

L-carnosine, a substrate of carnosinase-1, influences glucose metabolism.

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Abstract

Objective: Carnosinase 1 (CN1) is a secreted dipeptidase that hydrolyses L-carnosine. Recently, we have identified an allelic variant of hCN1, which results in increased enzyme activity and which is associated with susceptibility for diabetic nephropathy (DN) in human 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, the human CN1 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 HbA1c 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, non-transgenic db/db mice were substituted 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 beta-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 diabetes patients.

Keywords: gene-modified animals, glomerulopathy, late complications, gene, db/db mouse, diabetic nephropathy

Introduction

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% but is projected to rise over the next decade owing to increasing age of the population and 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 twins than among dizygotic twins [2]. There is also evidence for a genetic component to the risk of diabetic nephropathy (DN) [3, 4].

Our group is interested in identifying variants of genes that modify the risk for diabetic complications, such as DN. Recently, an association of DN 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 hydrolyses 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 DN and was associated with lower carnosinase 1 serum levels. Leader peptide variants containing more than 5 leucine repeats were associated with an increased risk for DN. 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].

So 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 but also in the central nervous system from 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 within their kidneys [8].

This study was undertaken to verify the genetic association of CN1 with diabetic disease and to test if CN1 determines the risk for DN 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: Endogenous L-carnosine serum levels were artificially lowered by over-expression of secreted human CN1 under the control of a liver-specific promoter mimicking the endogenous expression pattern of CN1 in humans. In a second experimental approach, diabetic mice were substituted with L-carnosine within their drinking water.

Research Design and Methods

Generation of experimental mice

The cDNA of human carnosinase 1 including the endogenous signal peptide of 6 leucines was amplified from IMAGE clone Acc# BX094414 using the primers CN1.fwd 5'-CACCATGGATCCCAAACCTCGGGA3' and CN1.rev 5'-TCAATGGAGCTGGGCCATCT3'. The PCR product was ligated into the *Stu*I site of plasmid pTTR1ExV3 [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 (The Jackson Laboratory, Maine, USA; line 000642) at the IBF,

University 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 directPCR Lysis Reagent Kit, Viagen Biotech, CA, USA, supplemented with proteinase K, Sigma, according to manufacturer's instructions. The transgene TTP-hCN1 was detected by PCR using the primers CN1Tail.fwd CCTCGCTTCAGACAAGAGCTCTTCAG AATGA and CN1Tail.rev GCTCTGAAGGCGCTCACAGCATTGAT CCAA (94°C 3 min.; annealing 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 Jackson.org. hCN1 transgenic db/wt mice were bred to non-transgenic db/wt littermates. Diabetic transgenic db/db mice were used as experimental animals (db/db CN1+). Diabetic non-transgenic (db/db water) as well as non-diabetic non-transgenic littermates (db/wt) served as controls. Transgenic mice without the Lepr mutation (wt/wt) were discarded.

A second experimental group of non-transgenic db/db mice (db/db L-carnosine, Jackson Laboratory, Maine, USA) was substituted with 4 mM of L-carnosine within the drinking water or with water only (db/db water). L-carnosine substituted 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 RT (data not shown). Daily drinking volumes were determined. Control animals were identical in every aspect (i.e. glucose metabolism, DN) to control animals derived from our own colony (not shown). Unless stated otherwise, each experimental group contained 8 mice, except for db/db water mice, which contained 16 mice. Experimental mice were housed at 22°C and a 12-hour light cycle and fed a standard rodent chow (SNIFF R/M-H diet for mice

and rats, Soest, Germany). Each group of experimental animals contained the same ratio of females and males. RT-PCR

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

Molecular biology techniques were performed as described according to standard protocols [12]. Immunoblotting or immunohistological stainings for carnosinase 1 was performed using the C17E antiserum (kind gift of Dr. Teufel, Strasbourg, France) [7] and anti-carnosine 1 polyclonal antiserum (R&D Systems, Wiesbaden-Nordenstadt, Germany) with identical results.

Analytical procedures

Total body weights or fasting plasma glucose levels (FPG) were measured each week at the beginning of the light cycle after an overnight fast until week 22 using Ascensia ELITE XL, Bayer, Germany. HbA1c levels were determined using MicromatTMII, Biorad, CA, USA. Insulin levels in mouse serum or INS-1e supernatants were determined using the mouse Insulin ELISA kit (EZRMI-13K, Linco Research, MO, USA). Glucose-tolerance and insulin-tolerance tests were performed on animals that had been fasted overnight for 16

hours as described [13]. In brief, animals were injected with either 2 gr./kg body weight of glucose or 4 IU/kg body weight of 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 minutes after the injection. Animals were placed in metabolic cages and received only water supplemented with 4% sucrose. 24-hour excretion of albumin and creatinine was measured at 20 weeks of age using Hitachi Analyzer 912, Roche, Mannheim, Germany.

Measurement of carnosine concentration in mouse EDTA-plasma was performed according to the method of Schonherr with modifications [14]. 80 μ l EDTA-plasma were mixed with 20 μ l 10% 5-sulfosalicylic acid (SSA). 10 μ l supernatant was mixed with 20 μ l 0,4M borate puffer (pH 9,5). 10 μ l supernatant were spiked with 10 μ M 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 μ l of each sample were mixed with 30 μ l of freshly prepared CFC solution (2.5 mM in acetone). After 90 s, 18 μ l of 1mM EDTA in 0.1M NaOH and 0.5M hydroxylamine hydrochloride in distilled water and 2-methylthioethanol was added. After additional 3 min, 42 μ l of quench solution, 20% (v/v) acetic acid in acetonitrile, was added. 40 μ l of diluted sample were injected for separation into the HPLC system LaChrom Elite HPLC system (Jupiter column, C18, 300 Å, 5 μ m particle size, Phenomenex, Aschaffenburg, Germany). The elution was performed with fluorescence detection at *extinction* 287 nm and *emission* 340 nm. The profile of the binary gradient was the 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; 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 euthanized at 24 weeks of age and perfused with 3% paraformaldehyde as described [15]. 1 μ m thick semi-thin kidney sections 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; 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 U/ml penicillin/streptomycin, 1 mM sodium pyruvate, and 100 μ M 2-mercaptoethanol [16]. The cells were exposed to various stimuli for 2 hours in KRBH buffer (n=3): 5 mM L-glutamine served as negative control; L-glutamine and 20 mM L-leucine or 30 mM KCL served as positive control (Sigma-Aldrich, Munich, Germany). Insulin levels were analyzed as ratio where basal insulin secretion in the presence of L-glutamine only was normalized to 1. To assess the proliferative index, INS-1e cells were pulsed with BrdU for 2 hours, fixed and stained using an anti-BrdU antiserum MAB3424, Chemicon International, Hampshire, UK.

Results

Generation of hCN1 transgenic db/db mice

In order to lower L-carnosine level within the serum of diabetic mice, the cDNA of human carnosinase-1 (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 alpha-cells of the pancreatic islets (not shown). Rodents do not endogenously express a secreted form of carnosinase 1 [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 model) from week 8. The BKS.Cg-m+/+ Lepr db /J 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. Out of 70 mice, 15 mice were transgenic for hCN1 (21%). All of the results described below were reproduced in at least 4 different founder lines. A stable line with robust CN1 expression was established from heterozygous db/wt, hCN1 transgenic founder mice by cross-breeding with non-transgenic db/wt littermates.

Analysis of hCN1 transgenic db/db mice

Messenger RNA was isolated from liver and kidney of hCN1 transgenic db/db mice and from non-transgenic littermates. Non-saturated RT-PCR analysis using primers specific for transgenic hCN1 demonstrated expression of transgenic mRNA within the liver but not in kidney (Fig. 2A, transgenic hCN1). TTP-hCN1 plasmid was used as positive control. Expression of endogenous cytosolic CN1 was detected in kidney, as described previously (Fig. 2A, endogenous mCN1). Gel electrophoresis of total RNA as well as non-saturating RT-PCR specific for endogenous beta-actin was used to control for equal loading. Negative controls omitting

reverse-transcription (RT-) or omitting mRNA (water only) were always negative.

To verify that the transgenic hCN1 is efficiently secreted into the serum, 10 μ l of serum derived from hCN1 transgenic (db/db CN1+) or non-transgenic controls (db/db water) were subjected to SDS-PAGE and subsequently immunoblotted with various anti-sera directed against CN1. No carnosinase 1 protein was detected in non-transgenic controls as described previously [7]. Significant amounts of CN1 protein were detected in hCN1 transgenic animals (arrow).

To verify the functional effects of hCN1 over-expression, L-carnosine levels were measured in EDTA-plasma obtained at the beginning of the light-cycle of transgenic and control db/db mice (Fig. 2C) at 12 weeks of age. In control mice, endogenous L-carnosine levels averaged around $1.2 \pm 0.5 \mu\text{M}$. In hCN1 transgenic mice, L-carnosine levels were significantly lower ($p < 0.01$), most values being below detection levels of $0.02 \mu\text{M}$ (Fig. 2C, db/db CN1+).

Analysis of db/db mice substituted with L-carnosine

A second experimental group of non-transgenic db/db mice was substituted with L-carnosine within the drinking water. L-carnosine plasma levels were significantly elevated to $6 \pm 1.1 \mu\text{M}$ in this group ($n = 6$ for each group) (Fig. 2C, db/db carnosine).

Fasting plasma glucose levels correlate inversely with L-carnosine levels

Normal db/db mice (db/db water) developed persistent hyperglycemia between weeks 8 – 10 (Fig. 3A, db/db water), as described [18]. hCN1 transgenic db/db mice (db/db CN1+) developed hyperglycemia earlier (week 6 – 7) and reached significantly higher FPG levels than the db/db water group. Conversely, FPG levels were significantly lower in L-carnosine substituted mice (Fig. 3A, db/db L-carnosine) and the onset of diabetes was delayed until week 17 – 20. Similarly, HbA1c levels were elevated in hCN1

transgenic mice (db/db CN1+) and significantly improved in L-carnosine substituted mice (db/db carnosine) compared to wild-type diabetic db/db mice (Fig. 3B, db/db water).

Obesity is important for the pathogenesis of altered glucose metabolism in the db/db mouse model. As expected, total body weights rose 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 non-diabetic db/wt control mice by week 22 (Fig. 3C, db/db CN1+). This progressive wasting ultimately led to the death of the mice between 24 – 28 weeks of age as was verified by autopsy (4 mice died because of dehydration, 1 mouse had an intraabdominal abscess). For this reason, the observation period of this study was limited to 23 weeks (Supplementary Figure 1.). 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 approx. 1.7 gr./day was observed in the db/db CN1+ group (Fig. 3D).

L-carnosine substituted mice continued to gain weight beyond week 15 until the end of the observation period (Fig. 3C, 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 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 to diabetic controls (db/db water), insulin levels were decreased in hCN1 transgenic mice despite higher FPG levels (Fig. 4A). The opposite pattern was observed in L-carnosine substituted mice (db/db L-carnosine), where serum insulin levels were significantly increased with lower FPG levels compared to diabetic controls (db/db water). These results

suggested that glucose metabolism was primarily affected by altered insulin secretion in hCN1 transgenic and L-carnosine substituted mice.

To validate these findings, insulin sensitivity tests were performed (Fig. 4B, age 12 weeks). FPG levels fell proportionally in all experimental groups (20% reduction after 30 min, 30% total reduction after 1 hour) indicating that peripheral insulin sensitivity is similar within the experimental groups. Glucose-tolerance tests were also performed (Fig. 4C). A similar rise in FPG levels was observed in L-carnosine substituted 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 substituted 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. 5A - E). Islet size was determined by morphometry (Fig. 5E). Overall, pancreatic islets were enlarged in diabetic mice compared to controls (db/wt). A striking difference in pancreatic islet size was observed in L-carnosine substituted animals (Fig. 5D, E). Hyperplastic islets of L-carnosine substituted mice were predominantly composed of insulin-producing beta-cells as demonstrated by immunohistological anti-insulin staining (Fig. 5F).

Next, it was tested whether L-carnosine exerts a direct effect on insulin-secretion or proliferation of beta-cells *in vitro*. For this purpose, INS-1e cells were exposed to varying concentrations of L-carnosine. INS-1e cells are derived from beta-cells and secrete insulin in a highly regulated glucose-dependent fashion

[16]. In the absence of L-carnosine, insulin secretion increased in the presence of increasing concentrations of L-glucose, as expected (Fig. 5G, 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. 5G, 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 3 independent experiments is shown in Fig. 5H. 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 substituted mice because of an increased or preserved mass of beta-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 human diabetics, it was investigated, if 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. 6A). Serum urea levels showed significantly elevated levels in hCN1 transgenic mice as a consequence of impaired renal function or dehydration (Fig 6B). A lbumin/creatinine ratios were

significantly increased in all diabetic experimental groups (db/db CN1+, db/db water, db/db Carnosine) compared to non-diabetic controls (db/wt), indicating the presence of diabetic nephropathy in all diabetic groups (Fig. 6E). However, no significant differences between L-carnosine substituted 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 to non-diabetic controls (Fig. 6C, db/wt). However, no significant differences were observed among the diabetic experimental groups. The ratio of kidney weight over total body weight (Fig. 6D) 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/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. 3C.

Finally, mesangial expansion as a sign for DN was assessed. A representative example for “no mesangial expansion” is shown in Fig. 6G, control, and for “significant mesangial expansion” in Fig. 6H. In agreement with the results described above, mesangial expansion was present in all three diabetic groups (Fig. 6F) compared to non-diabetic 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 carnosinase 1 activity with diabetic disease previously identified in human diabetes patients [6] could be confirmed in a type 2 diabetes mouse model. This association was mediated by the substrate of carnosinase 1, namely 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 not significantly affect diabetic nephropathy as suggested by the 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 5 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, L-carnosine levels were artificially raised in db/db mice in this study and this was associated with improved glucose metabolism. This indicates that the human genetic association between CN1 activity and diabetic complications is a consequence of a primary effect on glucose metabolism and that human carnosinase 1 is a modifier gene for diabetic disease. A direct effect of the quality of control on glucose metabolism and late diabetic complications has been established in human patients [19, 20]. In the initial study by Janssen et al., metabolic control of diabetic disease was evaluated during the later stages of diabetic disease (i.e. after more than 15 years) [6]. It is possible that the higher prevalence of diabetic complications in “high-risk” group of human diabetes patients reflected poor metabolic control of diabetic disease 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 fasting plasma glucose levels in families afflicted in part by obesity [21]. Significant linkages were found with a trait located at chromosome 18q22 (LOD 4.4 –

6.6), the region where CNDP1 (hCN1) is located together with approx. 150 other genes. No correlation of the identified locus with body mass index was observed by Li et al., 2004, 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 fasting plasma glucose levels but did not affect the prevalence of overt diabetic disease, as shown by Li et al., 2004 [21]. Likewise, L-carnosine levels only affected the extent and severity diabetic disease in our type 2 diabetes mouse model: Overt diabetes with FPG levels above 200 mg/dl manifested significantly earlier in hCN1 transgenic mice or later in L-carnosine substituted mice but eventually all mice became diabetic. Metabolic control in L-carnosine substituted mice eventually became worse similar to diabetic controls indicating that L-carnosine is not sufficient to prevent overt diabetic disease.

Our results are also in agreement with the initial finding by Janssen et al., 2005, that the risk for poor control of diabetic disease (reflected by a higher risk for DN) is inherited in an autosomal-dominant fashion [6]. A single CNDP1 allele with an efficient leader peptide for secretion (i.e. with more than 5 leucines) was sufficient to decrease serum L-carnosine levels, which were associated with poor diabetic control.

In this study, L-carnosine levels primarily affected serum insulin secretion as indicated by the inverse correlation of body weights to fasting plasma glucose (FPG), insulin levels and functional tests of glucose metabolism. Our data suggest that insulin secretion is preserved because of an increased beta-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, carnosinase 1 is a potential target for pharmacological interventions that

aim at manipulating glucose metabolism. Since only about 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 diabetes patients.

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