L-Carnosine, a Substrate of Carnosinase-1, Influences Glucose Metabolism

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OBJECTIVE—Carnosinase 1 (CN1) is a secreted dipeptidase that hydrolyzes L-carnosine. Recently, we have identified an allelic variant of human CN1 (hCN1) that results in increased enzyme activity and is associated with susceptibility for diabetic nephropathy in human diabetic patients. We therefore hypothesized that L-carnosine in the serum represents a critical protective factor in diabetic patients.

RESEARCH DESIGN AND METHODS—L-carnosine serum levels were manipulated in *db/db* mice, a model of type 2 diabetes. In a transgenic approach, hCN1 cDNA was expressed under the control of a liver-specific promoter in *db/db* mice, mimicking the expression pattern of hCN1 in humans.

RESULTS—Fasting plasma glucose as well as A1C levels rose significantly earlier and remained higher in transgenic animals throughout life. Body weights were reduced as a result of significant glucosuria. In an opposite approach, nontransgenic *db/db* mice were supplemented with L-carnosine. In these latter mice, diabetes manifested significantly later and milder. In agreement with the above data, serum fasting insulin levels were low in the transgenic mice and elevated by L-carnosine feeding. Insulin resistance and insulin secretion were not significantly affected by L-carnosine serum levels. Instead, a significant correlation of L-carnosine levels with β-cell mass was observed.

CONCLUSIONS—hCN1-dependent susceptibility to diabetic nephropathy may at least in part be mediated by altered glucose metabolism in type 2 diabetic patients. ***Diabetes*** **56:2425–2432, 2007**

Patients with type 2 diabetes often suffer serious complications of chronic hyperglycemia, including nephropathy, neuropathy, retinopathy, and accelerated development of cardiovascular disease. The prevalence of type 2 diabetes worldwide is 6% and projected to rise over the next decade, owing to the increasing age of the population and the surge of obesity (1). There is evidence of a genetic component in the risk for type 2 diabetes, including prevalence differences between racial groups and higher concordance rates among monozygotic than dizygotic twins (2). There is also evidence for a genetic component to the risk of diabetic nephropathy (3,4).

Our group is interested in identifying variants of genes that modify the risk for diabetes complications, such as diabetic nephropathy. Recently, an association of diabetic nephropathy and an allelic variant within the leader peptide of carnosine dipeptidase 1 (carnosinase 1 [CN1]), which affected the efficiency of enzyme secretion, was identified and subsequently confirmed (5,6). CN1 is a dipeptidase that hydrolyzes L-carnosine (β-alanyl-L-histidine) and to a lesser extent homocarnosine (7). The variant with the smallest number of leucine repeats was more common in the absence of diabetic nephropathy and was associated with lower CN1 serum levels. Leader peptide variants containing more than five leucine repeats were associated with an increased risk for diabetic nephropathy. From these genetic data in human patients, it was hypothesized that L-carnosine serum levels are associated with the risk for late complications of diabetic disease and that L-carnosine acts as a protective factor (6).

Thus far, the function of the atypical dipeptide L-carnosine remains unknown. L-carnosine is present intracellularly in most tissues. The highest levels of L-carnosine are found in muscle and also in the central nervous system, where it is released into the serum and cerebrospinal fluid, respectively. L-carnosine is also ingested into the serum from food. In humans, L-carnosine is degraded predominantly by CN1, which is secreted from the liver into the serum (7). In other mammals, including rodents, CN1 is expressed exclusively within the kidney and lacks a signal peptide. In these animals, L-carnosine is eliminated from the serum by filtration into the urine and reabsorption into tubular cells, which express CN1 within the cytosol (7). CN1 expression levels vary among different mouse strains, and C57BL/6J mice, used in this study, express particularly low levels of endogenous CN1 in their kidneys (8).

This study was undertaken to verify the genetic association of CN1 with diabetic disease and to test whether CN1 determines the risk for diabetic nephropathy through L-carnosine. L-carnosine levels were manipulated in the serum of a type 2 diabetes mouse model. This was achieved by two experimental approaches. First, endogenous L-carnosine serum levels were artificially lowered by overexpression of secreted human CN1 (hCN1) under the control of a liver-specific promoter mimicking the endogenous expression pattern of CN1 in humans. In a second

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CN1, carnosinase 1; FPG, fasting plasma glucose; hCN1; human CN1; TTP, transthyretin promoter.

experimental approach, diabetic mice were given L-carnosine in their drinking water.

RESEARCH DESIGN AND METHODS

Generation of experimental mice. The cDNA of hCN1 including the endogenous signal peptide of six leucines was amplified from IMAGE clone acc. no. BX094414 using the primers CN1 forward 5′P-CACCATGGATCCCAAACTCGGGA3′ and CN1 reverse 5′P-TCAATGGAGCTGGGCCATCT3′. The PCR product was ligated into the *Stu*I site of plasmid pTTR1ExV3, containing the transthyretin promoter (TTP) (9). The resulting plasmid, pTTP-hCN1, was sequenced. The transgene was liberated using *Hind*III and injected into the pronuclei of BKS.Cg-m$^{+/+}$ Lepr db/J mice (line 000642; The Jackson Laboratory, Bar Harbor, ME) at the Interfaculty Biomedical Research Institute, University of Heidelberg. All animal procedures were approved by the Regierungspräsidium Karlsruhe (AZ 35-9185.82/A-2/06, AZ53-9185.81/G-32/03).

Genomic DNA was extracted from tail biopsies using the mouse direct PCR Lysis Reagent Kit (Viagen Biotech, Los Angeles, CA) supplemented with proteinase K (Sigma-Aldrich) according to the manufacturer's instructions. The transgene TTP-hCN1 was detected by PCR using the primers CN1Tail forward CCTCGCTTCAGACAAGAGCTCTTCAGAATGA and CN1Tail reverse GCTCTGAAGGCGCTCACAGCATTGATCCAA (at 94°C for 3 min, annealing at 60°C, 2-min extension, 31 cycles) yielding a product of 343 bp (10). The point mutation within the leptin receptor was genotyped by PCR as described by http://jaxmice.jax.org. hCN1 transgenic db/wt mice were bred to nontransgenic db/wt littermates. Diabetic transgenic *db/db* mice were used as experimental animals (*db/db* CN1+). Diabetic nontransgenic (*db/db* water) as well as nondiabetic nontransgenic littermates (db/wt) served as controls. Transgenic mice without the Lepr mutation (wt/wt) were discarded.

A second experimental group of nontransgenic *db/db* mice (*db/db* L-carnosine; The Jackson Laboratory) was given drinking water supplemented with 4 mmol/l L-carnosine or water only (*db/db* water). L-carnosine–supplemented drinking water was replaced three times a week. Direct measurements of L-carnosine within the drinking bottles verified that L-carnosine was stable over a period of at least 5 days at room temperature (data not shown). Daily drinking volumes were determined. Control animals were identical in every aspect (i.e., glucose metabolism and diabetic nephropathy) to control animals derived from our own colony (not shown). Unless stated otherwise, each experimental group contained 8 mice, except for the group of *db/db* water mice, which contained 16 mice. Experimental mice were housed at 22°C on a 12-h light/dark cycle and fed standard rodent diet (R/M-H diet for mice and rats; Sniff, Soest, Germany). Each group of experimental animals contained the same ratio of females to males.

RT-PCR. RT-PCR was performed according to a previously described protocol (11). Photometric RNA concentrations and RNA volumes used for reverse transcription of exactly 5 μg RNA per tissue were as follows: *db/db* CN1+liver, 1.28 μg/μl per 3.9 μl; *db/db* water liver, 1.56 μg/μl per 3.2 μl; and *db/db* CN1+kidney, 1.52 μg/μl per 3.3 μl. cDNA generated from exactly 250 ng tissue RNA or 250 ng hCN1 plasmid were used per PCR. For detection of transgenic hCN1, endogenous mCN1, and β-actin, respectively, 30, 40, and 25 cycles of replication were used. Primers used for specific detection of the hCN1 transgene were forward 5′-cac ctc tgg agg tac cca tc-3′ and reverse gca tcc agg act ttg acc at-3′. Primers used for specific detection of endogenous mouse CN1, forward 5′-tca gga ccc gag tca tct tc-3′ and reverse 5′-ctt gct gat gtc agg gtt ga-3′, were directed against the 3′ untranslated region of the mRNA molecule, which was not present in the transgene.

Molecular biology techniques were performed as described according to standard protocols (12). Immunoblotting or immunohistological staining for CN1 was performed using C17E antiserum (kind gift from Dr. Teufel, Strasbourg, France) (7) and anti-CN1 polyclonal antiserum (R&D Systems, Wiesbaden-Nordenstadt, Germany) with identical results.

Analytical procedures. Total body weights and fasting plasma glucose (FPG) levels were measured each week at the beginning of the light cycle after an overnight fast until week 22 using Ascensia ELITE XL (Bayer, Leverkusen, Germany). A1C levels were determined using MicromatII (Bio-Rad, Hercules, CA). Insulin levels in mouse serum or INS-1e supernatants were determined using the Mouse Insulin ELISA kit (EZRMI-13K; Linco Research, St. Charles, MO). Glucose and insulin tolerance tests were performed on animals that had been fasted overnight for 16 h as described (13). In brief, animals were injected with either 2 g/kg body wt glucose or 4 IU/kg body wt human regular insulin (Novo Nordisk, Copenhagen, Denmark) into the peritoneal cavity. Glucose levels were determined in blood collected from the tail tip immediately before and 30, 60, and 120 min after the injection. Animals were placed in metabolic cages and received only water supplemented with 4% sucrose. Twenty-four–hour excretion of albumin, urea, and creatinine was measured at 20 weeks of age using Hitachi Analyzer 912 (Roche, Mannheim, Germany).

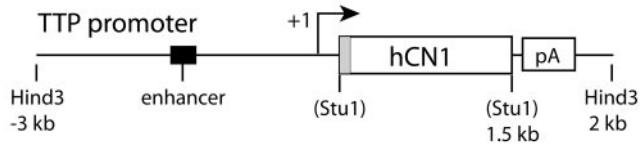

FIG. 1. Map of transgene. The human cDNA coding for CN1 including the endogenous signal peptide was expressed under the control of a 3-kb fragment of the TTP promoter, which drives expression specifically within the liver.

Measurement of carnosine concentration in mouse EDTA plasma was performed according to the method of Schonherr (14) with modifications. Moreover, 80 μl EDTA plasma was mixed with 20 μl 10% 5-sulfosalicylic acid, 10 μl supernatant was mixed with 20 μl 0.4 mol/l borate buffer (pH 9.5), and 10 μl supernatant was mixed with 10 μmol/l standard carnosine solution. Blank values, carnosine concentration standards, and carnosine composition standards (anserine, histidine) were prepared for each measurement in parallel with the samples.

For derivatization, 30 μl of each sample was mixed with 30 μl of freshly prepared CFC solution (2.5 mmol/l in acetone). After 90 s, 18 μl 1 mmol/l EDTA in 0.1 mol/l NaOH and 0.5 mol/l hydroxylamine hydrochloride in distilled water and 2-methylthioethanol was added. After an additional 3 min, 42 μl quench solution, 20% (vol/vol) acetic acid in acetonitrile, was added. Then 40 μl of diluted sample was injected for separation into the high-performance liquid chromatography system LaChrom Elite HPLC system (Jupiter column, C18, 300 Å, 5 μm particle size; Phenomenex, Aschaffenburg, Germany). The elution was performed with fluorescence detection at 287 nm extinction and 340 nm emission. The profile of the binary gradient was as follows: 0–45 min, 82% A and 18% B; 46–50 min, 18–100% B; 50–55 min, 100% B; 56–60 min, 100–18% B; and 60–80 min, 82% A and 18% B. The retention times strongly depended on the mobile phase composition.

Histological analysis. At the end of the observation period, animals were killed at 24 weeks of age and perfused with 3% paraformaldehyde as described (15). Semi-thin kidney sections, 1 μm thick, were evaluated by two renal pathologists in a blinded fashion for mesangial expansion. At least 50 glomeruli were evaluated from each experimental animal; mesangial expansion was classified into three groups: 0, no mesangial expansion; 1, mild; and 2, significant mesangial expansion.

Cell culture. The insulin-producing INS-1e cells were cultured in RPMI-1640 supplemented with HEPES, 10% heat-inactivated fetal bovine serum, 100 units/ml penicillin/streptomycin, 1 mmol/l sodium pyruvate, and 100 μmol/l 2-mercaptoethanol (16). The cells were exposed to various stimuli for 2 h in Krebs-Ringer bicarbonate HEPES buffer ($n = 3$); 5 mmol/l L-glutamine served as negative control, and L-glutamine and 20 mmol/l L-leucine or 30 mmol/l KCl served as positive control (Sigma-Aldrich, Munich, Germany). Insulin levels were analyzed as a ratio, where basal insulin secretion in the presence of only L-glutamine was normalized to 1. To assess the proliferative index, INS-1e cells were pulsed with BrdU for 2 h, fixed, and stained using an anti-BrdU antiserum (MAB3424; Chemicon International, Hampshire, U.K.).

RESULTS

Generation of hCN1 transgenic *db/db* mice. To lower L-carnosine level within the serum of diabetic mice, the cDNA of hCN1 was placed under the control of the human transthyretin promoter enhancer (TTP promoter) (Fig. 1). The TTP promoter was chosen because it drives expression in hepatocytes as well as the choroid plexus, recapitulating the expression pattern of endogenous hCN1 in humans (9). Low-level expression of transgenic hCN1 was also seen in α-cells of the pancreatic islets (Fig. 2*D–F*). Rodents do not endogenously express a secreted form of CN1 (7,8). The cDNA of hCN1 contained the endogenous signal peptide so that transgenic hCN1 was secreted into the serum and liquor in vivo as in humans.

The transgene was introduced by pronuclear injection into fertilized eggs derived from heterozygous *db/db* mice. This mouse line carries a point mutation within the leptin receptor that leads to its functional inactivation resulting in hyperphagia after weaning, obesity, hyperinsulinemia, and elevated plasma glucose levels (type 2 diabetes

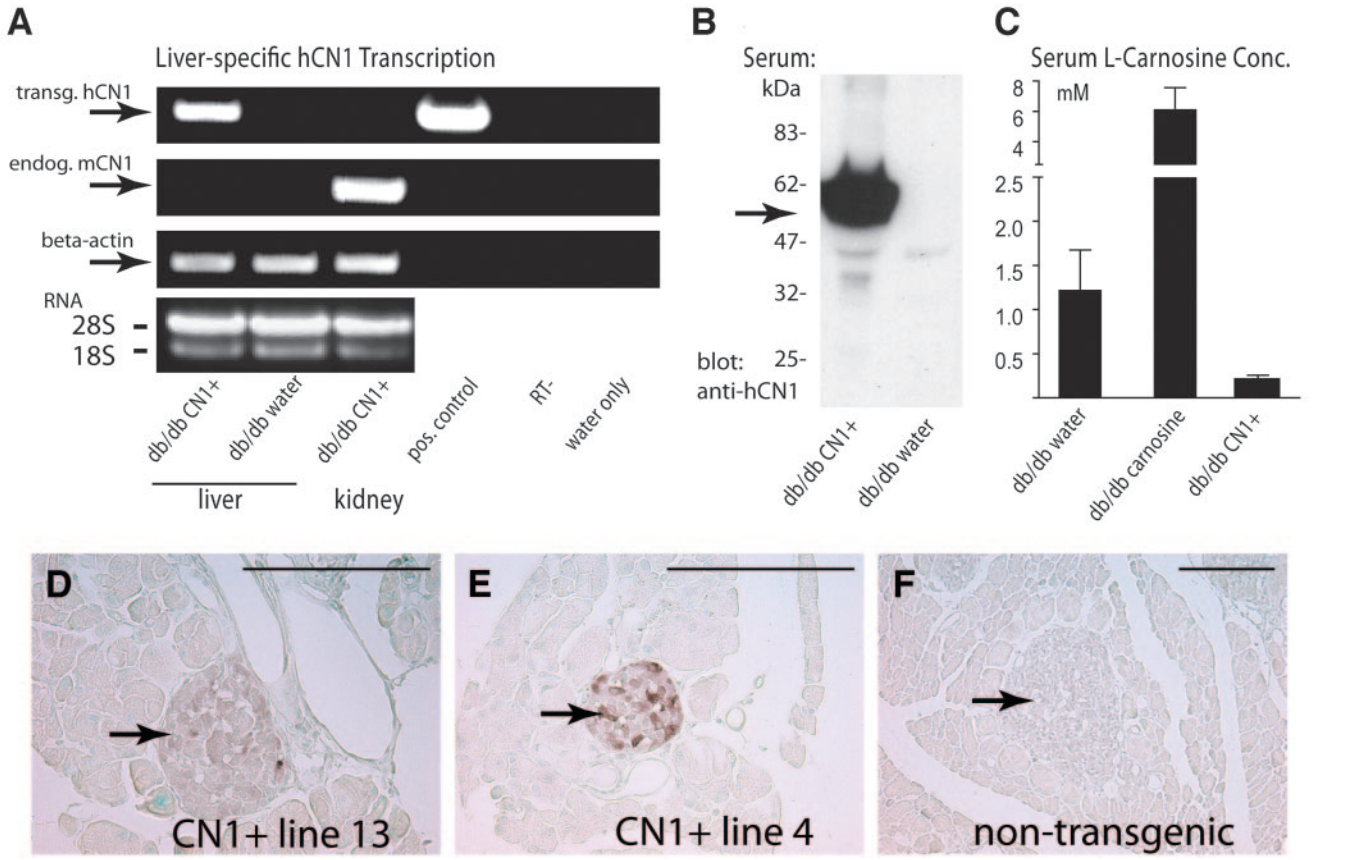

FIG. 2. Manipulation of L-carnosine serum levels in *db/db* mice. *A*: Messenger RNA expression of hCN1 and endogenous CN1 in liver and kidney of wild-type (*db/db* water) and transgenic *db/db* (*db/db* CN1+) mice was evaluated by RT-PCR. Expression of endogenous CN1, which is not secreted into the serum, was detected within the kidney, while transgenic hCN1 was expressed in liver. Total RNA and RT-PCR for β-actin verifies equal loading; hCN1 plasmid was used as positive control. Controls omitting reverse transcription (RT-) or using water only were negative. *B*: Serum (10 μl) of transgenic and wild-type *db/db* mice was submitted to SDS-PAGE and anti-hCN1 immunoblot analysis. Equal protein load was verified by Ponceau's staining. Transgenic hCN1 was detected at ~50 kDa and only in the serum of transgenic *db/db* CN1+ mice (arrow). *C*: L-carnosine concentrations were measured in the serum of hCN1-transgenic and wild-type *db/db* mice and in wild-type *db/db* mice that received L-carnosine in their drinking water (*db/db* carnosine) ($n = 5$ for each group). L-carnosine levels were lowered from baseline levels of 1.2 μmol/l to below minimal detection levels in transgenic *db/db* mice and elevated to 6 μmol/l in L-carnosine–supplemented mice. *D–F*: Immunohistological staining for CN1 on pancreatic sections revealed low-level ectopic transgene expression within the pancreatic islets of founder line 13 that was selected for this study (*D*). A single founder line (4) showed significant ectopic transgene expression within pancreatic islets (*E*). No staining was observed in nontransgenic littermates (*F*).

model) from week 8. The BKS.Cg-m$^{+/+}$ Lepr db/J mouse has been shown to be susceptible to diabetic nephropathy (17). Mice were genotyped for the transgene hCN1 as well as the leptin receptor mutation (Lepr) by PCR from genomic DNA preparations of tail biopsies. Of 70 mice, 15 mice were transgenic for hCN1 (21%). All of the results described below were reproduced in at least four different founder lines. A stable line with robust CN1 expression was established from heterozygous db/wt hCN1 transgenic founder mice by crossbreeding with nontransgenic db/wt littermates (line 13).

Analysis of hCN1 transgenic *db/db* mice. Messenger RNA was isolated from liver and kidney of hCN1 transgenic *db/db* mice and from nontransgenic littermates. Nonsaturated RT-PCR analysis using primers specific for transgenic hCN1 demonstrated expression of transgenic mRNA within liver but not kidney (Fig. 2*A*, transgenic hCN1). TTP-hCN1 plasmid was used as positive control. Expression of endogenous cytosolic CN1 was detected in kidney, as described previously (Fig. 2*A*, endogenous mCN1). Gel electrophoresis of total RNA as well as nonsaturating RT-PCR specific for endogenous β-actin was used to control for equal loading. Negative controls omitting reverse transcription (Fig. 2, RT−) or omitting mRNA (Fig. 2, water only) were always negative.

To verify that the transgenic hCN1 is efficiently secreted into the serum, 10 μl of serum derived from hCN1 transgenic (*db/db* CN1+) or nontransgenic (*db/db* water) controls were subjected to SDS-PAGE and subsequently immunoblotted with various anti-sera directed against CN1. No CN1 protein was detected in nontransgenic controls, as described previously (7). Significant amounts of CN1 protein were detected in hCN1 transgenic animals (arrow).

To verify the functional effects of hCN1 overexpression, L-carnosine levels were measured in EDTA plasma obtained at the beginning of the light cycle of transgenic and control *db/db* mice (Fig. 2*C*) at 12 weeks of age. In control mice, endogenous L-carnosine levels averaged ~1.2 ± 0.5 μmol/l. In hCN1 transgenic mice, L-carnosine levels were significantly lower ($P < 0.01$), most values below the detection level of 0.02 μmol/l (Fig. 2*C*, *db/db* CN1+).

Analysis of db/db mice supplemented with L-carnosine. A second experimental group of nontransgenic *db/db* mice was supplemented with L-carnosine in the drinking water. L-carnosine plasma levels were signifi-

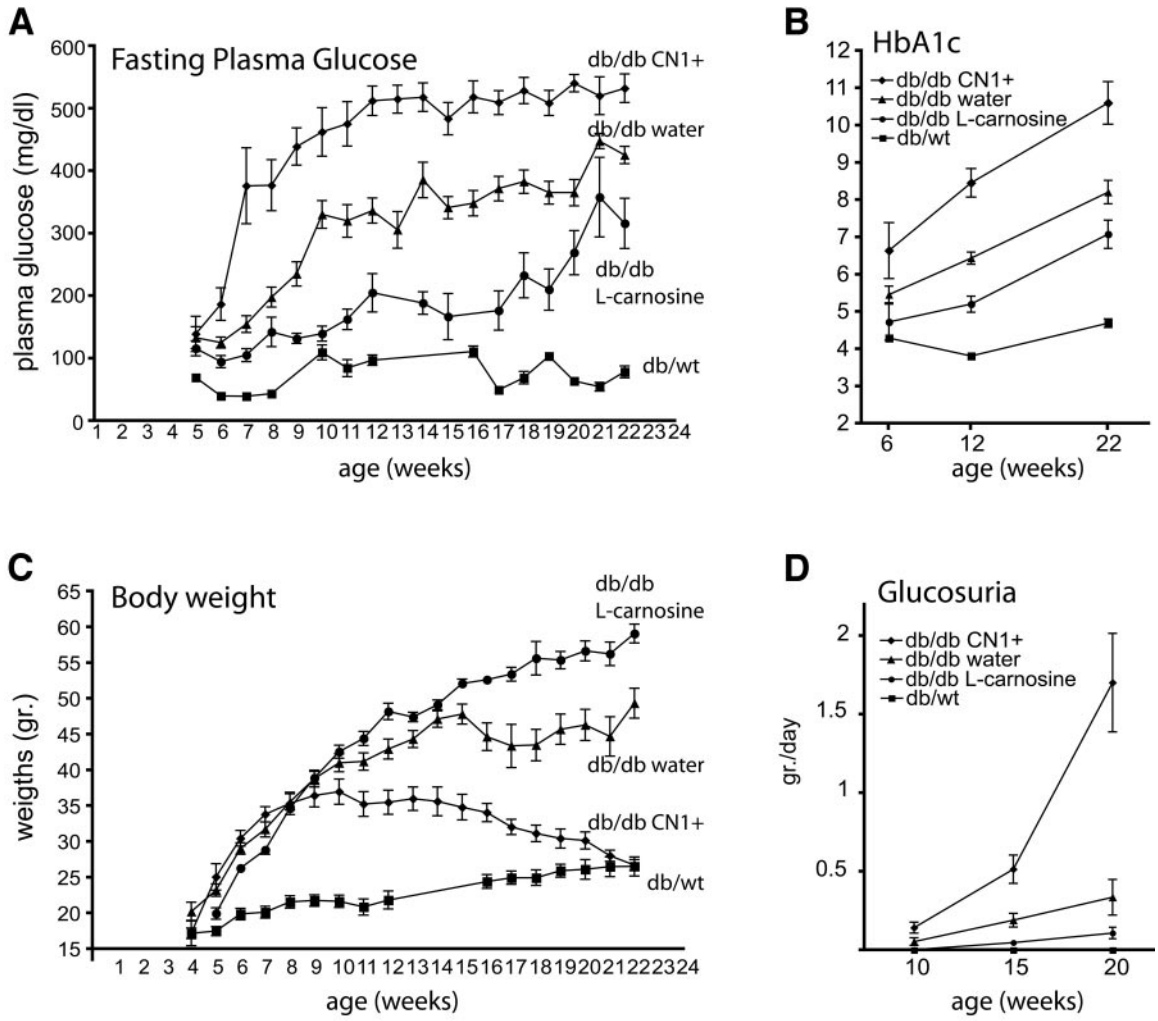

FIG. 3. Inverse correlation of L-carnosine levels with FPG levels. *A*: FPG levels were measured in nondiabetic controls (db/wt) or three experimental groups of diabetic *db/db* mice: hCN1 transgenic mice (*db/db* CN1+), L-carnosine–supplemented mice (*db/db* L-carnosine), and diabetic control mice that received water only (*db/db* water). FPG glucose levels rose significantly earlier and higher in CN1 transgenic mice until the age of 22 weeks, while L-carnosine–supplemented mice had significantly lower FPG levels. *B*: A1C levels (shown as percentages) were similarly elevated in diabetic CN1 transgenic mice and improved in L-carnosine–supplemented mice compared with diabetic controls (*db/db* water) over time. *C*: Body weights (in grams) were measured during 4–22 weeks of age. CN1 transgenic mice (*db/db* CN+) failed to gain weight from week 10, while L-carnosine supplemented *db/db* mice gained significantly more weight throughout the entire observation period until 22 weeks of age compared with diabetic controls (*db/db* water). *D*: Glucosuria was measured over 24 h at 10, 15, and 20 weeks of age. CN1 transgenic mice lost significantly more glucose (1.5–2 g glucose/day) due to poor metabolic control compared with the other experimental groups (n = 16 for *db/db* water mice; n = 8 for each remaining group).

cantly elevated to 6 ± 1.1 μmol/l in this group (n = 6 for each group) (Fig. 2*C*, *db/db* carnosine).

FPG levels correlate inversely with L-carnosine levels. Normal *db/db* mice (*db/db* water) developed persistent hyperglycemia between weeks 8 and 10 (Fig. 3*A*, *db/db* water), as described (18). hCN1 transgenic *db/db* mice (*db/db* CN1+) developed hyperglycemia earlier (weeks 6–7) and reached significantly higher FPG levels than the *db/db* water group. Conversely, FPG levels were significantly lower in L-carnosine–supplemented mice (Fig. 3*A*, *db/db* L-carnosine), and the onset of diabetes was delayed until weeks 17–20. Similarly, A1C levels were elevated in hCN1 transgenic mice (*db/db* CN1+) and significantly improved in L-carnosine–supplemented mice (*db/db* carnosine) compared with wild-type diabetic *db/db* mice (Fig. 3*B*, *db/db* water).

Obesity is important for the pathogenesis of altered glucose metabolism in the *db/db* mouse model. As expected, total body weights increased in parallel in all experimental diabetic groups until week 8. From week 15 onwards, hCN1 transgenic mice started to lose weight until they reached the weight of nondiabetic db/wt control mice by week 22 (Fig. 3*C*, *db/db* CN1+). This progressive wasting ultimately led to the death of the mice between 24 and 28 weeks of age, as verified by autopsy; four mice died of dehydration and one of an intra-abdominal abscess. The observation period of this study was therefore limited to 23 weeks (Supplementary Fig. 1 [available in an online appendix at http://dx.doi.org/10.2337/db07-0177]). No ketones were detected within spot-urine samples, and no significant differences in food or water intake were observed until week 20 (not shown). Significant glucosuria of ~1.7 g/day was observed in the *db/db* CN1+ group (Fig. 3*D*).

L-carnosine–supplemented mice continued to gain weight beyond week 15 until the end of the observation period (Fig. 3*C*, *db/db* carnosine, week 22), while diabetic control mice (*db/db* water) reached intermediate body weights by week 15. This data ruled out that glucose

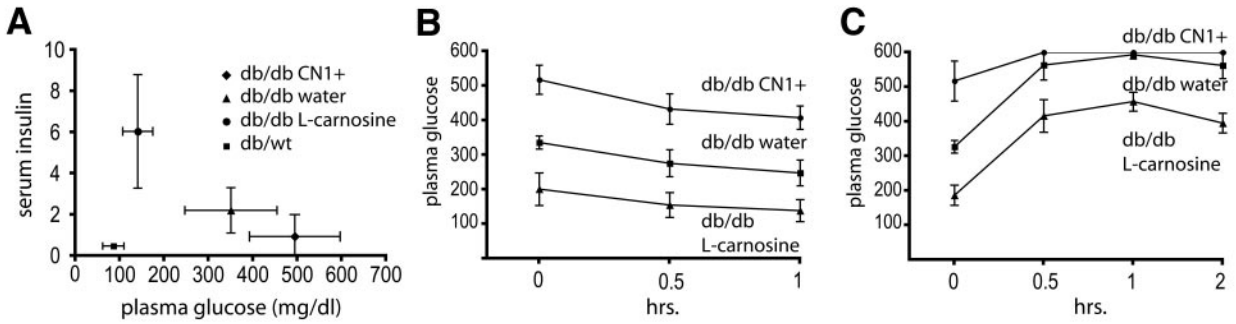

FIG. 4. Altered glucose metabolism due to differential serum insulin levels. *A*: Serum insulin and glucose levels were measured after 10 h of fasting in transgenic diabetic (*db/db* CN1+), diabetic (*db/db* water), L-carnosine–supplemented diabetic (*db/db* L-carnosine), and nondiabetic control (db/wt) mice (n = 8 for each group). Insulin levels were reduced in CN1 transgenic mice despite higher glucose levels and vice versa in L-carnosine–supplemented mice. *B* and *C*: Glucose tolerance test and insulin-sensitivity test in diabetic transgenic (*db/db* CN1+), L-carnosine–supplemented (*db/db* L-carnosine), and control *db/db* (*db/db* water) mice (n = 6 for each group aged 12 weeks). *B*: After an overnight fast, animals received 4 IU/g insulin i.p. FPG levels were reduced similarly in all experimental groups. *C*: After an overnight fast, animals received 1.5 mg/g L-glucose i.p. A similar increase in serum glucose levels was observed in control (*db/db* water) and L-carnosine–supplemented (*db/db* L-carnosine) mice. Glucose levels >600 mg/dl were counted as 600 mg/dl (*db/db* CN1+).

metabolism was aggravated in hCN1 transgenic *db/db* mice as a consequence of increased body mass.

Correlation of insulin secretion with L-carnosine serum levels. Compared with diabetic controls (*db/db* water), insulin levels were decreased in hCN1 transgenic mice despite higher FPG levels (Fig. 4*A*). The opposite pattern was observed in L-carnosine–supplemented mice (*db/db* L-carnosine), where serum insulin levels were significantly increased with lower FPG levels compared with those of diabetic controls (*db/db* water). These results suggested that glucose metabolism was primarily affected by altered insulin secretion in hCN1 transgenic and L-carnosine–supplemented mice.

To validate these findings, insulin sensitivity tests were performed (Fig. 4*B*, age 12 weeks). FPG levels fell proportionally in all experimental groups (20% reduction after 30 min, 30% total reduction after 1 h), indicating that peripheral insulin sensitivity is similar within the experimental groups. Glucose tolerance tests were also performed (Fig. 4*C*). A similar rise in FPG levels was observed in L-carnosine–supplemented mice (*db/db* L-carnosine) and diabetic control mice (*db/db* water). FPG levels in hCN1 transgenic mice (*db/db* CN1+) exceeded the range of the glucose test (600 mg/dl). In summary, a direct correlation of L-carnosine serum levels with insulin levels was detected in our experimental mice, whereas peripheral insulin sensitivity was unaltered.

Islet hyperplasia in L-carnosine–supplemented mice. To investigate the mechanism by which L-carnosine serum levels affect insulin secretion, pancreatic glands were removed from the experimental animals at 24 weeks of age, paraffin embedded, and subjected to histological analysis (Fig. 5*A*–*E*). Islet size was determined by morphometry (Fig. 5*E*). Overall, pancreatic islets were enlarged in diabetic mice compared with those in controls (db/wt). A striking difference in pancreatic islet size was observed in L-carnosine–supplemented animals (Fig. 5*D* and *E*). Hyperplastic islets of L-carnosine–supplemented mice were predominantly composed of insulin-producing β-cells, as demonstrated by immunohistological anti-insulin staining (Fig. 5*F*).

Next, it was tested whether L-carnosine exerts a direct effect on insulin secretion or proliferation of β-cells in vitro. For this purpose, INS-1e cells were exposed to varying concentrations of L-carnosine. INS-1e cells are derived from β-cells and secrete insulin in a highly regulated glucose-dependent manner (16). In the absence of L-carnosine, insulin secretion increased in the presence of increasing concentrations of L-glucose, as expected (Fig. 5*G*, open columns). Exposure to various concentrations of L-carnosine did not significantly affect glucose-dependent insulin secretion in INS-1e cells (gray, striped, and filled columns). Statistically significant increments of insulin secretion were detected for higher concentrations of L-carnosine in the absence of L-glucose (Fig. 5*G*, 0G, 1 mM and 20 mM L-carnosine). However, no significant influence of lower near-physiological levels of L-carnosine on insulin secretion was detected in the absence of L-glucose.

In a second set of experiments, INS-1e cells were cultured at low densities in the presence of varying concentrations of L-carnosine for 3 days. The percentage of BrdU-positive cells in three independent experiments is shown in Fig. 5*H*. A dose-dependent increase in BrdU-positive cells was observed with increasing L-carnosine concentrations.

These data suggested that insulin secretion was preserved in L-carnosine–supplemented mice because of an increased or preserved mass of β-cells within the pancreas. No significant stimulatory effect of L-carnosine on insulin secretion was observed in vitro, at least near physiological levels.

Diabetic nephropathy. Because CN1 has been identified as a risk factor for diabetic nephropathy in diabetic humans, it was investigated whether L-carnosine serum levels affect the kidney in our mouse model. Serum creatinine levels were lowest in hCN1 transgenic animals (*db/db* CN1+) and correlated with the extent of polyuria or glomerular filtration rate as a result of poor glycemic control (Fig. 6*A*). Serum urea levels showed significantly elevated levels in hCN1 transgenic mice as a consequence of impaired renal function or dehydration (Fig. 6*B*). Albumin-to-creatinine ratios were significantly increased in all diabetic experimental groups (*db/db* CN1+, *db/db* water, and *db/db* carnosine) compared with nondiabetic controls (db/wt), indicating the presence of diabetic nephropathy in all diabetic groups (Fig. 6*E*). However, no significant differences between L-carnosine–supplemented and CN1 transgenic animals were observed at 15 or 20 weeks of age.

Renal hypertrophy is an early sign of diabetic nephropathy. At 23 weeks of age, absolute kidney weights were significantly increased in all diabetic animals compared with those of nondiabetic controls (Fig. 6*C*, db/wt). How-

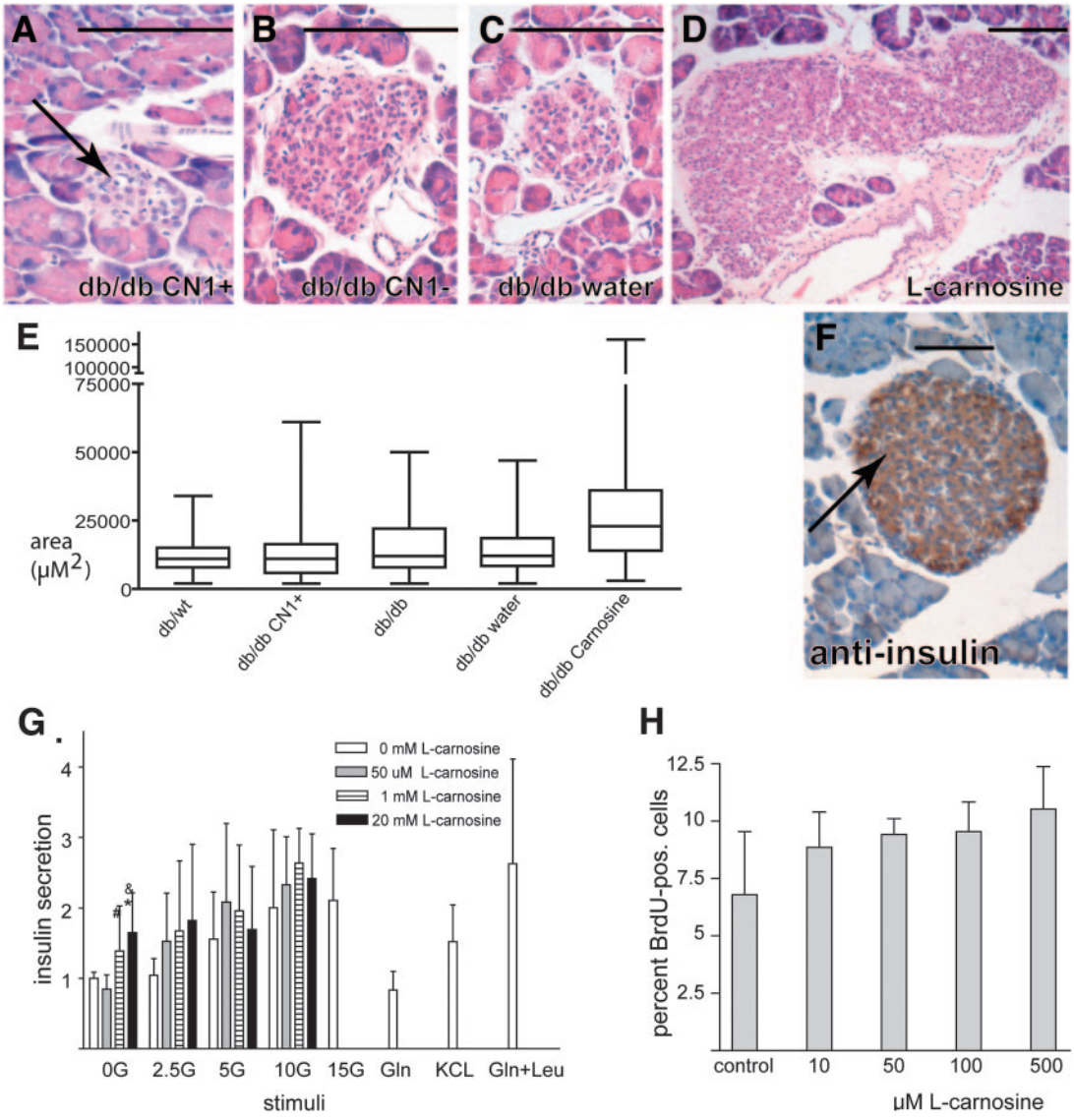

FIG. 5. Correlation of pancreatic islet size with L-carnosine levels. *A–D*: Representative histological sections derived from the pancreas of CN1 transgenic *db/db* mice (*A*), nontransgenic diabetic littermates (*db/db* CN1-) (*B*), diabetic controls (*db/db* water) (*C*), and L-carnosine–supplemented *db/db* mice (*D*) at 23 weeks of age (hematoxylin-eosin staining, size bar 75 μm). *E*: The area of 50 islets was determined in each experimental animal by morphometry. Pancreatic islets were significantly enlarged in L-carnosine–supplemented mice. (Box plots: mean, middle line; box, quartile; error bars, SD). *F*: Enlarged pancreatic islets of L-carnosine–supplemented mice contained predominantly insulin-producing β-cells (anti-insulin staining in brown). *G*: Insulin secretion in response to glucose and other stimuli in INS-1e cells. Cells were treated with different concentrations of L-glucose (G) (0, 2.5, 5, 10, and 15 mmol/l), 30 mmol/l KCl (KCL), 5 mmol/l glutamine (Gln), 20 mmol/l L-leucine (Leu), and different concentrations (0.05, 1, and 20 mmol/l) of L-carnosine, as indicated. Values are shown as means ± SD of three independent experiments. *P < 0.05 vs. 0 G + 0 L-carnosine; #P < 0.05 vs. 0 G + 0.05 L-carnosine; &P < 0.01 vs. 0 G + 0.05 L-carnosine. *H*: Proliferation of subconfluent Ins1e cells in the presence of varying concentrations of L-carnosine for 2 days. Data are shown as percentages of BrdU-positive cells in three independent experiments. A continuous increase of the proliferative index is observed with increasing L-carnosine concentrations (P = 0.059 between control and 500 μmol/l).

ever, no significant differences were observed among the diabetic experimental groups. The ratio of kidney weight over total body weight (Fig. 6*D*) suggested significant renal hypertrophy only in transgenic diabetic mice (*db/db* CN1+). However, the ratio failed to demonstrate renal hypertrophy in normal diabetic *db/db* mice (*db/db* water). It was concluded that kidney-to-body weight ratios are not a suitable parameter in this model because body weights were significantly affected by modulation of L-carnosine serum levels, as shown in Fig. 3*C*.

Finally, mesangial expansion as a sign for diabetic nephropathy was assessed. A representative example for "no mesangial expansion" is shown in Fig. 6*G* (control) and for "significant mesangial expansion" in Fig. 6*H* (mes. expansion). In agreement with the results described above, mesangial expansion was present in all three diabetic groups (Fig. 6*F*) compared with nondiabetic db/wt mice, but no significant differences between any of the diabetic groups could be detected in this mouse model.

DISCUSSION

In this study, an association of CN1 activity with diabetic disease, previously identified in human diabetic patients (6), was confirmed in a type 2 diabetes mouse model. This association was mediated by the substrate of CN1, L-carnosine. Interestingly, in the type 2 diabetes *db/db* mouse model described here, L-carnosine primarily affected glucose metabolism. L-carnosine serum levels did

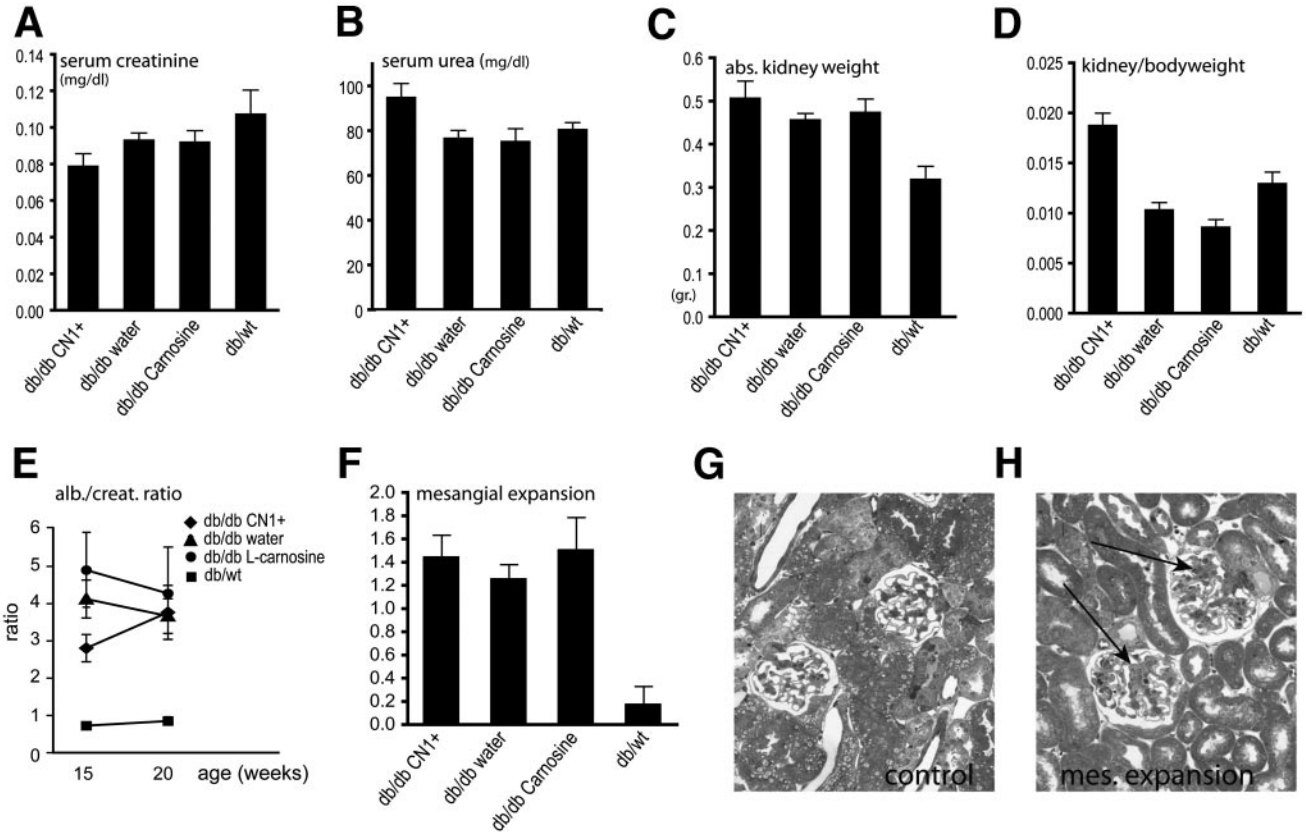

FIG. 6. L-carnosine levels do not correlate with diabetic nephropathy. *A* and *B*: Serum creatinine and urea were determined at 23 weeks of age. Lower creatinine and elevated urea levels were observed in transgenic mice (*db/db* CN1+). Serum creatinine levels were highest in nondiabetic db/wt control mice. *C*: Absolute kidney weight (in grams) and kidney-to-body weight ratio (*D*) were determined. Renal hypertrophy was observed in all diabetic experimental groups compared with nondiabetic controls (db/wt). No significant differences in kidney weight were observed among the diabetic groups *db/db* CN1+, *db/db* Carnosine, or *db/db* water. *D*: Kidney-to-body weight ratios showed significant differences among the diabetic experimental groups; however, this effect only reflected the differences in body weight among the groups. *E*: Albumin and creatinine concentrations were measured at 15 and 20 weeks of age in 24-h urine collections. Albumin-to-creatinine ratios (alb./creat. ratio) were significantly increased in all diabetic experimental groups compared with nondiabetic controls (db/wt). No significant differences were observed among CN1 transgenic (*db/db* CN1+), L-carnosine–supplemented (*db/db* Carnosine), and control diabetic (*db/db* water) mice. *F*: Semi-thin sections of the kidneys were evaluated in a blinded fashion by two independent renal pathologists for mesangial expansion (0, normal; 1, mild expansion; 2, strong expansion; 50 glomeruli for each animal). Representative images are shown in *G* (normal) and *H* (strong expansion, arrows). Significant mesangial expansion was observed in all diabetic experimental groups compared with nondiabetic controls (db/wt). No significant differences were observed among *db/db* CN1+, *db/db* Carnosine, and *db/db* water mice.

not significantly affect diabetic nephropathy, as suggested by an initial genetic study in human patients (6).

The results of this study contribute significantly to our understanding of the genetic association in humans. In humans, the "high-risk" group of patients carried more efficient signal peptides within the hCN1 gene (i.e., with more than five leucines), resulting in higher CN1 activity and lower L-carnosine levels with the serum (6). Consistent with the human data, hCN1 transgenic diabetic mice with lower L-carnosine serum levels were affected by more severe diabetic disease in this study. In a different experimental approach in this study, L-carnosine levels were artificially raised in *db/db* mice and associated with improved glucose metabolism. This indicates that the human genetic association between CN1 activity and diabetes complications is a consequence of a primary effect on glucose metabolism and that hCN1 is a modifier gene for diabetic disease. A direct effect of the quality of control on glucose metabolism and late diabetes complications has been established in human patients (19,20). In the initial study by Janssen et al. (6), metabolic control of diabetic disease was evaluated during the later stages of diabetes (i.e., after >15 years). It is possible that the higher prevalence of diabetes complications in the high-risk group of human diabetic patients reflected poor metabolic control of diabetes in this group (6).

Further support that hCN1 activity modifies glucose metabolism in human patients comes from a study that performed a genome scan for loci associated with increased FPG levels in families afflicted in part by obesity (21). Significant linkages were found with a trait located at chromosome 18q22 (logarithm of odds 4.4–6.6), the region where CNDP1 (hCN1) is located with ~150 other genes. No correlation of the identified locus and BMI was observed by Li et al. (21), supporting that decreased body weights in hCN1 transgenic mice were primarily a consequence of calorie loss in the urine in this study.

In humans, the trait on locus 18q22 only affected the FPG levels, not the prevalence of overt diabetic disease, as shown by Li et al. (21). Likewise, L-carnosine levels only affected the extent and severity diabetic disease in our type 2 diabetic mouse model—overt diabetes with FPG levels >200 mg/dl manifested significantly earlier in hCN1 transgenic mice or later in L-carnosine–supplemented mice, but eventually all mice became diabetic. Metabolic control in L-carnosine–supplemented mice eventually be-

came worse, similar to diabetic controls, indicating that L-carnosine is not sufficient to prevent overt diabetes.

Our results are also in agreement with the initial finding by Janssen et al. (6) that the risk for poor control of diabetes (reflected by a higher risk for diabetic nephropathy) is inherited in an autosomal-dominant fashion. A single CNDP1 allele with an efficient leader peptide for secretion (i.e., with more than five leucins) was sufficient to decrease serum L-carnosine levels, which were associated with poor diabetes control.

In this study, L-carnosine levels primarily affected serum insulin secretion, as indicated by the inverse correlation of body weights to FPG levels, insulin levels, and functional tests of glucose metabolism. Our data suggest that insulin secretion is preserved because of an increased β-cell mass as a consequence of increased L-carnosine serum levels. In addition to that, a discrete effect of L-carnosine on glucose-independent insulin secretion was observed in vitro, which can be expected from an amino acid or a small dipeptide such as L-carnosine.

In summary, CN1 is a potential target for pharmacological interventions aimed at manipulating glucose metabolism. Since only approximately one-third of the entire human population is homozygous for the "low-risk" CNDP1 allele (5,6,22), this approach has the potential for a broad applicability to diabetic patients.

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REFERENCES

1. Zimmet P, Alberti KG, Shaw J: Global and societal implications of the diabetes epidemic. *Nature* 414:782–787, 2001
2. Rich SS: Mapping genes in diabetes: genetic epidemiological perspective. *Diabetes* 39:1315–1319, 1990
3. Rincon-Choles H, Thameem F, Lehman DM, Arya R, Arar N, Duggirala R, Stern MP, Abboud HE: Genetic basis of diabetic nephropathy. *Am J Ther* 12:555–561, 2005
4. Placha G, Canani LH, Warram JHG, Krolewski AS: Evidence for different susceptibility genes for proteinuria and ESRD in type 2 diabetes. *Adv Chronic Kidney Dis* 12:155–169, 2005
5. Freedman BI, Hicks PJ, Sale MM, Pierson ED, Langefeld CD, Rich SS, Xu J, McDonough C, Janssen B, Yard BA, van der Woude FJ, Bowden DW: A leucine repeat in the carnosinase gene CNDP1 is associated with diabetic end-stage renal disease in European Americans. *Nephrol Dial Transplant* 22:1131–1135, 2007
6. Janssen B, Hohenadel D, Brinkkoetter P, Peters V, Rind N, Fischer C, Rychlik I, Cerna M, Romzova M, de Heer E, Baelde H, Bakker SJ, Zirie M, Rondeau E, Mathieson P, Saleem MA, Meyer J, Koppel H, Sauerhoefer S, Bartram CR, Nawroth P, Hammes HP, Yard BA, Zschocke J, van der Woude J: Carnosine as a protective factor in diabetic nephropathy: association with a leucine repeat of the carnosinase gene CNDP1. *Diabetes* 54:2320–2327, 2005
7. Teufel M, Saudek V, Ledig JP, Bernhardt A, Boularand S, Carreau A, Cairns NJ, Carter C, Cowley DJ, Duverger D, Ganzhorn AJ, Guenet C, Heintzelmann B, Laucher V, Sauvage C, Smirnova T: Sequence identification and characterization of human carnosinase and a closely related non-specific dipeptidase. *J Biol Chem* 278:6521–6531, 2003
8. Margolis FL, Grillo M: Inherited differences in mouse kidney carnosinase activity. *Biochem Genet* 22:441–451, 1984
9. Yan C, Costa RH, Darnell JE Jr, Chen JD, Van Dyke TA: Distinct positive and negative elements control the limited hepatocyte and choroid plexus expression of transthyretin in transgenic mice. *EMBO J* 9:869–878, 1990
10. Moeller MJ, Kovari IA, Holzman LB: Evaluation of a new tool for exploring podocyte biology: mouse Nphs1 5′ flanking region drives LacZ expression in podocytes. *J Am Soc Nephrol* 11:2306–2314, 2000
11. Braun GS, Veh RW, Segerer S, Horster MF, Huber SM: Developmental expression and functional significance of Kir channel subunits in ureteric bud and nephron epithelia. *Pflugers Arch* 445:321–330, 2002
12. Moeller MJ, Soofi A, Braun GS, Li X, Watzl C, Kriz W, Holzman LB: Protocadherin FAT1 binds Ena/VASP proteins and is necessary for actin dynamics and cell polarization. *EMBO J* 23:3769–3779, 2004
13. Bruning JC, Michael MD, Winnay JN, Hayashi T, Horsch D, Accili D, Goodyear LJ, Kahn CR: A muscle-specific insulin receptor knockout exhibits features of the metabolic syndrome of NIDDM without altering glucose tolerance. *Mol Cell* 2:559–569, 1998
14. Schonherr J: Analysis of products of animal origin in feeds by determination of carnosine and related dipeptides by high-performance liquid chromatography. *J Agric Food Chem* 50:1945–1950, 2002
15. Moeller MJ, Soofi A, Hartmann I, Le Hir M, Wiggins R, Kriz W, Holzman LB: Podocytes populate cellular crescents in a murine model of inflammatory glomerulonephritis. *J Am Soc Nephrol* 15:61–67, 2004
16. Asfari M, Janjic D, Meda P, Li G, Halban PA, Wollheim CB: Establishment of 2-mercaptoethanol-dependent differentiated insulin-secreting cell lines. *Endocrinology* 130:167–178, 1992
17. Breyer MD, Bottinger E, Brosius FC 3rd, Coffman TM, Harris RC, Heilig CW, Sharma K: Mouse models of diabetic nephropathy. *J Am Soc Nephrol* 16:27–45, 2005
18. Lee SM, Bressler R: Prevention of diabetic nephropathy by diet control in the db/db mouse. *Diabetes* 30:106–111, 1981
19. Tight blood pressure control and risk of macrovascular and microvascular complications in type 2 diabetes: UKPDS 38: UK Prospective Diabetes Study Group. *BMJ* 317:703–713, 1998
20. The effect of intensive treatment of diabetes on the development and progression of long-term complications in insulin-dependent diabetes mellitus: the Diabetes Control and Complications Trial Research Group. *N Engl J Med* 329:977–986, 1993
21. Li WD, Dong C, Li D, C Garrigan, Price RA: A quantitative trait locus influencing fasting plasma glucose in chromosome region 18q22–23. *Diabetes* 53:2487–2491, 2004
22. Zschocke J, Nebel A, Wicks K, Peters V, El Mokhtari NE, Krawczak M, van der Woude F, Janssen B, Schreiber S: Allelic variation in the CNDP1 gene and its lack of association with longevity and coronary heart disease. *Mech Ageing Dev* 127:817–820, 2006