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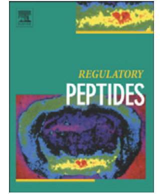

Carnosine treatment in combination with ACE inhibition in diabetic rats

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ABSTRACT

In humans, we reported an association of a certain allele of carnosinase gene with reduced carnosinase activity and absence of nephropathy in diabetic patients. CN1 degrades histidine dipeptides such as carnosine and anserine. Further, we and others showed that treatment with carnosine improves renal function and wound healing in diabetic mice and rats. We now investigated the effects of carnosine treatment alone and in combination with ACE inhibition, a clinically established nephroprotective drug in diabetic nephropathy.

Male Sprague–Dawley rats were injected i.v. with streptozotocin (STZ) to induce diabetes. After 4 weeks, rats were unilaterally nephrectomized and randomized for 24 weeks of treatment with carnosine, lisinopril or both. Renal CN1 protein concentrations were increased under diabetic conditions which correlated with decreased anserine levels. Carnosine treatment normalized CN1 abundance and reduced glucosuria, blood concentrations of glycosylated hemoglobin (HbA1c), carboxyl-methyl lysine (CML), N-acetylglucosamine (GlcNac; all $p < 0.05$ vs. non-treated STZ rats), reduced cataract formation ($p < 0.05$) and urinary albumin excretion ($p < 0.05$), preserved podocyte number ($p < 0.05$) and normalized the increased renal tissue CN1 protein concentration. Treatment with lisinopril had no effect on HbA1C, glucosuria, cataract formation and CN1 concentration, but reduced albumin excretion rate more effectively than carnosine treatment ($p < 0.05$). Treatment with both carnosine and lisinopril combined the effects of single treatment, albeit without additive effect on podocyte number or albuminuria.

Increased CN1 amount resulted in decreased anserine levels in the kidney. Both carnosine and lisinopril exert distinct beneficial effects in a standard model of diabetic nephropathy. Both drugs administered together combine the respective effects of single treatment, albeit without exerting additive nephroprotection.
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1. Introduction

Diabetic nephropathy (DN) is the most common cause of end stage renal disease in the Western world. Despite increasing knowledge on the pathogenesis of DN and potential therapeutic interventions, progression to end stage renal disease still occurs in a substantial number of diabetic patients [1,2]. At present, pharmacological nephroprotection is largely limited to tight glucose and blood pressure control and antiproteinuric intervention using antagonists of the renin–angiotensin system [3]. ACE inhibitors are known to reduce proteinuria and have been the first-line agents in the management of diabetic nephropathy [4,5] during the last years.

A genome-wide linkage study revealed strong evidence for linkage of DN to the CNDP1 gene, encoding serum carnosinase (CN1) [6]. (CTG)5 homozygous individuals display low CN1 activity in serum [6] and have a reduced susceptibility to DN. Carnosine, the natural substrate for CN1 [7–9] has several protective functions, such as antioxidant activity [10], scavenging of reactive oxygen species [11] and glycation inhibition [12]. In both in vitro and in vivo models high carnosine levels protect against diabetic complications by improving glucose metabolism, reduction of carbonyl stress and minimizing formation of advanced glycation end products (AGEs) [13–15]. An intriguing treatment approach in patients with diabetic nephropathy should be the combination of the direct nephroprotective action of ACE inhibitors and the carnosine mediated nephroprotection which is based on a variety of protective functions, such as scavenging of reactive oxygen species [11], inhibition of angiotensin converting enzyme [12], of protein glycation [12], of cellular senescence [16] and of matrix protein synthesis [6]. To assess the potential of ACE inhibition and pharmacological doses of carnosine in DN, we treated diabetic rats for 24 weeks with lisinopril, carnosine and a combination of both. Diabetes mellitus was

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induced by streptozotocin (STZ) which results in a well-defined nephropathy. The rats were moreover uninephrectomized in order to reduce the time until development of overt DN [17,18]. These animals underwent detailed analysis of glucose metabolism, carbonyl stress, renal morphology and renal function.

2. Material and methods

2.1. Animal and induction of experimental diabetes

Diabetes was induced in male Sprague–Dawley rats weighing 350 to 420 g by a single intravenous injection of 50 mg/kg bodyweight streptozotocin (STZ; Sigma-Aldrich Chemical, Deisenhofen, Germany). A second STZ injection was given in case blood glucose levels were below 400 mg/dl. Blood glucose levels were monitored regularly, i.e. daily in the first week after STZ-injection and weekly thereafter. Insulin glargine (Lantus, Sanofi-Aventis, Frankfurt, Germany) was injected s.c. in individually adjusted doses to maintain a blood glucose level of 400–600 mg/dl to allow for stable diabetes mellitus. Injection of insulin was daily in the first week after STZ-injection and thrice weekly thereafter. Rats were housed in a climate-controlled animal house with a 12 h light cycle and free access to standard diet and drug supplemented tap water.

2.2. Experimental design

Four weeks following STZ injection unilateral nephrectomy was performed and 52 rats were randomized to four groups, L-carnosine treatment (C, 1 g/kg bodyweight, Flamma S.p.A, Bergamo, Italy), lisinopril treatment (L, 2.5 mg/kg bodyweight, AstraZeneca GmbH, Wedel, Germany), the respective combined treatment (L + C) and untreated rats (STZ). Ten additional rats without preceding STZ injection served as age matched untreated controls (C). Treatment was administered via drinking water for 24 weeks. Blood glucose was controlled by thrice weekly insulin injections in all STZ groups. Drinking volumes were recorded daily, and bodyweight assessed twice weekly and drug dosing adjusted accordingly. Body weight and blood glucose levels and insulin doses administered did not differ between groups. Animals were placed in metabolic cages on week 28 post-STZ injection for 24 h. Following sacrifice, organs were removed immediately and either stored in liquid nitrogen or 4% w/v paraformaldehyde, respectively. Blood samples were collected for the determination of glycated hemoglobin concentration by affinity chromatography (MicromatII™; Bio-Rad Laboratories GmbH, Munich, Germany). Serum creatinine was measured by immunoassay (Dimension; Dade Behring, Germany), urinary albumin by ELISA as previously published [19] and serum carboxyl-methyl lysine was measured using a commercial ELISA (CML-ELISA Kit, Cell Biolabs, BIOCAT GmbH, Germany). All animal procedures were approved by the Regierungspräsidium Karlsruhe AZ 35-9185.81/G-5306.

2.3. Quantification of cataract formation

The formation of cataract was assessed semiquantitatively by macroscopic evaluation in a blinded setting using a score of 0–4, with 0 representing no cataract formation, 1 incipient cataract, 2 incipient cataract of both eyes or moderate cataract in one eye, 3 moderate cataract of both eyes and 4 with both eyes being completely opaque.

2.4. Histological analysis

Paraffin embedding of kidneys was performed using routine procedures and sections were stained with hematoxylin-eosin. Tissue sections were evaluated by two different, blinded individuals scoring a minimum of 20 microscopic fields per kidney (H. Köppel and R Waldherr). A semiquantitative score was used for grading of glomerular size, mesangial matrix expansion, interstitial fibrosis and tubular atrophy. The histologic grading scale ranged was from 0 to 3 (0 = not present, 1 = mild alteration, 2 = moderate alteration, and 3 = severe alteration). For determination of podocyte number, sections were stained for markers of podocytes (WT-1) and endothelial cells (CD31) according to standard protocols. Renal carnosinase abundance was assessed by immunohistochemistry, using an anti-CN1 (RYSK 173) monoclonal antibody (generated by our group; [9]). Staining intensity was classified as minute (=1), moderate (=2) and high (=3), respectively.

2.5. N-Acetylglucosamine immunoblotting

Shock frozen kidneys were homogenized in lysis buffer [150 mM NaCl, 10 mM Tris–HCl, 5 mM ethylenediaminetetraacetic acid (EDTA), 1% Triton X-100, 0 · 5% Na-deoxycholate, 1 μM dithiothreitol (DTT), 1 μg/ml aprotinin, 1 mM phenylmethylsulphonyl fluoride (PMSF)] using a Polytron homogenizer (IKA Labortechnik/Fischer Scientific, Schwerte, Germany) and incubated for 5 min on ice. Lysates were centrifuged (15 min at 16 000 *g*), protein concentrations in the supernatants measured using Coomassie Reagent (Pierce, Rockford, IL, USA). Proteins were separated on a 10% w/v sodium dodecyl sulfate–polyacrylamide gel by electrophoresis (SDS-PAGE) and semidry blotted onto a polyvinyl difluoride (PVDF) membrane (Roche Diagnostics, Mannheim, Germany). The membrane was incubated overnight in Tris-buffered saline (TBS) (10 mM Tris–HCl, pH 8 · 0, 150 mM NaCl) containing 5% w/v milk powder. Thereafter, the blots were incubated for 1 h with specific primary antibody for N-Acetylglucosamine-modified proteins (GlucNAc; Abcamplc, Cambridge, United Kingdom), followed by incubation with appropriate horseradish peroxidase (HRP) secondary antibody. Proteins were visualized by enhanced chemoluminescence technology according to the manufacturer's instructions (Pierce). To confirm equal protein loading, membranes were stripped with 62 · 5 mM Tris–HCl, 2% w/v SDS and 100 mM β-mercaptoethanol and incubated with antibodies against GAPDH (Abcamplc, Cambridge, United Kingdom). Intensity of specific bands was measured by chemiluminescence using the ImageJ 1.36b software and means of the absolute values of each group were expressed relative to WT.

2.6. Carnosine and anserine concentrations

Kidneys were removed and immediately homogenized in cold buffer containing 20 mM HEPES, 1 mM ethylene glycol-tetra-acetic acid (EGTA), 210 mM mannitol and 70 mM sucrose per gram tissue, pH 7.2. The homogenate was centrifuged at 1500 ×*g* for 5 min at 4 °C. Carnosine and anserine levels were measured in the supernatant by high-performance liquid chromatography as previously described [20]. Briefly, the samples were diluted with sulfosalicylic acid to precipitate proteins. After derivatization with carbazole-9-carbonyl chloride (CFC), the samples underwent liquid chromatography and quantification by fluorescence. All samples were measured twice, and one sample was spiked with the standard to identify anserine.

2.7. Statistical analyses

All values are shown as mean ± SD if not indicated otherwise. For survival, Kaplan–Meier survival estimates were calculated. Differences between treatment groups were analyzed by one-way ANOVA followed by t-test and Mann–Whitney in case of non-Gaussian distribution. Values of $p < 0.05$ were considered to be significant.

3. Results

STZ treatment increased glucosuria, glycosylated hemoglobin (HbA1c), carboxy-methyl lysine (CML) and N-Acetylglucosamine (GlcNAc) blood levels. Significant cataract developed over the study period, kidney morphology and function declined (Table 1), and renal

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