darmstadt, Germany). Sequence analysis of the *CNDP2* gene was performed by Synergene (Qormi, Malta). Techniques used for PCR-based genotyping of polymorphisms included restriction enzyme digest (rs2278161), fragment analysis using an ALF-Express DNA sequencer (Pharmacia-Biotech, Freiburg, Germany) (D18S58, rs10548323, rs3080862, DN31, rs10596720, D18S880, and D18S1161), TaqMan single nucleotide polymorphism genotyping using an ABI Prism 7000 (Applied Biosystems) (rs12965928 and rs2241509), denaturing high-performance liquid chromatography analysis (Transgenomic, Berlin, Germany) (rs1046407 and rs22778156), or sequence analysis (rs1559803, rs7237740, rs3764509, rs890332, rs4892247, and rs2887). Primer sequences are available on request. For D18S880, we used a standard PCR protocol with primers AGGCAGCTGTGTGAGGTAAC (forward) and GGGTGAGGAGAACATGCC (reverse). The annealing temperature was 60°C. Sequence analysis confirmed that a PCR product length of 167 corresponds to 5 CTG repeat units (five leucine codons).

Measurement of carnosine and carnosinase activity in serum. For measurement of serum-carnosinase activity, serum samples were withdrawn between 9:00 and 10:00 A.M., cooled on ice immediately, and stored at −80°C. A total of 150 μl carnosine (1 mg/ml in 50 mmol/l Tri-HCl buffer) (Sigma Aldrich Chemie, Deisenhofen, Germany) was added to 600 μl serum, divided into aliquots of 100 μl, and incubated at 30°C. The reaction was stopped after 5, 10, 15, 20, 30, and 40 min by adding 25 μl 10% 5-sulfosalicylic acid (Sigma Aldrich Chemie). After shaking, the samples were incubated at 4°C for 30 min and centrifuged at 13,000 rpm for 5 min to remove the precipitated proteins. Carnosine concentrations were assayed by fluorometic determination after derivatization with carbazole-9-carbonyl chloride. Separation was performed by liquid chromatography according to the method described by Schonherr (14).

In vitro studies

Cell culture. Human SV 40–transformed mesangial cells as well as human thermosensitive SV 40–transformed podocytes were used to investigate the influence of L-carnosine on the increased production of extracellular matrix proteins and transforming growth factor-β (TGF-β)1 and -2 under hyperglycemic conditions. SV 40–transformed, temperature-sensitive podocytes were characterized as previously described (15). These cells proliferate at the "permissive" temperature (33°C). After transfer to the "nonpermissive" temperature (37°C), they enter growth arrest and express markers of differentiated in vivo podocytes, including nephrin, podocin, CD2AP, and synaptopodin. Human SV 40–transformed mesangial cells were characterized as previously described (16). All cell lines were cultivated in uncoated culture flasks in Dulbecco's modified Eagle's medium (PAA, Coelbe, Germany) supplemented with 10% FCS (Greiner, Frickenhausen, Germany), insulin-transferin selenium (all in concentrations of 5 ng/ml), epidermal growth factor (5 ng/ml), and penicillin/streptomycin (10 units/ml, all from Sigma, St. Louis, MO). Methods for indirect immunofluorescence, TGF-β1 and -β2, enzyme-linked immunosorbent assays, RNA isolation, and real-time PCR on human glomeruli, CNDP1 antibodies, and immunohistochemistry are available from www.klinikum.uni-heidelberg.de/index.php?id = 6,791.

Computer analyses and statistical tests. We used the PSORT II program (19) for functional analysis of 5′ sequences (calculation of G. von Heijne [GvH] scores). Statistical analyses were performed with SAS V8.02 (PC version). Fisher's exact test and χ^2 analyses were performed to test for association within each population group. The Mantel-Haenszel test was used to analyze data obtained from multiple populations. The Wilcoxon rank-sum test was used to compare groups of samples. P values <0.05 were regarded as significant.

RESULTS

Patient data. A total of 5,500 patient files were screened to identify the patients included in this study. One hundred thirty-five patients with diabetic nephropathy and 107 diabetic patients without diabetic nephropathy were selected for the study. The most common cause for exclusion was microalbuminuria (~30%). An equal number of patients had normoalbuminuria but a diabetes duration of <15 years. Six percent had normoalbuminuria and a sufficient diabetes duration but were on an ACE inhibitor or angiotensin II receptor 1 antagonist, which they were not willing to stop for reevaluation of albuminuria. Four percent of macroalbuminuric and diabetic dialysis patients had to be excluded because they did not have diabetic retinopathy at the onset of renal disease. Other types of diabetes (e.g., gestational diabetes or diabetes due to pancreatic disease) led to the exclusion of 10% of patients. Another 10% were unwilling to participate or unable to give informed consent. Missing data were the reason for exclusion in only 6% of patients. For exact numbers on excluded patients and decision making on inclusion and exclusion see the addendum of this article available from www.klinikum.uni-heidelberg.de/index.php?id = 6,791. If we compared the number of excluded patients per center, a similar pattern of exclusion was observed for each center. Demographic data and medication of the included patients are given in Tables 1 and 2.

Sequence analysis of coding regions. All 12 exons of the *CNDP1* gene, all 12 exons of the *CNDP2* gene, and the intronic regions flanking these exons were completely sequenced in three German patients with diabetic nephropathy and in two German patients without diabetic nephropathy after 15 years of diabetes. All patients had

TABLE 1
Demographic data of the examined groups*

	Group 1 (trios with type 2 diabetes)		Group 2 (type 2 diabetes)		Group 3 (type 1 diabetes)	
	With diabetic nephropathy	Without diabetic nephropathy	With diabetic nephropathy	Without diabetic nephropathy	With diabetic nephropathy	Without diabetic nephropathy
n	37	8	77	63	21	36
Age (years)	68.2 ± 10.5	64.3 ± 9.3	61.8 ± 10.6	65.4 ± 11.6	48.0 ± 11.7	41.9 ± 11.3
Sex (male/female)	26/11	4/4	38/39	30/33	11/10	13/23
Retinopathy (%)						
Proliferative	56.8	50.0	58.4†	4.8†	95.2†	5.6†
Nonproliferative	37.8	25.0	37.7†	15.9†	4.8†	11.1†
None	5.4	25.0	3.9†	79.4†	0†	83.3†
Arterial hypertension (%)	79.2†	87.5†	70.1†	46.0†	90.5	2.8
Diabetes duration (years)	12.5 ± 7.0†	23.1 ± 8.0†	14.9 ± 8.6†	22.3 ± 6.9†	25.5 ± 10.7	23.8 ± 8.1
A1C (%)	7.6 ± 0.7	7.4 ± 1.2	7.5 ± 2.0	7.3 ± 1.7	7.0 ± 1.5	7.5 ± 1.3
Ethnicity (*n*)						
German‡	37	8	42	18	2	21
Czech	0	0	11	36	0	0
Dutch	0	0	7	4	19	15
Arabic	0	0	11	5	0	0
Other	0	0	6	0	0	0

Data are means ± SD, unless otherwise indicated. *Shown are the demographic data of the three examined groups. Patients without retinopathy in the groups with diabetic nephropathy had biopsy-proven diabetic nephropathy. †Significant differences between the groups with and without diabetic nephropathy. ‡All German patients were of German extraction.

type 2 diabetes. Several polymorphic sequence variants were detected (Table 3), but no truncating or obviously obliterating mutations were identified.

Association analysis. We performed an explorative search for polymorphisms associated with the diabetic nephropathy trait upon the first group of patients: the "trio-group" (37 patients with type 2 diabetes and diabetic nephropathy and 8 diabetic control subjects). All polymorphisms listed in Table 3 were tested. The most significant association was found with marker D18S880, a trinucleotide repeat in *CNDP1* exon 2. Since this polymorphism lies in the 5′ coding part of the transcript, the number of trinucleotide repeats is directly related to the number of leucine residues in the leader peptide of the carnosinase precursor: five, six, or seven leucines. The five-leucine allele was found on 88% of chromosomes in patients without diabetic nephropathy but had an allele frequency of only 59% in the diabetic nephropathy group. In contrast to diabetic nephropathy patients, most patients without diabetic nephropathy were homozygous for this allele ($P = 0.041$) (odds ratio 5.54 [95% CI 0.98–31.45]). The genotype frequencies are given in Table 4. Since the first individual found to be homozygous for the five-leucine allele came from the city of Mannheim, we designated this allele the "*CNDP1* Mannheim allele."

The second patient group consisted of patients with type 2 diabetes with ($n = 77$) and without ($n = 63$) diabetic nephropathy from Germany, The Netherlands, Qatar, and the Czech Republic. In this group, we tested whether homozygosity for the *CNDP1* Mannheim allele was less frequent among patients with diabetic nephropa-

TABLE 2
Medication of the patients

	Group 1 (trios with type 2 diabetes)		Group 2 (type 2 diabetes)		Group 3 (type 1 diabetes)	
	With diabetic nephropathy	Without diabetic nephropathy	With diabetic nephropathy	Without diabetic nephropathy	With diabetic nephropathy	Without diabetic nephropathy
n	37	8	77	63	21	36
Antidiabetic treatment						
Oral antidiabetics	28.6	12.5	18.3	44.1*	0	0
Insulin	57.1	50.0	60.0	18.6*	100	100
Combination	14.3	37.5	18.3	23.7	0	0
Other medication						
ACE inhibitor	43.5	25.0	57.6	23.7*	42.9	0.0*
AT1 antagonist	8.7	25.0	6.1	1.7	28.6	0.0*
Combination (ACE + AT1)	8.7	0.0	7.6	0.0*	0.0	0.0
β-Blockers	47.8	75.0	53.3	27.8*	33.3	0.0*
Statins	42.1	75.0	36.1	36.4	81.0	0.0*

Data are percent of patients on medication. Patients in the groups without diabetic nephropathy on ACE inhibitor or AT1 antagonist had undergone a 6-week washout phase before evaluation of albuminuria. *Significant differences between the groups with and without diabetic nephropathy.

TABLE 3
Selected polymorphisms in and near the *CNDP2* and *CNDP1* loci

Variant	Gene	Change	Allele most associated with diabetic nephropathy*	Odds ratio (95% CI)
D18S58	5′ of *CNDP2*	CA repeat	149 bp	3.75 (0.45–31.26)
Rs10548323 (“DN33”)	5′ of *CNDP2*	Complex repeat	208 bp	1.40 (0.46–4.23)
Rs3080862 (“DN12”)	5′ of *CNDP2*	CA repeat	259 bp	2.53 (0.74–8.62)
Rs1046407	5′ of *CNDP2*	C → T	C	2.33 (0.58–9.61)
Rs22778156	5′ of *CNDP2*	C → T	C	1.86 (0.33–10.56)
Rs1559803	5′ of *CNDP2*	T → A	T	2.94 (0.93–9.26)
Rs12965928	5′ of *CNDP2*	C → T	C	1.57 (0.38–6.64)
DN31 (unpublished repeat 17.9 kb 3′ from rs22778156)	5′ of *CNDP2*	TA repeat	341 bp	1.56 (0.52–4.72)
rs7237740 (“PM”)	5′ of *CNDP2*	C → T*	C	1.40 (0.46–4.23)
rs3764509	*CNDP2* (intron)	C → G	C	1.42 (0.43–4.76)
rs2278161 (Y126H)	*CNDP2*	A → G	A	1.79 (0.53–6.06)
rs2241509	*CNDP2* (intron)	T → A	T	1.08 (0.35–3.26)
rs890332	5′ of *CNDP1*	T → C*	T	1.88 (0.43–8.26)
rs10596720 (“DN13”)	*CNDP1* (intron)	CA repeat	179 bp	1.78 (0.36–8.85)
D18S880	*CNDP1*	CTG repeat	6–7 Leu	4.77 (1.01–22.5)†
rs4892247	*CNDP1* (intron)	T → C	C	1.80 (0.53–6.15)
D18S1161	*CNDP1* (intron)	CA repeat	96 bp	1.16 (0.37–3.64)
rs2887	*CNDP1* (3′UTR)	C → T	C	1.22 (0.22–6.76)

*Test for association on 37 patients with diabetic nephropathy and 8 patients with no sign of diabetic nephropathy and a duration of type 2 diabetes >15 years. †D18S880 is a three-allele repeat encoding five, six, or seven leucine residues. It is the only variant significantly associated with diabetic nephropathy in this study. Hence, this variant was selected for further investigations.

thy compared with patients without the complication. A significant difference was confirmed, with only 20 of 77 diabetic nephropathy cases being homozygous for the Mannheim allele compared with 25 of 63 cases without diabetic nephropathy ($P = 0.024$, single-sided Mantel-Haenszel test). The frequencies of the observed genotypes are shown in Table 4. There was no evidence of heterogeneity between the four populations.

In a third group, it was tested whether the predominance of the Mannheim allele in diabetic nephropathy–resistant patients also applies to type 1 diabetes. Homozygosity for the Mannheim allele was found in only 4 of 21 (19%) patients with diabetic nephropathy, compared with 15 of 36 (42%) case subjects without nephropathy ($P = 0.098$). Combined analysis of this group with the type 2 diabetic groups mentioned above showed an odds ratio of 2.56 (95% CI 1.36–4.84) ($P = 0.0028$). As shown in Table 4, the distribution of D18S880 genotypes is very similar among diabetic nephropathy patients with type 1 and type 2 diabetes; the Mantel-Haenszel test showed no evidence for heterogeneity.

Sequence comparisons on CNDP1 leader peptide. The leader peptide at the NH_2-terminal end of the serum-carnosinase precursor protein has only little homology to the consensus pattern, as published by von Heijne (20). The GvH score, used to measure homology to the consensus, is the original weight-matrix score introduced by von Heijne subtracted by 3.5. The seven-leucine allele showed a GvH score of 0.83, whereas the *CNDP1* Mannheim allele showed a GvH score of −1.76 (Fig. 1*A*).

Measurement of serum carnosinase activity in normal volunteers. Serum-carnosinase activities and leucine repeat D18S880 genotypes were determined in 45 healthy volunteers. The enzyme activities (12–46 nmol · ml^{-1} · h^{-1}, mean 26.6) were plotted against the genotype (Fig. 1*B*). There is a clear correlation between leucine repeat number and serum-carnosinase activity ($P = 0.018$). The lowest activity is associated with homozygosity for the five-

TABLE 4
Genotype frequencies

	Group 1 (trios with type 2 diabetes)		Group 2 (type 2 diabetes)		Group 3 (type 1 diabetes)		All groups	
Genotype	With diabetic nephropathy	Without diabetic nephropathy	With diabetic nephropathy	Without diabetic nephropathy	With diabetic nephropathy	Without diabetic nephropathy	With diabetic nephropathy	Without diabetic nephropathy
5–5	13 (35)	6 (75)	20 (26)	25 (40)	4 (19)	15 (42)	37 (27)	46 (43)
5–6	16 (43)	2 (25)	44 (57)	27 (43)	10 (48)	13 (36)	70 (52)	42 (39)
5–7	2 (5)	—	2 (3)	1 (2)	—	2 (6)	4 (3)	3 (3)
6–6	5 (14)	—	11 (14)	9 (14)	7 (33)	4 (11)	23 (17)	13 (12)
6–7	1 (3)	—	—	1 (2)	—	2 (6)	1 (1)	3 (3)
7–7	—	—	—	—	—	—	—	—
Total	37	8	77	63	21	36	135	107

Data are *n* (%).

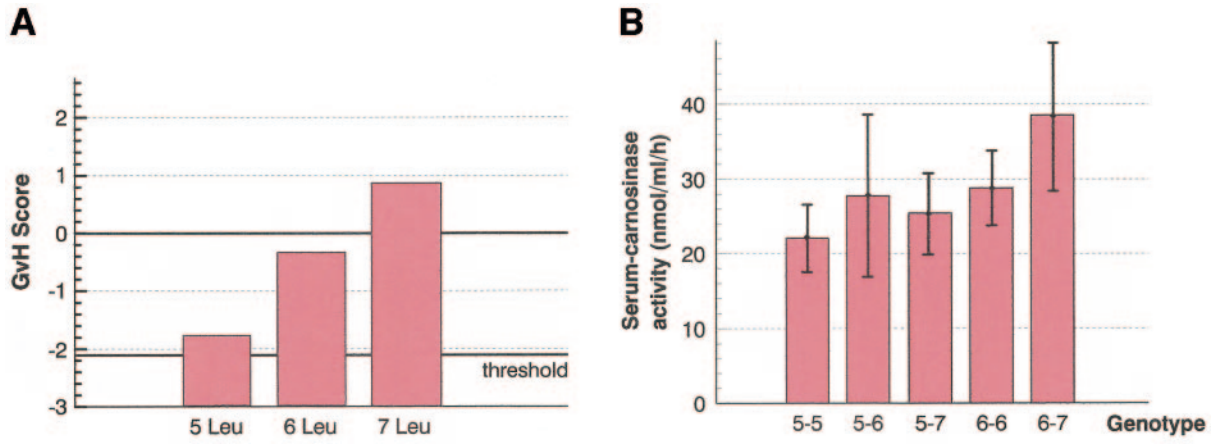

FIG. 1. ***A*****: The predicted efficacy of secretion and cleavage expressed as GvH score. This score quantifies the resemblance of 5′ sequences to a leader peptide consensus. A value >0.0 is likely to represent a cleavable leader peptide. A sequence with a value below −2.1 is assumed to be nonfunctional as a cleavable leader peptide. Since the *CNDP1* gene product has to be secreted and cleaved to become a serum-carnosinase, the higher GvH scores suggest that the 6 Leu and 7 Leu alleles are gain-of-function mutations.** ***B*****: The correlation between D18S880 genotype (leucine repeat length) and serum-carnosinase activity measured in 45 healthy control individuals. Shown are the mean enzyme activity and the SD for each observed genotype. The lowest activity was associated with homozygosity for the five-leucine allele (*P* = 0.018) (Wilcoxon rank-sum test).**

leucine allele. This suggests that six and seven leucine alleles can be regarded as gain-of-function mutations associated with a higher enzyme activity. Despite the immediate stabilization of serum carnosine after blood withdrawal, the measured carnosine levels were very low. In the control subjects, the carnosine concentrations ranged from 0.08 to 0.81 μmol/l (mean 0.33) and are close to our detection threshold.

Confirmation of the protective role of carnosine by cell culture experiments. Carnosine inhibited the effects of high glucose on cultured human podocytes (Fig. 2) and on cultured human mesangial cells (Fig. 3). The addition of carnosine blocked the glucose-induced increase in production of extracellular matrix components fibronectin and collagen type VI by podocytes and of TGF-β2 in mesangial cells. TGF-β1 production was not influenced by the addition of glucose or carnosine (data not shown).

CNDP1 expression in kidneys. Real-time measurement of *CNDP1* expression was performed on isolated glomeruli from four healthy kidneys and three diabetic nephropathy kidneys. The relative expression of *CNDP1* mRNA in

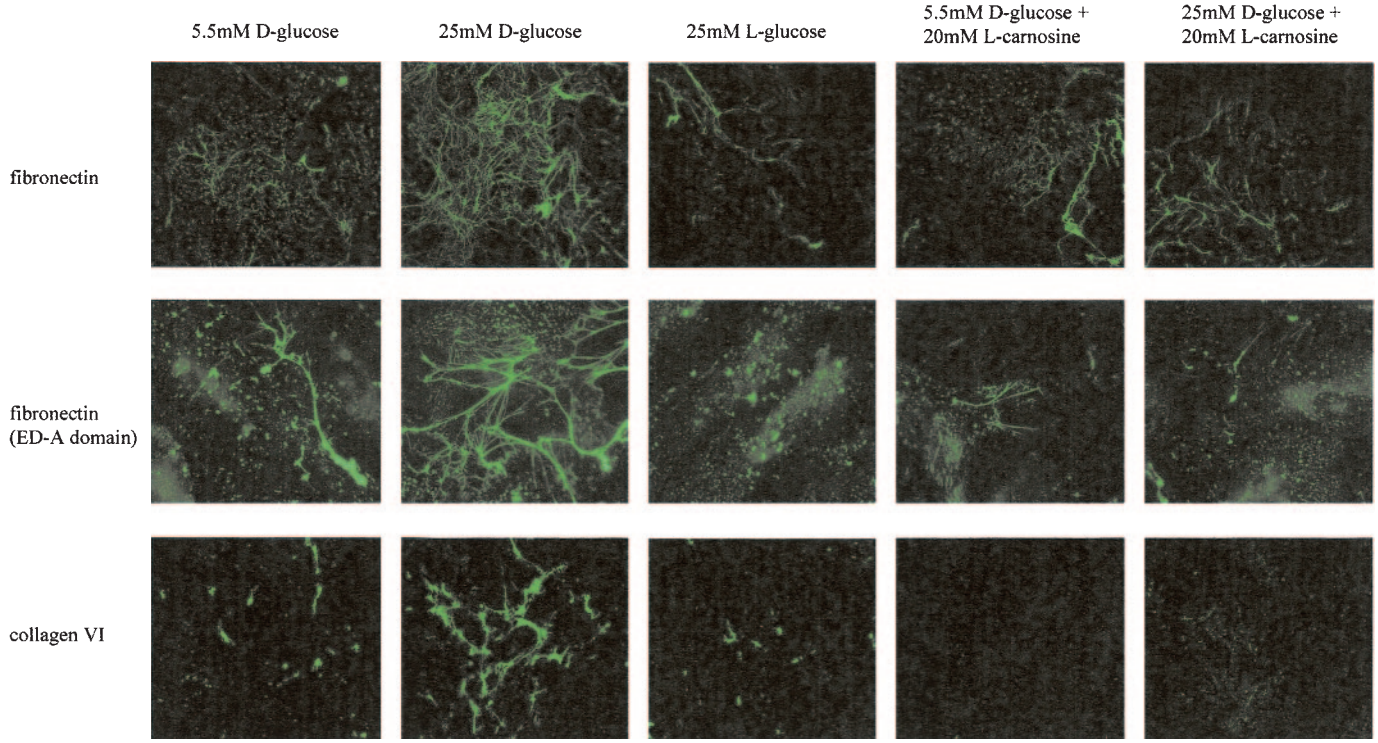

FIG. 2. Human thermosensitive SV 40–transformed podocytes were cultured at nonpermissive conditions (37°C) under various concentrations of D-glucose in the presence or absence of 20 mmol/l L-carnosine. A total of 25 mmol/l L-glucose was used as osmotic control. After 14 days, indirect immunofluorescence stainings were performed. The figure shows an increased production of fibronectin and collagen type VI in podocytes cultured with 25 mmol/l glucose compared with normal glucose (5.5 mmol/l). The panels on the right show that carnosine inhibits this increase.

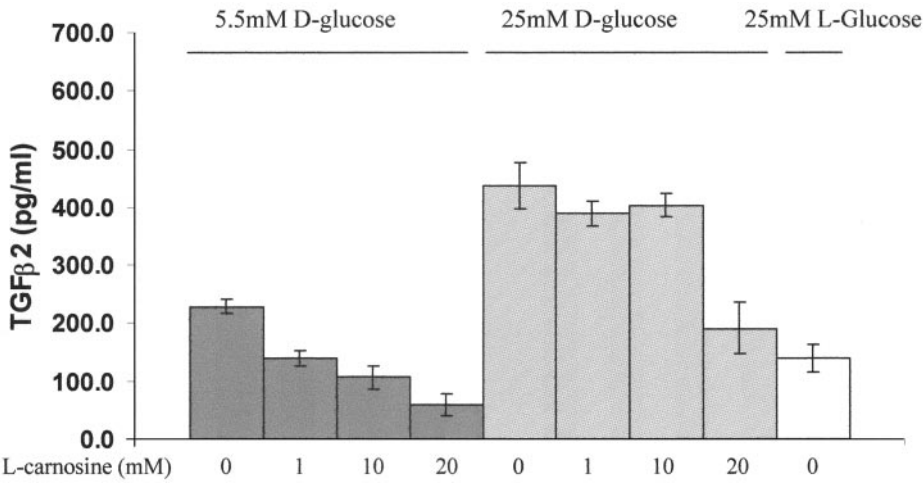

FIG. 3. Human SV 40–transformed mesangial cells were cultured under various concentrations of D-glucose in the presence or absence of 20 mmol/l L-carnosine. A total of 25 mmol/l L-glucose was used as osmotic control. After 10 days, the production of TGF-β2 in the culture supernatant was assessed by an enzyme-linked immunosorbent assay technique. Three experiments per group were performed. In 5 mmol glucose, the decrease of the TGF-β2 signal is significant with all three carnosine concentrations; in 25 mmol D-glucose, only 1 and 20 mmol/l L-carnosine lead to a significant decrease (Kruskal-wallis test modified according to Conover-Inman). All pairwise comparisons (*P* values) are available from www.klinikum.uni-heidelberg.de/index.php?id=6791.

glomeruli from patients with diabetic nephropathy was 1.0 (range 0.4–1.4) and 2.9 (2.7–3.4) in control glomeruli.

Human kidney specimens from seven patients with diabetic nephropathy and five from control individuals (three pretransplantation biopsies, two from patients after undergoing tumor-related nephrectomy) were stained for *CNDP1* using two different antibodies (C17E and Y18K). Both antibodies revealed a similar staining pattern for *CNDP1*. Representative stainings using C17E are depicted in Fig. 4. The stainings show *CNDP1* expression especially in podocytes and confirm an elevated expression in all kidneys from patients with diabetic nephropathy.

DISCUSSION

This study shows that a repeat expansion in the leader peptide of the *CNDP1* gene is associated with susceptibility for diabetic nephropathy in patients with type 1 and type 2 diabetes. Diabetic patients with two copies of *CNDP1* Mannheim, the gene variant with the lowest number of leucine repeats in the leader peptide, are less susceptible to diabetic nephropathy. Our data confirm that susceptibility for diabetic nephropathy is a dominant trait.

We stringently excluded from this study patients with an unclear cause of renal damage, such as patients with microalbuminuria, patients in which protein excretion was only determined while they were on ACE inhibitors or ATR1 blockers, and patients without renal symptoms with a duration of diabetes <15 years. The patients in this study were white and from central and western Europe or the Middle East. These findings therefore confirm and extend earlier data obtained in Turkey, in North-American Pima Indians, and in African-American patients (7,8).

As we hypothesized, individuals with a higher number of *CNDP1* leucine repeats in the leader peptide had higher serum carnosinase activity levels. Our in vitro data strengthen the plausibility of our hypothesis: the substrate of the enzyme encoded by the *CNDP1* gene, carnosine, protects renal cells against the deleterious effects of high glucose levels. The carnosine concentrations used in the cell culture experiments are in the same order of magnitude as has been reported in tissues of long-lived species (12). The read-out parameters of the in vitro studies are relevant: the ED-A domains of fibronectin and collagen type VI are known to accumulate in human diabetic nephropathy (21–23), and several in vitro and in vivo studies (24–27) have implicated mesangial TGF-β as a key mediator in diabetic renal disease. In our experiments, glucose only induced TGF-β2, not -β1, in cultured human mesangial cells. Hill et al. (28) have suggested that TGF-β2 is closely linked to fibrogenesis in diabetic nephropathy.

Carnosine, β-alanyl-L-histidine, was first described in 1900 and functions as a natural ACE inhibitor (29,30), a natural radical oxygen species (ROS) scavenger (31), an AGE breaker (32), and, thus, as a natural antiaging substance (29). The mitochondrial overproduction of ROS has been demonstrated to be fundamental in the vascular

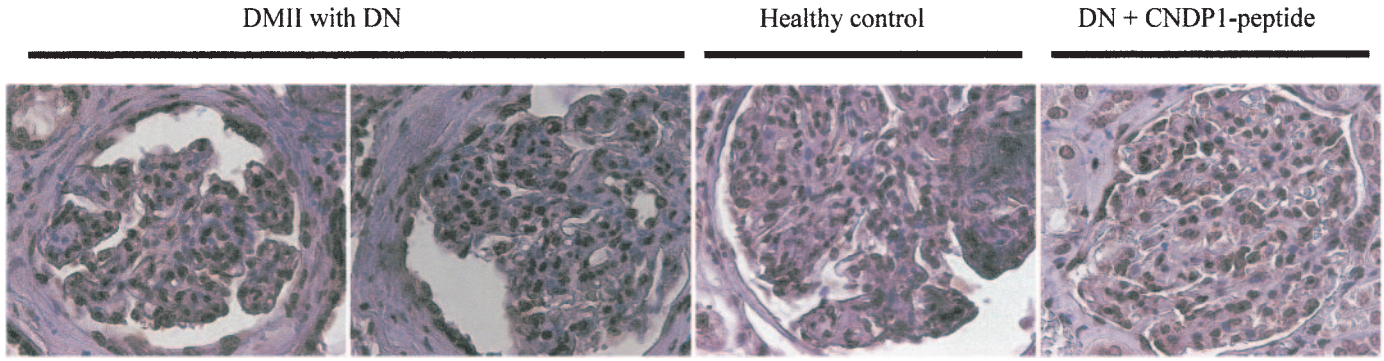

FIG. 4. Human kidney specimen from patients with diabetic nephropathy (DMII with DN) and control individuals (Healthy control) were stained for *CNDP1*. In the kidneys with diabetic nephropathy, there is increased staining in the glomerulus and in parietal epithelial cells. Representative stainings using the C17E antibody are depicted. A C17E antibody preincubated with the respective peptide was used as control (*last panel*).

pathobiology of diabetic microvascular complications (33). In light of the properties of carnosine, it is important to note that several studies have also shown that pharmacologic inhibition of the renin-angiotensin system effectively delays disease progression in patients with diabetic nephropathy (34,35), that binding of AGEs to the receptor for AGEs is thought to mediate the release of ROS in mesangial cells in kidneys with diabetic nephropathy (36), and that nondiabetic parents of children with diabetic nephropathy have been reported to die earlier than parents of diabetic children without nephropathy (37). Because AGEs induce the production of angiotensin II in mesangial cells and carnosine may function as an ACE inhibitor (30,36), our results are compatible with the notion that the renin-angiotensin system plays a crucial role in the pathogenesis of diabetic nephropathy (rev. in 38). It remains to be determined where in the cascade of AGE–receptor for AGE binding, ROS generation, activation of TGF-β–Smad signaling, fibronectin synthesis, and autocrine production of angiotensin II carnosine has its major effects. The effects of carnosine on life expectancy have been shown both in vitro (39) and in vivo (40), and the findings reported here may help to explain the shortened life expectancy in both patients with diabetic nephropathy and their relatives (37,41). Since carnosines have been reported to be generated in vivo by exercise (42), our findings provide a possible molecular basis for the previously reported beneficial effects of exercise on the incidence of diabetic nephropathy in patients with diabetes (43,44).

Although the association between *CNDP1* Mannheim and protection against diabetic nephropathy was robust in the four populations tested, patients with *CNDP1* Mannheim occasionally developed diabetic nephropathy. This indicates that additional modifying genes contribute to the genetic predisposition to develop diabetic nephropathy. A recent report by Bowden et al. (8) confirms the existence of four more loci, some of which may be restricted to African-American patients with diabetes. We assume that the occurrence of diabetic nephropathy is the net result of two counteracting mechanisms: protein glycation (mainly determined by glucose levels) and protective factors such as tissue carnosine content. It has been shown that in patients with type 1 and type 2 diabetes, there is a good correlation between diabetes regulation (as documented by HbA_{1c} [A1C] levels) and the chances of developing diabetic nephropathy (45,46). It is therefore conceivable that patients with *CNDP1* Mannheim may still develop diabetic nephropathy when blood glucose levels are very poorly controlled. Further clinical studies will be required to address this issue in more detail.

CNDP1 Mannheim was associated with lower serum carnosinase activity. However, we were unable to detect any meaningful serum carnosine levels; this is most likely explained by the high activity of serum carnosinase. It is unknown how polymorphisms in the *CNDP1* gene relate to carnosine levels in kidney tissues. Our study did not confirm the absence of *CNDP1* expression in kidneys, as suggested by Teufel et al. (13) but clearly showed *CNDP1* mRNA and protein expression in the glomerulus. The increased presence of the gene product in glomeruli with diabetic nephropathy is compatible with a crucial role of this molecule in the pathogenesis of diabetic nephropathy.

We conclude that the number of leucine repeats in the leader peptide of the *CNDP1* gene is associated with susceptibility for diabetic nephropathy. The findings implicate carnosine as an important protective factor in diabetes. The value of the leucine repeat as a tool to determine the risk of a patient with type 1 or type 2 diabetes for developing diabetic nephropathy has to be investigated in a prospective setting. Our study suggests that carnosine or carnosine derivates may possibly be used to design new therapeutic strategies to optimize renoprotection in diabetes.

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Contributors: Recruitment and screening of records of patients was performed by H.K., M.R., M.Z., and D.H., with assistance from M.C., I.R., S.J.L.B., and P.N. Participating centers and contact persons were Fifth Medical University Clinic, University Clinic at Mannheim, Germany; Diabetes Polyclinics, Dialysis Unit, and Kidney Transplantation Unit, University Clinic at Groningen, The Netherlands, (R.O.B. Gans); Diabetologist Practice, Speyer, Germany (T. Segiet); Diabetes Polyclinic, University Clinic at Heidelberg, Germany (A. Hamann); Medical Center, Leeuwarden, The Netherlands (J. Broekroelofs); Second Medical Department, Third Medical Faculty, Charles University Prague, Czech Republic (J. Psottova); University Clinic at Tübingen, Germany (T. Risler and B. Friedrich); Communal Clinic, Ludwigshafen, Germany, kidney biopsy records (R. Bergner); and Hamad Medical Corporation, Doha, Qatar. B.J., D.H., and F.V.D.W. were the lead investigators in this project and were principally responsible for the data analysis, interpretation, and preparation of this report. Together with the lead investigators, C.R.B and H.-P.H. were involved in study design and logistical matters. E.D.H and H.B. determined gene expression levels in kidneys. N.R. and J.M. performed the genetic analyses. V.P., N.R., and J.Z. were responsible for determination of carnosine levels and enzyme activities. The in vitro studies were performed by P.B., S.S., and B.A.Y., E.R., P.M., and M.A.S. provided the cell lines. C.F. provided statistical expertise.

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