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RESEARCH ARTICLE

Carnosinase-1 overexpression, but not aerobic exercise training, affects the development of diabetic nephropathy in BTBR *ob/ob* mice

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Everaert I, He J, Hanssens M, Stautemas J, Bakker K, Albrecht T, Zhang S, Van der Stede T, Vanhove K, Hoetker D, Howsam M, Tessier FJ, Yard B, Baba SP, Baelde HJ, Derave W. Carnosinase-1 overexpression, but not aerobic exercise training, affects the development of diabetic nephropathy in BTBR *ob/ob* mice. *Am J Physiol Renal Physiol* 318: F1030–F1040, 2020. First published March 9, 2020; doi:10.1152/ajprenal.00329.2019.—Manipulation of circulating histidine-containing dipeptides (HCD) has been shown to affect the development of diabetes and early-stage diabetic nephropathy (DN). The aim of the present study was to investigate whether such interventions, which potentially alter levels of circulating HCD, also affect the development of advanced-stage DN. Two interventions, aerobic exercise training and overexpression of the human carnosinase-1 (hCN1) enzyme, were tested. BTBR *ob/ob* mice were either subjected to aerobic exercise training (20 wk) or genetically manipulated to overexpress hCN1, and different diabetes- and DN-related markers were compared with control *ob/ob* and healthy (wild-type) mice. An acute exercise study was performed to elucidate the effect of obesity, acute running, and hCN1 overexpression on plasma HCD levels. Chronic aerobic exercise training did not affect the development of diabetes or DN, but hCN1 overexpression accelerated hyperlipidemia and aggravated the development of albuminuria, mesangial matrix expansion, and glomerular hypertrophy of *ob/ob* mice. In line, plasma, kidney, and muscle HCD were markedly lower in *ob/ob* versus wild-type mice, and plasma and kidney HCD in particular were lower in *ob/ob* hCN1 versus *ob/ob* mice but were unaffected by aerobic exercise. In conclusion, advanced glomerular damage is accelerated in mice overexpressing the hCN1 enzyme but not protected by chronic exercise training. Interestingly, we showed, for the first time, that the development of DN is closely linked to renal HCD availability. Further research will have to elucidate whether the stimulation of renal HCD levels can be a therapeutic strategy to reduce the risk for developing DN.

carnosinase-1; carnosine; diabetes; diabetic nephropathy; exercise

INTRODUCTION

Twenty to forty percent of patients with type 2 diabetes develop diabetic nephropathy (DN). As no optimal treatment for DN is currently available, this frequently results in end-stage renal disease and dependence on dialysis. A growing body of evidence suggests a protective role of carnosine against DN. Carnosine and its methylated analog anserine are histidine-containing dipeptides (HCD), consisting of amino acids β-alanine and histidine and π-methylhistidine, respectively, which are available in the diet of omnivorous subjects. They possess some beneficial biochemical properties, such as antioxidative, metal chelating, and antiglycation properties, which enable them to attenuate the development of DN (for a review, see Ref. 6).

Genetic studies have revealed an association of a polymorphism in the carnosinase-1 gene (*CNDP1*), the enzyme responsible for the hydrolysis of carnosine in the circulation, and the prevalence of DN, especially in female patients with type 2 diabetes (13, 21, 29). Although carnosine is only present in the low nanomolar range in the fasted state (because of the very active carnosinase-1 enzyme) (41), it has been hypothesized that a higher circulating carnosine content upon dietary intake was the underlying mechanism for protection against DN (12, 42).

Long-term supplementation of carnosine in drinking water has repeatedly been shown to protect against early signs of DN in different diabetic rodent models [*db/db* mice (35), obese Zucker rats (2), and streptozotocin-induced diabetic rats (34, 52)] and recently also in BTBR *ob/ob* mice, which have DN with severe glomerular lesions (1). Reducing the amount of circulating carnosine levels, on the other hand, has been achieved by overexpressing the human carnosinase-1 (hCN1) enzyme, which is only present in humans and not in rodents. This resulted in a more pronounced diabetic state (as reflected by hyperglycemia, HbA1c, and insulin) in *db/db* mice (42). However, since *db/db* mice are slow to develop advanced DN, the effect of reduced circulating carnosine levels on DN in a model with more pronounced DN (BTBR *ob/ob*) was investigated in the present study.

HCD are mainly present in skeletal muscle but also in lower amounts in kidney tissue. Owing to its high concentration in skeletal muscles and their total volume, it is estimated that ~99% of the carnosine body burden is stored in skeletal muscle. Therefore, a potential alternative way to manipulate circulating carnosine levels is hypothesized to be the release of carnosine from skeletal muscle as a result of physical exercise.

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Both mice and horses were characterized by higher carnosine content in their circulation when being physically active (11, 30). Human evidence is scarce, but muscle interstitium carnosine levels were increased in response to submaximal leg exercise (32).

Interestingly, it has been established that prolonged aerobic exercise training could protect different diabetic rodent models against the increase in urinary albumin and other pathophysiological characteristics of DN (39, 49, 51). Intervention studies investigating the effects of exercise training on the development of DN in patients with diabetes are lacking (10); however, one prospective study revealed that moderate- and high-intensity leisure-time physical activity attenuate the initiation and progression of DN (50). We thus hypothesized that part of the protective effect of exercise training on DN is mediated by enhanced circulating carnosine and/or anserine, which are released from muscular storage sites upon muscle contractile activity. This could be a novel therapeutic insight on the benefits of exercise, in addition to the more commonly proposed mechanism that exercise training can improve glycemic control (43).

The aim of the present study was to investigate whether overexpression of the hCN1 enzyme or chronic aerobic exercise training, interventions potentially resulting in decreased and increased circulating HCD levels, respectively, could affect the development of DN in mice that develop glomerular lesions that closely resemble an advanced stage of human DN [BTBR *ob/ob* mice (19)]. An acute study was performed to evaluate the effect of obesity, acute exercise, and hCN1 overexpression on plasma and muscle HCD levels. A chronic study further examined the development of diabetes and DN in *ob/ob* mice compared with that in *1*) *ob/ob* mice that underwent a treadmill-based aerobic exercise training protocol for 20 wk and *2*) *ob/ob* mice that were genetically manipulated to overexpress the hCN1 enzyme.

METHODS

The experimental protocol was approved by the Ethics Committee for Animal Research at Ghent University and followed the Principles of Laboratory Animal Care.

Generation and Genotyping of Experimental Mice

Human carnosine dipeptidase 1 (h*CNDP1*) transgenic mice were generated in the BTBR *wt/ob* (Black and Tan, BRachyuric) background as previously described in *db*/*db* mice (42). Briefly, the cDNA of h*CNDP1* including the endogenous signal peptide of six leucines was amplified from IMAGE clone Accession No. BX094414 with the primers 5′-PCACCATGGATCCCAAACTCGGGA-3′ (*CNDP1* forward) and 5′-PTCAATGGAGCTGGGCCATCT-3′ (*CNDP1* reverse). PCR products were ligated behind the transthyretin promoter into the StuI site of plasmid pTTR1ExV3. Thereafter, DH5α-competent *Escherichia coli* were transformed with h*CNDP1* containing pTTR1ExV3. Transformed *E. coli* were propagated with standard protocols. For isolation of the hCNDP1-pTTR1ExV3 plasmid, a Maxi-Prep plasmid purification kit (Qiagen, Hilden, Germany) was used. Thereafter, the fragment containing hCNDP1 and the TTR1 promoter was cut from the plasmid by HindIII digestion. The purified fragment was injected into the pronuclei of the fertilized ovum of a BTBR *ob/wt* female mouse (stock no. 004824, Jackson Laboratories, Bar Harbor, ME). All animal procedures were approved by the Regierungspräsidium Karlsruhe (AZ 35-9185.81/G-116/14 and G-108/13). The fertilized ovum was transplanted into the oviduct of a pseudopregnant mouse that had been induced to act as a recipient by mating with a vasectomized male mouse. In separate experiments from our research group, we previously observed that hCN1 overexpression in *ob*/*wt* mice did not result in metabolic disturbances, reflected by similar levels of HbA1c and the albumin-to-creatinine ratio in transgenic versus nontransgenic *ob*/*wt* mice (B. Yard et al., unpublished data).

hCN1 transgenic BTBR mice were crossbred with BTBR *ob*/*wt* mice (line 004824, The Jackson Laboratory). The Lep^{ob} point mutation and overexpression with hCN1 was checked by quantitative PCR (high-resolution melting analysis, based on Ref. 47). DNA was isolated from tails of 7- to 11-day-old pups with the Invisorb Spin tissue mini kit (Stratec Molecular). DNA content was quantified with NanoDrop (ThermoFisher Scientific) and diluted with elution buffer to a final concentration of 25 ng/μL. For the ob point mutation, primer mix (0.4 μL, final concentration: 2 μM, forward: 5′-CAGATAGCCAATGACCTGGAG-3′ and reverse: 5′-TCTTGGAGAAGGCCAGCAGAT-3′), JumpStart Taq ReadyMix (6 μL, Sigma-Aldrich), SYBR Green PCR Master Mix (1.2 μL, Life Technologies), and DNA (0.5 μL) were added to a LightCycler 480 multiwell plate. For the CN1 transgene, primer mix (0.4 μL, final concentration: 2 μM, forward 5′-CCTCGCTTCAGACAAGAGCTCTTCAGAATGA-3′ and reverse 5′-GCTCTGAAGGCGCTCACAGCATTGATCCAA-3′), JumpStart Taq ReadyMix (3.6 μL, Sigma-Aldrich), SYBR Green PCR Master Mix (3.6 μL, Life Technologies), and DNA (0.5 μL) were used. Amplification and melt curve analysis was performed on a Roche LightCycler 480 instrument using the following parameters: detection format: SYBR green I, preincubation (95°C for 3 min), amplification/quantification (95°C for 30 s, 58°C for 30 s, and 72°C for 1 min with single acquisition; 38 cycles), high-resolution melting (95°C for 1 min, 40°C for 1 min, 65°C for 1 s, and 90°C with 25 acquisitions per °C). The genotyping was validated by evaluating the phenotypes, i.e., obesity and plasma CN1 activity, respectively.

Experimental Design

Acute study. Four- to six-week-old wild-type (WT; $n = 17$), *ob/ob* ($n = 29$), and *ob/ob* hCN1 ($n = 5$) mice were included in these experiments (Table 1). After five familiarization sessions (in 2 wk), mice were placed on either a running treadmill (*ob/ob* EX; 10 m/min, $n = 14$) or a stationary treadmill (*ob/ob*; $n = 14$) for 1 h. Mice were immediately sedated (80% Ketalar-20% Rompun) after 1 h, blood was collected as quickly as possible in precooled EDTA tubes and centrifuged at 14,000 *g* for 5 min, and collected plasma was stored, after deproteinization with sulfosalicylic acid (SSA), at −20°C. Gastroc-

Table 1. *Acute study: basic group characteristics*

	Number of Mice (Male/Female)	Age, wk	Body Weight, g	Carnosinase-1 Activity, $\mu mol \cdot mL^{-1} \cdot h^{-1}$
WT	17 (8/9)	8.3 ± 0.7	28.1 ± 3.0	<LOD
ob/ob	15 (6/9)	7.5 ± 1.5	47.3 ± 6.5	<LOD
ob/ob EX	14 (7/7)	7.0 ± 1.3	47.7 ± 8.3	<LOD
ob/ob hCN1	5 (2/3)	6.6 ± 1.1	44.2 ± 8.7	6.04 ± 0.94

Values are means ± SD for the numbers of mice indicated. The following groups are shown: wild type (WT) mice, *ob*/*ob* mice, *ob/ob* mice with acute exercise (EX), and *ob*/*ob* human carnosinase-1 transgenic (hCN1) mice. LOD, limit of detection.

nemius muscle was immediately collected after cervical dislocation, snap frozen in liquid nitrogen, and stored at −80°C.

Chronic study. Four-week-old BTBR mice were randomly allocated to the following four experimental groups: WT ($n = 15$), *ob/ob* ($n = 19$), *ob/ob* with exercise training (EX-T; $n = 19$), and *ob/ob* hCN1 ($n = 16$) (Table 2). Male and female mice were included equally. Exercise training, on a Columbus Exer-3/6 open treadmill, was initiated at the age of 4 wk with a familiarization period of 2 wk, in which running time and intensity were gradually increased up to 10 m/min for 1 h. Mice were closely monitored during running and were removed from the treadmill when they refused (e.g., because of injury) to leave the electrical grid. At the age of 4 and 24 wk, daily food and water intake (mean value per cage, mice having been placed in separate cages per group, sex, and age) and body weight were monitored. At *weeks 4*, *14*, and *24*, fasted (4–6 h) serum was collected via the tail vein and diluted with 0.9% NaCl for further analysis. Fasted (4–6 h) spot urine was collected at *weeks 4*, *8*, *12*, *14*, and *24*. Euthanasia took place at the age of 24 wk. One kidney was removed (snap frozen and stored at −80°C) under isoflurane anesthesia, after which whole body perfusion (heart left ventricle) with saline was performed. The remaining, perfused kidney was placed in formalin solution (neutral buffered, 10%, Sigma-Aldrich) for 24 h, after which it was stored in 70% ethanol at room temperature embedded in paraffin. The perfused gastrocnemius was further dissected and stored at −80°C until analysis. Fasted serum glucose, triglycerides, and cholesterol were measured in the Clinical Laboratory of University Hospital UZ Ghent with a Roche/Hitachi Cobas C according to the manufacturer's procedures. Serum protein levels were evaluated by means of the Total Protein Kit Micro-Lowry, Onishi & Barr Modification (catalog no. TP0200, Sigma).

Histological Analysis

Paraffin-embedded kidney tissue was cut on a Leica microtome (Wetzlar). Sections (4-μm thickness) were deparaffinized and rehydrated according to a standard protocol. Sections were stained with periodic acid-Schiff as well as hematoxylin and eosin. Stained slides were digitalized with the Philips Ultra Fast Scanner 1.6 RA (Philips Electronics) for morphological measurement. The surface areas (μm^2) of the whole glomerulus, Bowman's space, and glomerular tuft of 25 randomly chosen glomeruli were measured with ImageJ software to examine glomerular hypertrophy. Periodic acid-Schiff-stained kidney tissue was graded in a blinded manner by two independent observers on a scale of 0−3, depending on the amount of the mesangial matrix (0: 0–25%, 1: 25–50%, 2: 50–75%, and 3: >75%), in 25 randomly chosen glomeruli. Interstitial fibrosis was determined by sirius red staining.

Immunohistochemistry

Sections were deparaffinized, rehydrated, and thereafter stained for Wilms' tumor-1 protein. Antigen retrieval was performed with Tris-EDTA buffer. Sections were incubated with rabbit anti-Wilms' tumor-1 (1:1,000, Santa Cruz Biotechnology, Santa Cruz, CA) followed by an anti-rabbit-Envision, horseradish peroxidase-labeled secondary antibody (Dako). As a negative control, normal rabbit serum was used as the primary antibody. Immunostaining was visualized with diaminobenzidine (DAB+, Dako) as the chromogen. Thereafter, sections were counterstained with hematoxylin and eosin, dehydrated, and mounted. Sections were imaged with a Philips Ultra-Fast Scanner 1.6 RA (Philips Electronics). The number of podocytes in each sample was determined by counting the number of Wilms' tumor-1-positive nuclei per glomerulus in 25 randomly chosen glomeruli.

Urinary Albumin

Urine albumin levels were measured with rocket immunoelectrophoresis against rabbit anti-mouse albumin, and purified mouse serum albumin (Sigma-Aldrich) was used as the standard. Urine creatinine levels were measured with a creatinine assay with picric acid, sodium hydroxide, and creatinine standards (Sigma-Aldrich). Thereafter, the albumin-to-creatinine ratio was calculated. The rabbit anti-mouse albumin antibody was produced in our laboratory and tested by Western blot for specificity.

Kidney Carboxymethyllysine

Carboxymethyllysine (CML), a widely studied marker of advanced glycation, was analyzed in kidneys with the method described by Niquet-Léridon and Tessier (31) with the instrument parameters detailed by Guilbaud et al. (16).

Carnosine and Anserine Determination

Carnosine and anserine were quantified based on the protocol of Ref. 17. Deproteinized plasma (acute exercise study) and spot urine (chronic exercise study) were diluted 1:20 and 1:25, respectively, in a 75% acetonitrile-25% water solution containing 10 μM tyrosine-histidine (mass-to-charge ratio: 319 Da) as an internal standard. Samples were centrifuged at 13,000 *g* for 10 min to eliminate excessive precipitate. The supernatant was used for further analysis. Gastrocnemius muscle (acute study) and kidney tissue (chronic study) were homogenized in extraction solution containing 10 mM HCl, 100 μM phenanthroline (specific CN1 inhibitor), and tyrosine-histidine internal standard (25 μM for muscle tissue and 2 μM for kidney tissue) with a BeadBug D1030 tissue homogenizer. Homogenates were sonicated on ice for 10 s and centrifuged at 13,000 *g* for 10 min. The supernatant was diluted 1:1,500 (muscle homogenate) and 1:100 (kidney homogenate) in a 75% acetonitrile-25% water solution before further analysis.

High-resolution ultraperformance liquid chromatography (UPLC; Waters ACQUITY UPLC H-Class System) coupled with a Xevo TQ-S micro triple quadrupole MS (LC-MS/MS) was used to identify and quantify carnosine, anserine, and carnosine-aldehyde conjugate

Table 2. *Chronic study: basic group characteristics*

	Number of Mice (Male/Female)	Body Weight, g	Water Intake, mL/day	Carnosinase-1 Activity, $\mu mol \cdot mL^{-1} \cdot h^{-1}$
Week 4				
WT	15 (7/8)	19.9 ± 4.6	12.7 ± 9.4	ND
ob/ob	19 (10/9)	28.8 ± 5.0*	13.0 ± 3.7	ND
ob/ob EX-T	19 (9/10)	27.2 ± 2.9	15.1 ± 4.3	ND
ob/ob hCN1	16 (7/9)	26.4 ± 7.9	15.1 ± 5.8	33.4 ± 20.2
Week 24				
WT	14 (7/7)	38.8 ± 6.8	15.7 ± 5.2	0.15 ± 0.15
ob/ob	17 (9/8)	74.0 ± 10.8*	29.4 ± 15.3*	0.54 ± 0.75
ob/ob EX-T	16 (7/9)	67.7 ± 7.4	25.0 ± 14.1	0.62 ± 0.75
ob/ob hCN1	14 (6/8)	72.2 ± 12.2	30.6 ± 11.7	145.8 ± 61.5†

Values are means ± SD for the numbers of mice indicated. The following groups are shown: wild type (WT) mice, *ob/ob* mice, *ob/ob* mice with chronic exercise training (EX-T), and *ob/ob* human carnosinase-1 transgenic (hCN1) mice. ND, not determined. *$P \leq 0.05$, *ob/ob* vs. WT mice; †$P > 0.05$ and < 0.1 vs. *ob/ob* mice.

levels. Carnosine, anserine, and carnosine-aldehyde conjugates were separated with a Waters ACQUITY BEH HILIC column (1.7 μm, 2.1 × 50 mm) equipped with an in-line frit filter unit. The elution of analytes was achieved with a binary solvent system consisting of 10 mM ammonium formate and 0.125% formic acid in 5% acetonitrile-95% water for *mobile phase A* and 10 mM ammonium formate and 0.125% formic acid in 95% acetonitrile-5% water for *mobile phase B* at a flow rate of 0.55 mL/min. Initial conditions were 0.1:99.9 *A*:*B* ramping to 99.9:0.1 *A*:*B* over 5 min before rapidly returning to 0.1:99.9 *A*:*B* over 0.5 min. This mobile phase composition was held from 5.5 to 8 min to equilibrate the column for the next injection (5 μL). Dipeptides were quantified with external standard curves, and carnosine-aldehyde conjugates were quantified with the peak ratio of histidyl-dipeptide to the tyrosine-histidine internal standard.

CN1 Activity

CN1 activity was quantified by fluorometric determination of liberated histidine after carnosine addition [based on Teufel et al. (48)]. Briefly, the reaction was initiated by the addition of 10 mM carnosine (Flamma) to serum and stopped after a 10-min incubation at 37°C by the addition of 600 mM trichloroacetic acid (TCA). For controls, TCA was added before carnosine. After centrifugation (4,500 *g*, 15 min) supernatant was added to a mixture of *o*-phthalaldehyde (OPA; incomplete phthalaldehyde with 0.2% 2-mercaptoethanol) and 4 M sodium hydroxide, and fluorescence was determined after 40 min (excitation: 360 nm and emission: 465 nm).

Citrate Synthase Activity

Citrate synthase activity assay was based on the binding of coenzyme A (CoA-SH) to 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB), after initiation by oxaloacetic acid, and absorbance measured at 450 nm. In short, gastrocnemius muscle was homogenized in ice-cold buffer consisting of 250 mM sucrose, 2 mM Na-EDTA, and 5 mM Tris base (pH 7.4) and centrifuged for 10 min at 10,000 rpm. Protein content was measured with a Total Protein Kit (Merck, Micro-Lowry, Onishi & Barr Modification). Reagent solution (100 mM Tris base, 100 μM DTNB, and 0.05 mM acetyl CoA, pH 8, 250 μL) was added to the samples (5 μL, ~0.5 g/L protein) or standards (5 μL CoA-SH), after which the reaction was initiated by the addition of 5 μL of 25 mM oxaloacetic acid. The linear part of the enzymatic reaction was used to calculate, with a standard curve, the amount of CoA-SH that was formed.

Statistical Analysis

All data are expressed as means ± SD. For the acute study, one-way ANOVA was performed to evaluate the effect of the interventions on plasma and muscle carnosine and anserine. Significant results were further evaluated by means of independent *t* tests. For the chronic study, body weight and water intake were analyzed by means of repeated measures with time (2) as within-subject and group (4) as between-subjects factor. The evolution over time of serum CN1 activity of the hCN1 group was evaluated by means of a paired *t* test. Repeated-measure parameters were evaluated with time as within factor (3 or 4) and group (4) as between factor. In case of significant interaction effects, the test was repeated per two groups and an independent *t* test was performed per time point. Parameters measured at *week 24* were checked with one-way ANOVA with group (4) as between factor and post hoc analysis when necessary. Correlations were evaluated by means of Pearson correlations. Differences were considered statistically significant at $P < 0.05$.

RESULTS

Acute Study

Because CN1 hydrolyzes carnosine and anserine, we first determined whether hCN1 overexpression in *ob/ob* mice enhanced CN1 activity and affected circulating and skeletal muscle HCD levels. As expected, no CN1 activity was detected in the serum of WT or *ob/ob* mice, whereas CN1 hydrolyzing activity was significantly enhanced in *ob/ob* hCN1 mice (6.04 ± 0.94 $\mu mol \cdot mL^{-1} \cdot h^{-1}$; Table 1). This CN1 activity was higher compared with the CN1 activity of healthy subjects (mean: ~1.5 $\mu mol \cdot mL^{-1} \cdot h^{-1}$, unpublished data). Analysis of the plasma by LC-MS/MS showed that HCD levels were significantly decreased in *ob/ob* mice (0.28 ± 0.16 μM) compared with WT mice (0.65 ± 0.38 μM, $P < 0.05$). This decrease can be attributed to both carnosine (WT: 0.36 ± 0.32 μM vs. *ob/ob*: 0.11 ± 0.08 μM) and anserine (WT: 0.29 ± 0.15 μM vs. *ob/ob*: 0.16 ± 0.08 μM). As expected, overexpression of hCN1 in *ob/ob* mice resulted in a >100-fold decrease in circulating HCD (0.003 ± 0.007 μM, $P < 0.05$) compared with *ob/ob* mice. Acute running did not significantly affect circulating carnosine and anserine levels in *ob/ob* mice ($P > 0.1$; Fig. 1*A*). Circulating carnosine-propanal levels (carnosine bounded to the reactive carbonyl acrolein) were significantly higher in *ob/ob* mice (7.73 ± 2.10 nM) versus WT mice (5.03 ± 1.22 nM) and tended ($0.05 > P < 0.1$) to be higher in exercising *ob/ob* mice (8.83 ± 1.45 nM) compared with resting (6.63 ± 2.18 nM) obese control mice (data not shown).

In these young animals, carnosine and anserine gastrocnemius levels were not dependent on obesity (Fig. 1*B*). Furthermore, acute exercise or overexpression of hCN1 had

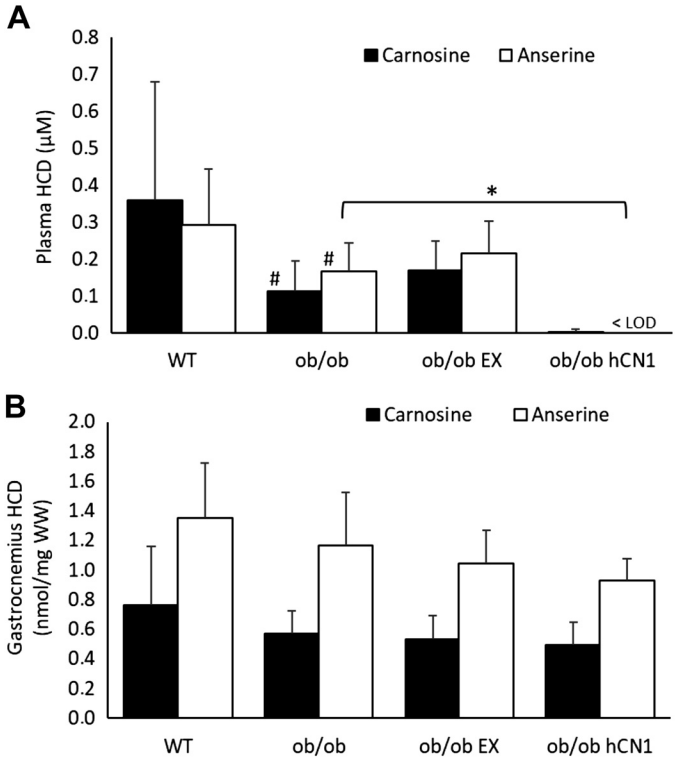

Fig. 1. Acute study: metabolism of histidine-containing dipeptides (HCD). *A* and *B*: plasma (*A*) and gastrocnemius (*B*) carnosine and anserine content in wild-type (WT) mice, *ob/ob* mice, *ob/ob* mice with acute exercise (EX), and *ob/ob* human carnosinase-1 (hCN1) transgenic mice. LOD, limit of detection; WW, wet weight. Values are means ± SD. #$P \leq 0.05$, *ob/ob* vs. WT mice; *$P \leq 0.05$ vs. *ob/ob* mice.

no effect on skeletal muscle HCD levels in these BTBR *ob/ob* mice (Fig. 1*B*).

Chronic Study

To determine the effect of exercise training, *ob/ob* mice were exercised at an aerobic intensity for 20 wk, running 5 days/wk for 1 h at 10 m/min. The body weight gain after 20 wk was more than twofold higher in *ob/ob* versus WT mice. Exercise training or hCN1 overexpression did not influence the evolution of body weight over time (Table 2). Serum carnosinase activity (*week 24*) was markedly higher in hCN1 transgenic mice (145.8 ± 61.5 $\mu mol \cdot mL^{-1} \cdot h^{-1}$) versus nontransgenic mice (0.54 ± 0.75 $\mu mol \cdot mL^{-1} \cdot h^{-1}$) and increased approximately fourfold from 4 to 24 wk in transgenic mice (Table 2).

Development of diabetes in BTBR ob/ob mice. To determine the effect of exercise training and CN1 overexpression on the development of diabetes, we measured fasting serum glucose levels. The results showed that serum glucose levels were three- to fourfold higher in *ob/ob* mice at *weeks 14* and *24* compared with WT mice (*week 14*: 674.02 ± 297.54 vs. 170.76 ± 40.49 mg/dL and *week 24*: 724.90 ± 502.58 vs. 265.67 ± 66.75 mg/dL; Fig. 2*A*) but were not affected by exercise training or hCN1 overexpression.

Plasma fructosamine content, a long-term biomarker of glucose overload, was significantly higher at *weeks 14* (+57%) and *24* (+48%) in *ob/ob* versus WT mice. Fructosamine levels returned to baseline levels from *week 14* to *week 24* in *ob/ob* mice ($P < 0.05$; Fig. 2*B*). The increase in fructosamine levels was independent of exercise training or hCN1 overexpression (Fig. 2*B*). Total serum protein levels were higher in *ob/ob* mice (71.58 ± 21.92 g/L) compared with WT mice at *week 24* (61.90 ± 8.48 g/L; data not shown). Therefore, it is suggested that reduced circulating protein levels were not the cause of the decline in serum fructosamine levels between *weeks 14* and *24*.

In contrast to the glucose-based markers, serum triglycerides and cholesterol levels were significantly affected by hCN1 overexpression. Serum triglycerides evolved differentially over time in *ob/ob* versus *ob/ob* hCN1 mice, with a trend to significant higher values at *week 14* (+57%) but not at *week 24* (Fig. 2*C*). Similar to triglycerides, cholesterol levels were significantly increased (+109%) at 14 wk of age in *ob/ob* hCN1 mice compared with *ob/ob* mice but not at 24 wk. No effect of exercise training on the evolution over time of serum triglycerides and cholesterol was observed (Fig. 2, *C* and *D*).

The effect of the interventions (hCN1 and exercise training) on the different diabetes-related parameters were not dependent on sex (data not shown) except for the development of hyperglycemia. The development of hyperglycemia was more pronounced in male (*week 24*: 1,001.41 ± 309.48 mg/dL) versus female (*week 24*: 462.55 ± 253.30 mg/dL, $P < 0.05$) obese mice. Furthermore, male *ob/ob* EX-T and *ob/ob* hCN1 mice had significantly lower serum glucose levels (−20% and −18%, respectively) compared with male *ob/ob* mice at *week 14*, whereas no differences were found between different obese groups in female mice (data not shown).

Development of DN in BTBR ob/ob mice. The development of DN was evaluated by measuring the urinary albumin-to-

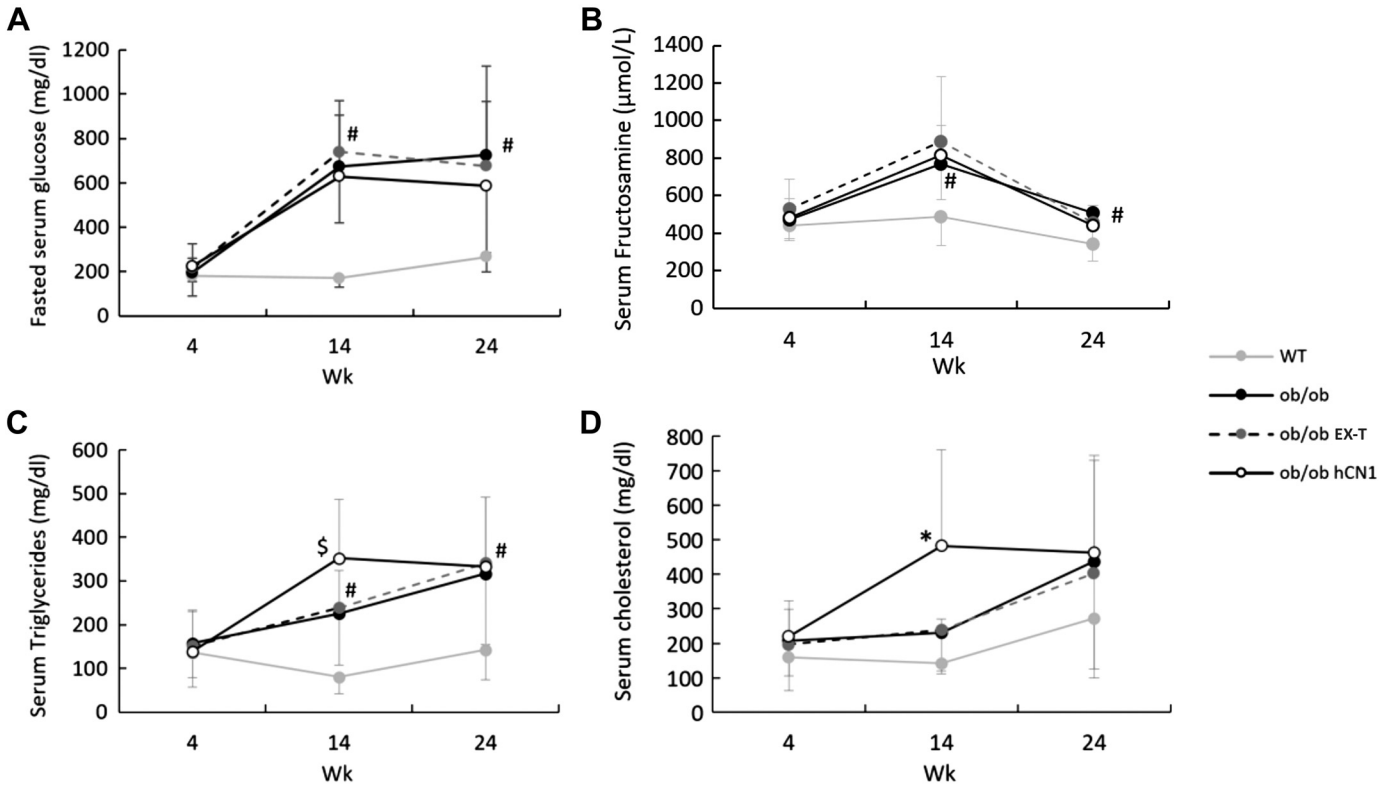

Fig. 2. Development of diabetes in BTBR *ob/ob* mice. *A*−*D*: fasted serum glucose (*A*), fructosamine (*B*), triglycerides (*C*), and cholesterol (*D*) were quantified at 4, 14, and 24 wk of age in wild-type (WT) mice, *ob/ob* mice, *ob/ob* mice with exercise training (EX-T), and *ob/ob* human carnosinase-1 (hCN1) transgenic mice. Values are means ± SD. #$P \leq 0.05$, *ob/ob* vs. WT mice; *$P \leq 0.05$ vs. *ob/ob* mice; \$$P > 0.05$ and < 0.1 vs. *ob/ob* mice at the same time point.

creatinine ratio. At the end of the intervention, the albumin-to-creatinine ratio of *ob/ob* mice was 1,194.62 ± 963.86 μg/mg, i.e., ~25-fold higher in *ob/ob* versus WT mice (47.86 ± 21.87 μg/mg), which corresponds with a stage of advanced DN. The albumin-to-creatinine ratio was not affected by the exercise intervention, but *ob/ob* hCN1 mice (2,182.24 ± 1,667.66 μg/mg) had significantly higher levels (+82%) compared with *ob/ob* mice (Fig. 3*B*).

Renal lesions were evaluated by scoring the degree of mesangial matrix expansion. This expansion was 3.8-fold more pronounced in *ob/ob* versus WT mice and was further aggravated by hCN1 overexpression (+22%, $P < 0.05$; Fig. 3*C*). Representative images of a WT, an *ob/ob*, and a *ob/ob* hCN1 mouse are shown in Fig. 3*A*. The number of podocytes was not affected by obesity or by hCN1 overexpression ($P > 0.1$; Fig. 3*D*). Also, weak to mild interstitial fibrosis was seen both in the *ob/ob* mouse and *ob/ob* hCN1 mouse.

Glomerular hypertrophy was evaluated by measuring the surface areas (μm^2) of the glomerular tuft, Bowman's space, and whole glomerulus (Fig. 3, *E–G*). The three different areas were expanded in *ob/ob* versus WT mice ($P < 0.05$), and overexpression of hCN1 further aggravated the hypertrophy in the glomerular tuft ($P < 0.05$; Fig. 3*E*) and whole glomerulus ($P < 0.05$; Fig. 3*G*) but not in Bowman's space ($P > 0.1$; Fig. 3*F*).

None of the above-mentioned DN-related parameters was affected by the chronic aerobic exercise training protocol (Fig. 3).

Metabolism of HCD and acrolein adducts. To further elucidate the effect of hCN1 overexpression, HCD levels were quantified in the kidney, skeletal muscle, and urine in 24-wk-old mice. Carnosine and anserine were remarkably lower in both the kidney (Fig. 4*A*) and muscle (Fig. 4*B*) in *ob/ob* versus WT mice ($P < 0.05$). Kidney (−95%, $P < 0.05$) and muscle (−20%, $P < 0.1$) anserine were further declined in hCN1 transgenic *ob/ob* versus nontransgenic *ob/ob* mice (Fig. 4, *A* and *B*).

The evolution of urinary excretion of HCD was not dependent on obesity or the different interventions ($P > 0.1$; Fig. 4*C*). Urinary carnosine-propanal adducts were not detectable throughout the intervention period. In contrast, urinary carnosine-propanol levels were above the limit of detection and similar between WT and *ob/ob* mice at 4 and 24 wk of age and slightly higher at 14 wk of age in *ob/ob* versus WT mice. In *ob/ob* hCN1 mice, urinary carnosine-propanol levels were not different at 4 and 14 wk from *ob/ob* mice but, however, were 20-fold lower in *ob/ob* hCN1 at 24 wk of age ($P < 0.05$; Fig. 4*D*).

Kidney carnosine-propanol levels were not detectable in any of the groups. In contrast, kidney carnosine-propanal levels were detectable (overall mean ± SD: 3.09 ± 0.79 pmol/mg

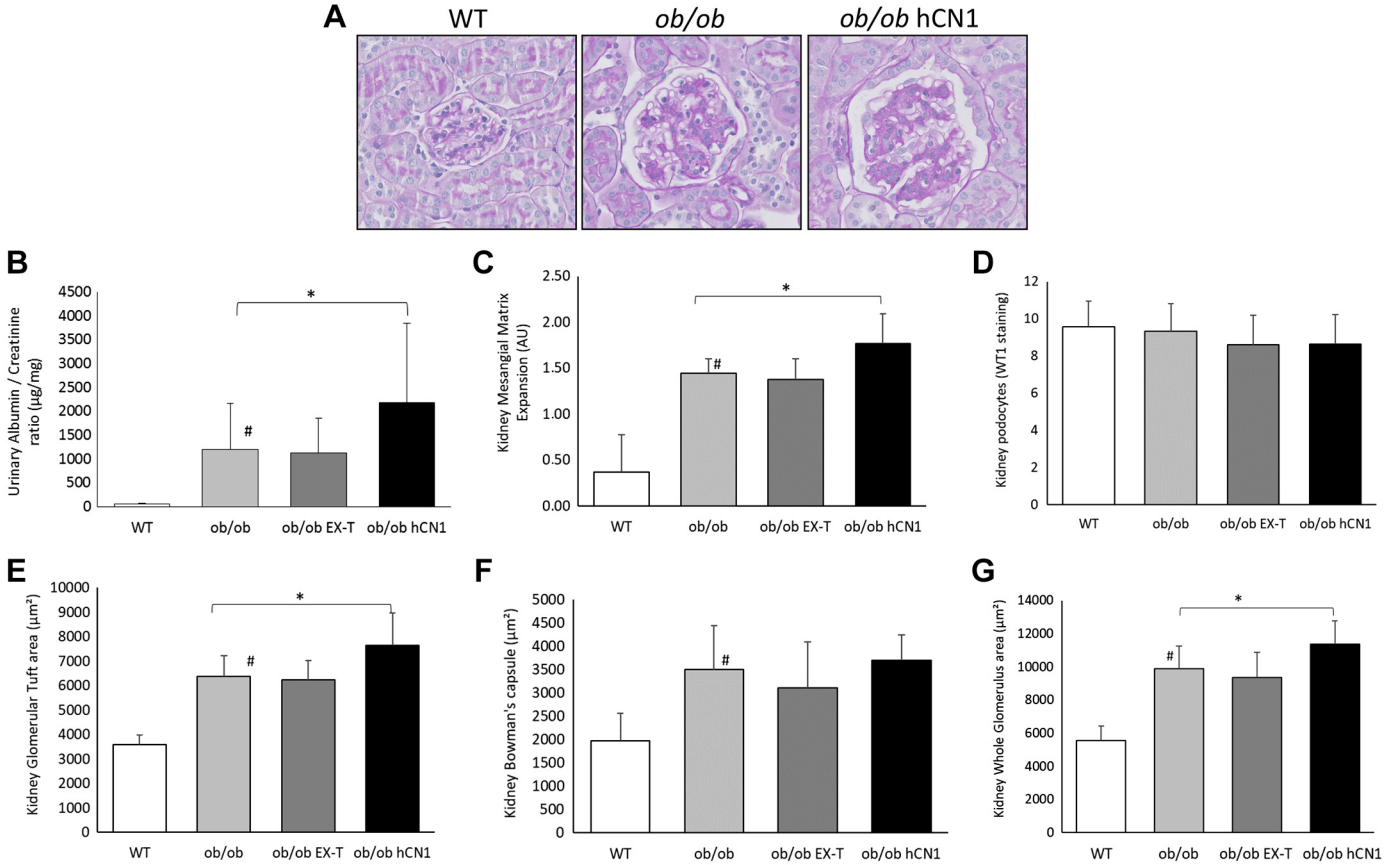

Fig. 3. Development of diabetic nephropathy in BTBR *ob/ob* mice. *A*: representative images of glomeruli (periodic acid-Schiff-stained sections) of (from *left* to *right*) wild-type (WT) mice, *ob/ob* mice, and *ob/ob* human carnosinase-1 (hCN1) transgenic mice. *B–G*: urinary albumin-to-creatinine ratio (*B*), degree of mesangial matrix expansion (*C*), number of podocytes (*D*), and surface area of the glomerular tuft (*E*), Bowman's space (*F*), and whole glomerular area (*G*) at *week 24* for WT mice, *ob/ob* mice, *ob/ob* mice with exercise training (EX-T), and *ob/ob* hCN1 mice. AU, arbitrary units; WT1, Wilms' tumor-1 protein. Values are means ± SD. #$P \leq 0.05$, *ob/ob* vs. WT mice; *$P \leq 0.05$ vs. *ob/ob* mice.

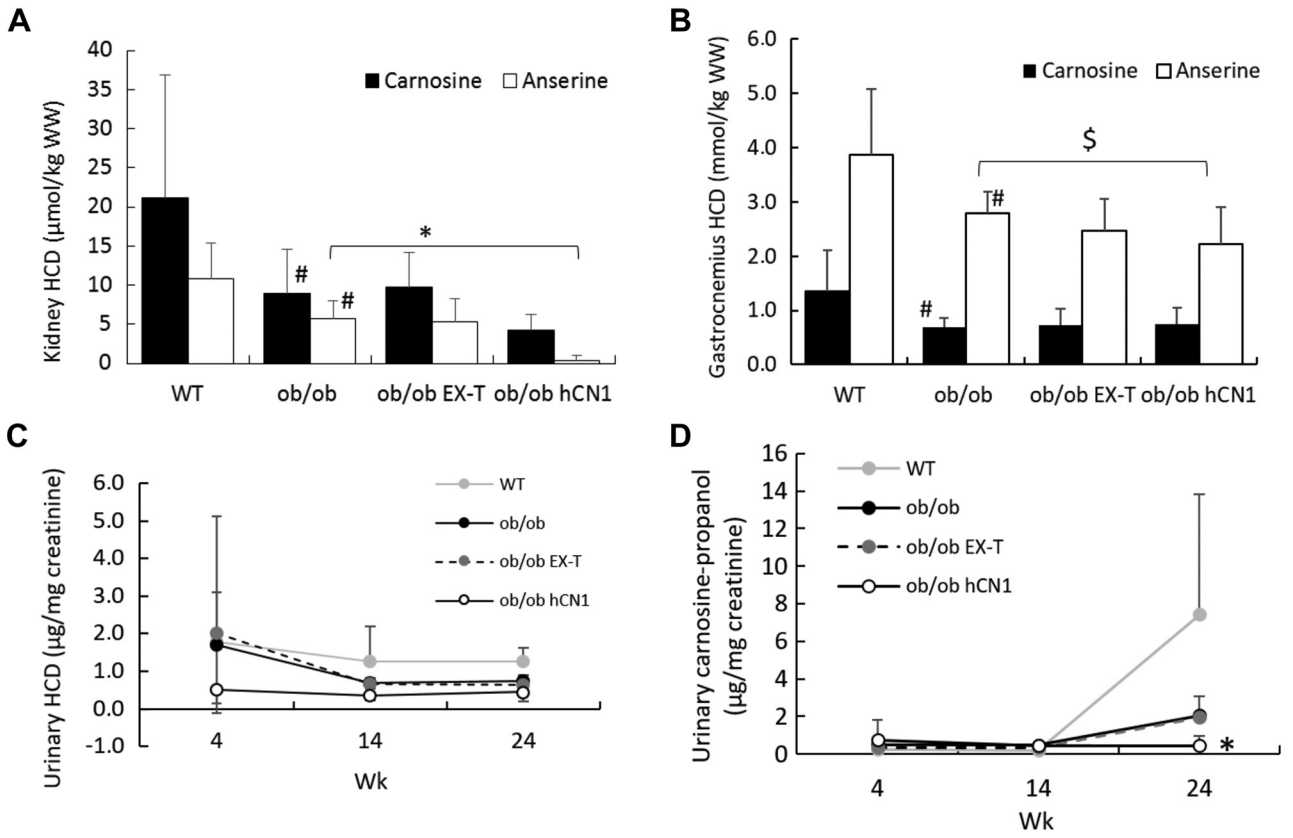

Fig. 4. Metabolism of histidine-containing dipeptides (HCD) in BTBR *ob/ob* mice. *A–C*: kidney (*A*; *week 24*), gastrocnemius (*B*; *week 24*), and urinary (*C*; *weeks 4*, *14*, and *24*) HCD content in wild-type (WT) mice, *ob/ob* mice, *ob/ob* mice with exercise training (EX-T), and *ob/ob* human carnosinase-1 (hCN1) transgenic mice. Values are means ± SD. #$P \leq 0.05$, *ob/ob* vs. WT mice; *$P \leq 0.05$ vs. *ob/ob* mice; \$$P > 0.05$ and < 0.1 vs. *ob/ob* mice. *D*: urinary carnosine-propanol adducts (*weeks 4*, *14*, and *24*) in WT, *ob/ob*, *ob/ob* EX-T, and *ob/ob* hCN1 mice. WW, wet weight. Values are means ± SD. *$P \leq 0.05$ vs. *ob/ob* mice at the same time point.

wet wt) but not affected by obesity, hCN1 overexpression, or the chronic exercise intervention (data not shown).

Correlations. The amount of HCD in the muscle ($r = -0.38$ and $r = -0.38$, $P < 0.05$) and especially in the kidney ($r = -0.76$ and $r = -0.69$, $P < 0.05$) was negatively correlated to mesangial matrix expansion (Fig. 5*A*) and glomerular hypertrophy (Fig. 5*B*), respectively.

Carboxymethyllysine. The amount of protein-bound kidney CML was quantified as a marker of glycation damage in this mouse model. At the age of 24 wk, kidney CML levels (overall: 203.77 ± 39.33 μmol/mol lysine) were independent of obesity, exercise training, and hCN1 overexpression (data not shown).

Citrate synthase activity. The activity of the citrate synthase enzyme was measured in gastrocnemius muscles to elucidate the effect of chronic exercise training on the aerobic capacity of BTBR *ob/ob* mice. After 20 wk of aerobic exercise training, citrate synthase activity was approximately twofold higher in *ob/ob* EX-T mice (132.9 ± 61.1 $\mu mol \cdot min^{-1} \cdot g\ protein^{-1}$)

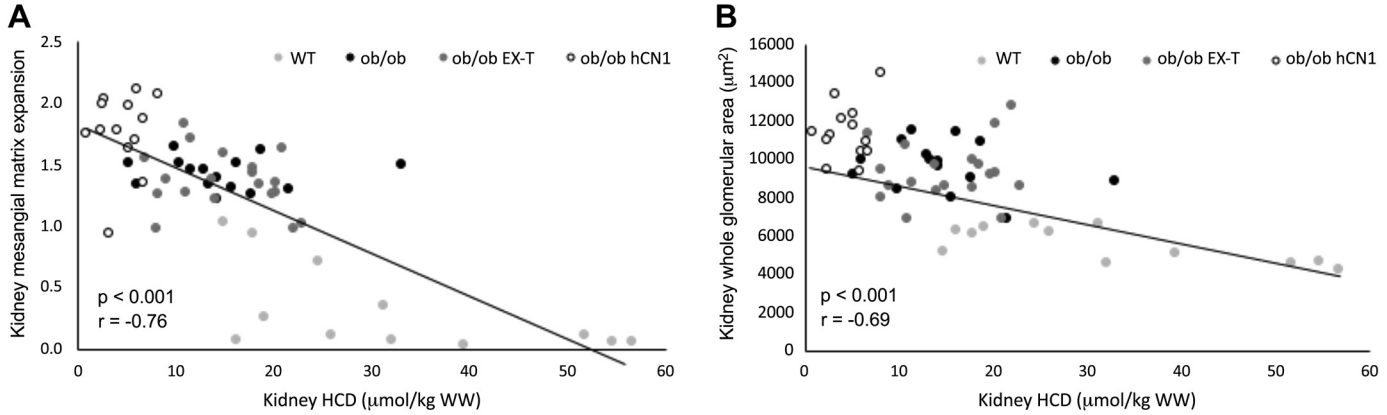

Fig. 5. Correlations. *A* and *B*: correlations between kidney histidine-containing dipeptide (HCD) content and mesangial matrix expansion (*A*) and whole glomerular area (*B*) in wild-type (WT) mice, *ob/ob* mice, *ob/ob* mice with exercise training (EX-T), and *ob/ob* human carnosinase-1 (hCN1) transgenic mice. WW, wet weight.

compared with control *ob/ob* mice (66.6 ± 50.0 μmol·min^{-1}·g protein^{-1}; $P < 0.05$) and not affected by obesity (WT: 45.2 ± 46.6 μmol·min^{-1}·g protein^{-1}; $P > 0.1$) or hCN1 overexpression (*ob/ob* hCN1: 75.7 ± 56.6 μmol·min^{-1}·g protein^{-1}; $P > 0.1$).

DISCUSSION

The main finding of the present study is that changes in renal HCD availability are accompanied with variations in the development of DN in BTBR *ob/ob* mice. Kidney HCD content is already compromised in the diabetic state, and DN is significantly accelerated and aggravated when it is further reduced by hCN1 overexpression. The underlying mechanism seems lipid rather than sugar related, since biochemical markers indicate that this does not coincide with hyperglycemic state (Fig. 2, *A* and *B*) or glycation damage but rather with an attenuation of hyperlipidemic state and a diminished capacity to quench, eliminate, and protect against lipid-derived reactive carbonyl species, such as acrolein. Interestingly, overexpression of hCN1 had opposite effects on the development of DN (albumin-to-creatinine ratio and glomerular hypertrophy) as carnosine supplementation, which has been investigated in the same mouse model by Albrecht et al. (1). The second hypothesis, i.e., that exercise training attenuates the development of DN through the release of HCD from muscular depots, was not supported by our findings. There was no substantial elevation of circulating HCD during acute exercise in this animal model, and there was no protective effect of exercise training on DN.

The results of the present study suggest that the amount of body burden of HCD determines the potential for scavenging reactive carbonyl species such as acrolein and thereby plays a (key) role in the development of DN. Diabetes in itself resulted in strongly reduced gastrocnemius (−48% and −28%, respectively; Fig. 4*B*), kidney (−58% and −47%, respectively; Fig. 4*A*), and plasma (−69% and −44%, respectively; Fig. 1*A*) carnosine and anserine levels. This is in accordance with reduced HCD levels in the kidney [from −90% to −98% (34, 35, 38)], retina [−31% (36)], liver [−78% (28)], and brain [−64% (5)] in different type 1 and type 2 diabetic models but not with increased levels in skeletal muscle of patients with type 2 diabetes [+39% (46)]. Different reports have suggested that carnosine can act as a sacrificial dipeptide by binding and eliminating deleterious agents such as reactive carbonyl species. A disproportional accumulation of reactive carbonyl species, which takes place, for example, during diabetes, can therefore result in a shortage of tissue carnosine or anserine. Compared with the reducing effect of diabetes on carnosine and anserine levels, hCN1 overexpression resulted not only in lower circulating HCD levels (carnosine: −97% and anserine: more than −99%) but in strongly reduced whole body carnosine and anserine stores. Diminished levels of carnosine and anserine were observed in kidney (*week 24*: −53% and −95%, respectively) and gastrocnemius tissue (*week 24*: anserine: −20%) in hCN1 transgenic versus nontransgenic *ob/ob* mice (Fig. 4, *A* and *B*).

Importantly, there was a positive correlation between the amount of carnosine ($r = 0.64$, $P < 0.05$) and anserine ($r = 0.33$, $P < 0.05$) in the kidney and urinary carnosine-acrolein adducts. Kidney HCD were further also negatively correlated with different markers of DN (Fig. 5). Unfortunately, it is not known whether kidney HCD levels were enhanced after carnosine supplementation in BTBR *ob/ob* mice in the study of Albrecht et al. (1) and could therefore be the main underlying mechanism for an attenuated development of DN in carnosine-supplemented mice. However, as *1*) different rodent studies have shown that kidney HCD are enhanced upon oral supplementation of carnosine (34), β-alanine (unpublished data), D-carnosine (2), and carnosinol (3) and as *2*) the PEPT2 transporter is highly abundant in renal cells (22, 26), it is highly likely that kidney HCD levels were enhanced in carnosine-supplemented BTBR *ob/ob* mice. Enhancing the dietary intake of HCD, which potentially maintains kidney carnosine and anserine during periods of increased exposure of reactive carbonyl species, should therefore be further explored as a promising therapeutic strategy to offset DN. Unfortunately, our study is limited by the lack of a direct comparison between the effect of hCN1 overexpression and carnosine supplementation on both kidney HCD levels and DN.

The observed effect of hCN1 overexpression on DN in mice confirms the genetic link between polymorphisms of the *CNDP1* gene, the gene encoding the CN1 enzyme, and the prevalence of DN in patients with diabetes (21). The dramatic decline in kidney carnosine and anserine levels in hCN1 transgenic mice together with the strong correlation between kidney HCD levels and the degree of DN (Fig. 5) brings us to the hypothesis that the underlying protective mechanism of low CN1 activity (21) could not only be higher circulating carnosine levels but also higher renal carnosine and anserine levels. Indeed, in this study, serum CN1 activity was negatively correlated with renal carnosine ($r = -0.33$, $P < 0.05$) and especially anserine ($r = -0.70$, $P < 0.05$) levels. These results can therefore be a start to further elucidating the exact role of renal HCD in (diabetic) kidney diseases. The development of a mouse model with podocyte-specific CN1 overexpression, which would probably only result in decline in the kidney and not full body HCD levels, would be able to further unravel the contribution of renal HCD to the development of DN. Future research will have to elucidate whether serum CN1 activity and the risk for developing DN are also related to renal HCD levels in humans.

The present study is the first to document that overexpression of the hCN1 enzyme results in a faster development of hyperlipidemia in diabetic mice (as reflected by higher fasting triglycerides and cholesterol at *week 14*; Fig. 2, *C* and *D*). These results are in line with an improved lipidemic profile in different diabetes or metabolic syndrome rodent models after long-term carnosine supplementation (2, 4, 8, 25). The levels of serum triglycerides were furthermore also significantly reduced in patients with type 2 diabetes who received oral supplements of 1 g carnosine/day for 12 wk (18). Although evidence is accumulating for an antihyperlipidemic effect of carnosine, no underlying mechanism has been reported so far. Novel approaches, such as lipidomics and proteomics, are needed to further explain this lipid regulation mechanism. Anyhow, it is likely that the favorable effects of carnosine (and adverse effects of CN1) on dyslipidemia and hypercholesteremia will contribute to a lower risk of developing diabetes-induced microvascular and macrovascular complications.

Different therapeutic approaches are currently being developed to encounter the negative effects of CN1 upon carnosine supplementation. The administration of carnosinol, a chemi-

cally synthetized carnosine analog that is well absorbed in the intestinal wall but not degraded by CN1, strongly and dose dependently counteracted several consequences of metabolic syndrome [inflammation, dyslipidemia, insulin resistance, and steatohepatitis (3)]. Anserine, a naturally occurring carnosine analog that is also less vulnerable to degradation by CN1 (9), efficiently reduces fasting plasma glucose levels, proteinuria, and vascular permeability in *db/db* mice (33). In parallel to the development of carnosinase-resistant carnosine analogs, CN1 blockers are also currently under investigation (37).

Surprisingly, 20 wk of chronic aerobic exercise training did not retard the progression of diabetes or DN in the BTBR *ob/ob* mouse model. This is in contrast with previous reports on treadmill-trained *db/db* mice (14, 45) and different high-fat (7, 40), heminephrectomized (49), and streptozotocin-induced (23) diabetic rat models. All studies reported a slower development of early DN (mostly based on the albumin-to-creatinine ratio and mesangial matrix expansion) after 5–12 wk of aerobic treadmill running. The lack of an exercise-induced amelioration of the development of DN is especially surprising as the chronic exercise training program enhanced citrate synthase enzyme activity, a marker of mitochondrial content (24). Our intervention doubled citrate synthase activity, whereas others have reported increases of ~30–50% following chronic exercise intervention in obese mice models (14, 15, 20). It can be concluded that a doubling in citrate synthase enzyme activity did not ameliorate the disturbance in glucose and lipid metabolism, or the development of DN, in our BTBR *ob/ob* model. A possible reason for the absence of a protective effect of chronic exercise in our study (Fig. 2, *A* and *B*) could be the very high glucose levels, possibly leading to severe ketoacidosis, lower exercise tolerance, or dehydration during exercise training. This is in accordance with the absence of an antihyperglycemic effect of exercise in *db/db* mice with very high circulating glucose levels (14, 27) compared with *db/db* mice with lower glucose levels (45) and in accordance with absence of an exercise-induced improvement in glycemic control in subjects with high (>13 mmol/L) fasting glucose levels (44).

In contrast to previous reports (11, 30), circulating carnosine and anserine were not significantly enhanced after an acute bout of exercise in mice lacking the serum CN1 enzyme. The degree of release could be dependent on different exercise modalities, such as intensity and duration, but can also be dependent on other factors, such as perceived stress (treadmill vs. running wheel) or mouse strain. The lack of exercise-induced release of carnosine and anserine may also partially explain the absence of a protective effect of the chronic exercise program on diabetes and DN.

In conclusion, we have shown, for the first time, that overexpression of the hCN1 enzyme in BTBR *ob/ob* mice aggravates the development of advanced glomerular lesions. However, a chronic aerobic exercise training program did not affect diabetes or DN. Our data further suggest that the amount of renal HCD is closely linked to the severity of DN. Developing a supplementation regimen that efficiently enhances renal carnosine and anserine levels in humans should be explored as potential therapeutic target to reduce the risk of developing DN.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

I.E., M. Hanssens, and W.D. conceived and designed research; I.E., J.H., M. Hanssens, J.S., K.B., T.A., S.Z., T.V.d.S., K.V., D.H., M. Howsam, B.Y., S.P.B., H.J.B., and W.D. performed experiments; I.E., J.H., M. Hanssens, J.S., K.B., T.A., S.Z., T.V.d.S., K.V., D.H., M. Howsam, F.J.T., B.Y., S.P.B., H.J.B., and W.D. analyzed data; I.E., J.H., M. Hanssens, J.S., F.J.T., H.J.B., and W.D. interpreted results of experiments; I.E. and M. Hanssens prepared figures; I.E. and W.D. drafted manuscript; I.E., J.H., M. Hanssens, J.S., K.B., T.A., S.Z., T.V.d.S., K.V., D.H., M. Howsam, F.J.T., B.Y., S.P.B., H.J.B., and W.D. edited and revised manuscript; I.E., J.H., M. Hanssens, J.S., K.B., T.A., S.Z., T.V.d.S., K.V., D.H., M. Howsam, F.J.T., B.Y., S.P.B., H.J.B., and W.D. approved final version of manuscript.

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