Carnosine Prevents Apoptosis of Glomerular Cells and Podocyte Loss in STZ Diabetic Rats

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Key Words
Diabetic nephropathy • Carnosine • Apoptosis • Podocytes

Abstract
Background/Aims: We identified carnosinase-1 (CN-1) as risk-factor for diabetic nephropathy (DN). Carnosine, the substrate for CN-1, supposedly is a protective factor regarding diabetic complications. In this study, we hypothesized that carnosine administration to diabetic rats might protect the kidneys from glomerular apoptosis and podocyte loss. Methods: We examined the effect of oral L-carnosine administration (1g/kg BW per day) on apoptosis, podocyte loss, oxidative stress, AGEs and hexosamine pathway in kidneys of streptozotocin-induced diabetic Wistar rats after 3 months of diabetes and treatment. Results: Hyperglycemia significantly reduced endogenous kidney carnosine levels. In parallel, podocyte numbers significantly decreased (-21% compared to non-diabetics, p<0.05), apoptotic glomerular cells numbers increased (32%, compared to non-diabetic, p<0.05) and protein levels of bax and cytochrome c increased (175% and 117%). Carnosine treatment restored carnosine kidney levels, prevented podocytes loss (+23% compared to diabetic, p<0.05), restrained glomerular apoptosis (-34% compared to diabetic; p<0.05) and reduced expression of bax and cytochrome c (-63% and -54% compared to diabetics, both p<0.05). In kidneys of all diabetic animals, levels of ROS, AGEs and GlcNAc-modified proteins were increased. Conclusion: By inhibition of pro-apoptotic signaling and independent of biochemical abnormalities, carnosine protects diabetic rat kidneys from apoptosis and podocyte loss.

Introduction
Diabetic kidney disease (DKD) is a frequent complication of both diabetes mellitus type 1, and type 2. About one third of patients with DKD will progress to overt diabetic nephropathy (DN) within 5-15 years. ROS pro-
duction, non-enzymatic glycosylation and an increased flux through the hexosamine pathway are important biochemical changes in hyperglycaemic patients and that are causally linked to the development of DN [1-3]. Early in the development of DN, these biochemical changes cause apoptosis of different glomerular cells [4-6], thereby promoting the development of DN [7, 8]. Apoptosis of glomerular cells is supposed to be causally related to DN, as development of DN can completely be prevented by inhibiting apoptosis in the glomerulus [9].

At the onset of DN, podocytes are key targets for glomerular injury [10-13]. Podocytes loss occurs either by apoptosis or by detachment from the glomerular basement membrane [5]. Hence, numbers of podocytes per glomerulus gradually decline in the course of DN [11] and this change is highly predictive of both progressive glomerular injury and long-term albumin excretion in diabetic patients [14]. Thus, podocyte apoptosis seems to precede the development of DN and determines at least in part kidney function in diabetic patients.

In contrast to other diabetic complications, only 20-40% of diabetic patients are susceptible to develop DN which seems to be genetically determined [15, 16]. We recently identified a polymorphism in the signal sequence of the CNDP1 gene, encoding the serum carnosinase (CN-1), to be associated with susceptibility to DN. Since DN occurs significantly less frequent in diabetic patients that are homozygous for the shortest allelic form, i.e. (CTG)\textsubscript{5} (the so-called Mannheim allele), the Mannheim-allele appears to be protective in terms of susceptibility to DN [17]. This can be explained by the fact that the (CTG)\textsubscript{5} allele corresponds with a low CN-1 secretion efficiency [18] and hence with a low serum CN-1 enzyme activity [17, 19]. Because carnosine, the natural substrate for CN-1, has potential intrinsic renal protective properties [20-24] a low CN-1 expression or activity is beneficial for diabetic patients. Carnosine may prevent apoptosis [25-27] and up-regulates defensive mechanisms such as heat shock proteins [28].

Short-term carnosine administration has been shown to protect against ischemia-reperfusion damage [29-33], and chronic oral carnosine administration can retard onset of diabetes in db/db mice [34]. However, whether carnosine can protect against DN has not been investigated yet. In the present study we therefore tested the effect of oral carnosine administration on early diabetic changes in STZ-induced diabetic rat kidneys, i.e. biochemical abnormalities, glomerular cell apoptosis and podocyte loss.

Materials and Methods

Animals and Diabetes Induction

The study was performed in accordance with the ARVO statement for the use of animals in ophthalmic and vision research and all animal procedures were approved by the Regierungsräsidium Karlsruhe. Male Wistar rats (Harlan Laboratories, Inc.) were housed in a 12h light/dark cycle with free access to food and drinking water. Animals were randomly divided into three groups: 24 non-diabetic rats, 46 diabetic rats and 32 diabetic rats substituted with carnosine. Rats of the diabetic groups were rendered diabetic by intravenous injection of streptozotocin (45mg/kg, Roche, Mannheim, Germany) diluted in citrate buffer (pH 4.5). Animals were considered diabetic when blood glucose reached stable levels over 250 mg/dl. Insulin was occasionally given to individual diabetic rats to prevent critical weight loss. Metabolic data (body weight and blood glucose) were measured every week and blood samples for the determination of glycated haemoglobin concentration by affinity chromatography (MicromatIT™; Bio-Rad Laboratories GmbH, Munich, Germany) were collected at the end of the study (n= 7-9 per group). All rats were sacrificed after 3 month of diabetes. Kidneys were taken out immediately and washed carefully in ice cold PBS. For histology and immunohistochemistry, kidneys were subsequently fixed in 4% formalin. For western blot analysis, kidney cortex was isolated, washed thoroughly and frozen at -80°C until further processing.

Carnosine Supplementation

Diabetic Wistar rats were divided into two groups one week after diabetes induction. One diabetic control group, receiving pure water and a second diabetic group receiving water supplemented with 1 g of carnosine/kg body weight (Flamma S.p.a., Chignolo d’Isola, Italy). Carnosine was dissolved in the daily drinking volume. Water consumption and body weights of each cage were assessed once a week. L-carnosine–supplemented drinking water and lightproof bottles were replaced every day. Carnosine concentration in kidney tissue was measured fluorometrically by high-performance liquid chromatography as previously described by Janssen et al. [17] and Schonherr [35].

Renal histology

2 micrometer kidney paraffin sections were stained with periodic acid-Schiff (PAS) and hematoxylin according to standard staining protocols. An investigator evaluated kidney histopathology in a blinded fashion and graded the glomerular damage including mesangial matrix expansion, hyalinosis with focal adhesion, capillary dilation, glomerular tuft occlusion and sclerosis.

Detection of glomerular cell apoptosis

DNA strand breaks in apoptotic cells were visualized in 4 micrometer kidney paraffin sections using terminal transferase dUTP nick-end labelling (TUNEL, In Situ Cell Death Detection Kit, POD; Roche, Mannheim, Germany) according to the manufacturer’s instructions. In brief, sections were deparaffinised and treated with 2% H\textsubscript{2}O\textsubscript{2} /Methanol to block endogenous

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Quantification of podocyte loss
Paraffin-embedded, formalin-fixed, 3-µm-thick kidney sections were deparaffinised, treated with 2% H<sub>2</sub>O<sub>2</sub>/methanol to block endogenous peroxidase, and microwaved at 500 W and 270 W for 10 min each in citrate buffer. The sections were then incubated with a polyclonal antibody to Wilms' tumor 1 protein (Santa Cruz biotechnology Inc., Heidelberg, Germany) for 1 h at room temperature. After incubation with an appropriate POD coupled secondary antibody, POD was visualized by a substrate reaction using the DAB Peroxidase substrate kit (Vector Laboratories/LINARIS Biologische Produkte GmbH, Wertheim, Germany), followed by nuclear counterstaining. TUNEL positive nuclei were counted in 10 randomly selected kidney glomeruli per animal by two independent persons and their numbers were expressed relative to the corresponding glomerular area as assessed by an image analyzing system (CUE-2; Olympus Opticals, Hamburg, Germany).

Western Blot
In order to isolate proteins from kidney cortex, frozen tissue was homogenized mechanically on ice. Proteins were extracted from lysates using a lysis buffer containing 125 mM NaCl, 10 mM EDTA, 25 mM Hepes, 10 mM Na<sub>2</sub>VO<sub>4</sub>, 0.5 % deoxycholic acid, 0.1 % SDS, 1% Triton-X-100, phosphatase-Inhibitor (Sigma, Steinheim, Germany) and Protease-Inhibitor (Roche, Mannheim, Germany). Insoluble debris was removed by centrifugation at 14,000g at 4°C for 10 minutes. Protein extracts were heated in Laemmli-sample buffer (Bio-Rad Laboratories GmbH, Munich, Germany) for 5 min at 100°C prior to separation by SDS-PAGE. Thereafter, proteins were electrophoretically transferred to a PDVF membrane. Depending on the primary antibody applied, membranes were blocked with either 5% skimmed milk or 5% BSA in TBS according to the manufacturer’s recommendations. For detection of apoptosis signalling, antibodies against cytochrome c and Bax (Cell Signalling Technology, Danvers, USA) were utilized. Biochemical changes in the kidney were detected by antibodies directed against carboxymethyl-lysine- (CML, [36]), nitrotyrosine- (Alexis Biochemicals, Axxora Deutschland GmbH, Germany), methylglyoxal (methylglyoxal-H1, [37]) and N-Acetylglucosamine-modified proteins (Abcam plc, Cambridge, United Kingdom). All antibodies were diluted and incubated as recommended by the manufacture. Immunoblots were made visible on Amersham Hyperfilms (Amersham, Buckinghamshire UK) by incubating the membranes with appropriate HRP-coupled secondary antibodies (Santa Cruz Biotechnology, Heidelberg, Germany) and by using enhanced chemiluminescence (PerkinElmer, LAS, USA). For loading control, membranes were stripped and re-probed with antibodies directed against GAPDH (GAPDH antibody - Loading Control, Abcam plc, Cambridge, United Kingdom). Intensity of the bands was measured by densitometry using the ImageJ 1.36b software.

Statistical analysis
All data are expressed as mean ± SD. Data analysis was performed by using unpaired two-tailed student T-test and a P value less than 0.05 was considered as statistically significant.

Results
All diabetic rats developed stable hyperglycemia of more than >250 mg/dl two weeks after diabetes induction. Average blood glucose levels were significantly elevated in the diabetic rats (Fig. 1B; measurement at 3 month: non-diabetic vs. diabetic: 123 ± 36 mg/dl and 585 ± 26 mg/dl; p<0.05 and non-diabetic vs. diabetic with carnosine: 123 ± 36 mg/dl and 592 ± 14 mg/dl; p<0.05; diabetic vs. diabetic with carnosine not significant). The increase in body weight was significantly impaired after the onset of hyperglycemia resulting in reduced body weights at the end of the study (non-diabetic vs. diabetic: 457 ± 35 g and 282 ± 25 g; p<0.05 and non-diabetic vs. diabetic with carnosine: 457 ± 35 g and 292 ± 38 g; p<0.05; diabetic vs. diabetic with carnosine not significant; Fig. 1A). Glycated haemoglobin levels were 2.3 fold increased in diabetic rats and 1.9 fold in diabetic rats supplemented with carnosine (non-diabetic vs. diabetic: 5.9 ± 0.4 % and 13.4 ± 3.4 %; p<0.05 and non-diabetic vs. diabetic with carnosine: 5.9 ± 0.4 % and 11.2 ± 2.8; p<0.05; diabetic vs. diabetic with carnosine not significant; Fig. 1C). As depicted in Fig. 1D, carnosine levels were significantly decreased in diabetic kidneys (-98% compared to non-diabetic; non-diabetic vs. diabetic: 150.0 µg/g ± 3.5 and 3.3 µg/g ± 0.7; p<0.05) two weeks after diabetes induction. In diabetic rats that were fed with carnosine, renal carnosine concentrations increased significantly (diabetic vs. diabetic with carnosine: 3.3 µg/g ± 0.7 and 300.0 µg/g ± 102.8; p<0.05) and were higher than in non-diabetic rats (+89% compared to non-diabetic; non-diabetic vs. diabetic with carnosine, p not significant). Kidney histopathology was examined in PAS and hematoxylin stained kidney sections. Mild glomerulosclerotic changes,
indicating an early and not advanced stage of a diabetic kidney damage, were found in the diabetic animals. No significant difference was found between the treated and the non treated group (Fig. 2).

To evaluate the effect of carnosine treatment on glomerular apoptosis, DNA strand breaks were assessed by in situ TUNEL staining. Three months after the onset of diabetes the numbers of TUNEL positive cells were significantly increased in non-treated diabetic rats (+32% compared to non-diabetics, non-diabetics: 0.0014 ± 0.0007 TUNEL+ cells/glomerular area and diabetics: 0.0019 ± 0.00018 TUNEL+ cells/glomerular area, p<0.05, Fig. 3A, B). In carnosine treated diabetic animals, the numbers of TUNEL positive cells were reduced by -40% compared to diabetic animals (diabetics: 0.0019 ± 0.000018 TUNEL+ cells/glomerular area and diabetics with carnosine: 0.0013 ± 0.00007 TUNEL+ cells/glomerular area, p<0.05 compared to diabetics, diabetics with carnosine vs. non-diabetics not significant, Fig. 3A, B).

We further investigated the anti-apoptotic potential of carnosine by western blot analysis of kidney cortex lysates. Immunoblots stained for pro-apoptotic bax and cytochrome c displayed an increased expression of +175%, and +117% respectively in the diabetic kidneys of non-treated diabetic rats (p<0.05 for both, Fig. 3C-F). Carnosine treatment significantly inhibited hyperglycemia-induced upregulation of bax (-63% compared to diabetics, p<0.05) and cytochrome c (-54% compared to diabetics, p<0.05) in diabetic kidneys (Fig. 3C-F).

To investigate whether carnosine treatment can prevent podocyte loss in diabetic rats, we determined glomerular podocyte numbers using wt-1 as podocyte marker in histological kidney sections. Podocytes numbers were significantly reduced in diabetic kidneys (-21% compared to non-diabetics, non-diabetics: 0.0018 ± 0.00023 WT-1+ cells/glomerular area and diabetics: 0.0014 ± 0.00018 WT-1+ cells/glomerular area, p<0.05, Fig. 4A, B), while in diabetic rats fed with carnosine, podocyte numbers were normalized (+23% compared to diabetics, diabetics: 0.0014 ± 0.00018 WT-1+ cells/
glomerular area and diabetic with carnosine: 0.0017 ± 0.00022 WT-1+ cells/glomerular area, p<0.05 and -4% compared to non-diabetics, p not significant, Fig. 4A, B).

To examine the influence of carnosine on the biochemical abnormalities induced by diabetes, the activity of three cells damaging mechanisms investigated by western blotting. To this end, antibodies directed against nitrosylated (NT, Fig. 5A) and carboxymethyl-lysine (CML, Fig. 5C)-modified proteins as markers of oxidative stress were used. Moreover, immunoblots were incubated...
Fig. 4. The influence of carnosine treatment on diabetic podocyte loss. Podocytes were stained with antibodies directed against wt-1 in kidney sections of non-diabetic (NC), diabetic (DC), and carnosine treated diabetic (DC+C) rats. Panel A: Representative pictures of kidney sections stained for wt-1. Arrows indicate wt-1 positive cells. Original magnification 400x. Panel B: Quantification of wt-1 positive cells was performed in a blinded fashion on ten randomly selected glomeruli per animal (n=4, for each group) by two independent investigators. Results are expressed as mean podocyte numbers relative to the corresponding glomerular area ± SD. No significant difference (n.s.) in podocyte numbers were found between NC and DC+C, whereas podocyte numbers were significantly reduced in DC. *=p<0.05 compared to non-diabetic controls and #=p<0.05 compared to diabetics.

with antibodies directed against methylglyoxal- (MG, Fig. 5E) and GlcNAc-modified proteins (Fig. 5G) in order to assess AGE-production and the activity of the hexosamine pathway. In diabetic kidney cortex, the levels of NT- (Fig. 5B) and of CML- (Fig. 5D) modified proteins were significantly increased (+167% and +88%, respectively, p<0.05 for both compared to non-diabetics). Likewise, the levels of MG- and GlcNAc-modified proteins were increased in diabetic kidney lysates (+250% and +326%, respectively compared to non-diabetics, p<0.05, Fig. 5F and H). Carnosine treatment of diabetic rats had no effect on hyperglycemia-induced increase in NT-, CML-, MG- and N-Acetylglucosamine-protein levels (diabetic with carnosine vs. non-diabetics: +204% for NT, +122% for CML, +333% for MG and +230%, all p<0.05 compared to non-diabetics, diabetes with carnosine vs. diabetics not significant).

Discussion

Experimental evidence suggests that the carnosine-carnosinase system might play an important role in diabetes associated complications, in particular in DN [17, 34]. Diabetic patients that are homozygous for the Mannheim allele (CTG) are less affected by DN. This genotype is associated with a low serum CN-1 activity, most likely because the (CTG) repeat in the signal peptide of CN-1 influences CN-1 secretion [18]. Inasmuch as individuals homozygous for the Mannheim allele develop less frequently DN, it is conceivable that the protective effect of the Mannheim allele is directly related to the amount of CN-1 found in serum.

A number of studies conducted in recent years have suggested that carnosine, the substrate of CN-1, might be beneficial in relation to diabetic complications diabetes [17, 24, 34, 38]. Hence, a low carnosine turnover in (CTG), homozygous patients could be a plausible explanation why these patients develop less frequently DN. Yet, definitive proof for the salutary effect of carnosine in terms of preventing hyperglycaemia mediated damage in renal tissue is still lacking. In the present study, we therefore investigated whether oral carnosine treatment can ameliorate early changes in kidneys of streptozotocin-induced diabetic rats. The main findings of this study are the following. Carnosine treatment prevents the up-regulation of pro-apoptotic molecules in the kidneys of diabetic rats. This is independent of major biochemical abnormalities that occur as a consequence of diabetes, since carnosine did not influence NT, CML, methylglyoxal or GlcNAc expression in kidneys of diabetic rats.

Microvascular complications in diabetic patients are for a great deal causally related to an increased production of ROS, AGEs and GlcNAc-modified proteins, evoked by chronic hyperglycemia. Based on the intrinsic
Fig. 5. The effect of carnosine treatment on oxidative stress, AGE formation and activity of the hexosamine pathway in diabetic kidney cortex. In order to assess the effect of carnosine treatment on hyperglycemia-induced biochemical changes, kidney cortex lysates from non-diabetic (NC), diabetic (DC), and diabetic rats treated with carnosine (DC+C) were probed with antibodies against nitrotyrosine- (NT, Fig. 4A, B) and carboxymethyl-lysine- (CML, Fig. 4C, D) modified proteins as markers of oxidative stress, methylglyoxal- (MG, Fig. 4E, F) modified proteins as a marker for AGE-production and N-acetyl-D-glucosamine- (GlcNAc, Fig. 4G, H) modified proteins as a marker for hexosamine pathway activation. Representative immunoblots (Fig. 4A, C, E, G) and corresponding densitometrical quantifications (Fig. 4B, D, F, H) show a significant increase of all the markers in diabetic kidneys, irrespective of carnosine treatment. Ratios between the different biochemical markers and GAPDH were assessed to avoid loading dissimilarity errors. A total of 3 animals per group were evaluated for each marker. Results are expressed as mean ratio ± SD. *=p<0.05 compared to non-diabetic.

biochemical properties of carnosine, i.e. antioxidant effects and inhibition of AGE formation [22, 24, 39-42], we expected that carnosine feeding would counteract these factors in kidneys of diabetic rats. Yet surprisingly, our results clearly show that carnosine feeding of diabetic rats is neither capable of preventing oxidative stress and AGE formation, nor could carnosine prevent the activation of the hexosamine pathway in our experimental setting, despite of carnosine feeding significantly increased renal carnosine concentrations. However, it is wise to take some caution in stating that carnosine has antioxidant activity, as most of the data that support this assumption are derived from in vitro cell culture studies [43, 44]. Similar to our findings, Aruoma et al [43] have questioned the anti-oxidative property of carnosine in vivo and they concluded from their study that it is possible that carnosine could act as physiological antioxidant by scavenging hydroxyl radicals, but it does not have a broad spectrum of antioxidant activity, and its ability to inhibit lipid peroxidation is not well established.

Similarly, the ability of carnosine to inhibit AGE formation was mostly demonstrated in in vitro experiments. Nonetheless, it has been repeatedly shown that carnosine is protective in models of ischemia reperfusion [29-33]. In terms of oxidative challenge, these models clearly differ from the diabetic model where sustained severe hyperglycemia over a period of 3 months may yield a higher oxidative burden compared to a relative short period of ischemia. It might well be that the protective biochemical characteristics of carnosine to counteract oxidative stress and hyperglycemia-induced protein modification in the diabetic kidney are exhausted by severity and duration of experimental diabetes. Besides, it has to be admitted that the antioxidant and AGE inhibiting capacity of carnosine can still be sufficient to protect the glomerular capillaries the surrounding parenchymal cells. Such a localized effect would be bound to go unno-
ticed in western blot analysis.

Nonetheless, this study does show that carnosine has a cell protective effect in the context of diabetes, as apoptosis of glomerular cells and podocyte loss were significantly diminished by carnosine. Since carnosine is a multifunctional dipeptide involved in numerous physiological processes, e.g. pH buffering [45, 46], enzyme regulation [47], and induction of heat shock proteins [28], these properties may equally contribute to the cytoprotective effect of carnosine.

Hyperglycaemia causes mitochondrial stress, an eligible condition for the induction of apoptosis. Apoptosis is believed to play an important role in a number of glomerular diseases including DN [48], and is most likely caused by changing the balance in the expression of the anti- and pro-apoptotic molecules Bcl-2 and Bax respectively. While Bcl-2 expression may account for maintaining glomerular hypercellularity, Bax expression might be more important in cell loss leading to glomerulosclerosis. Bax forms oligomers thereby increasing mitochondrial permeability and facilitating the release of cytochrome c from the mitochondrial intermembrane space. Once released from the mitochondria, cytochrome c further activates apoptosis. In our model both bax as well as cytochrome c expression were increased in the cortex of diabetic rats, pointing out that pro-apoptotic signalling is active in the diabetic kidneys. In line with this observation, quantification of TUNEL stained kidney sections revealed increased glomerular apoptosis in diabetic rats. This shift towards apoptosis was restrained by carnosine treatment, indicating a cytoprotective effect of carnosine on glomerular cells. Recently, Isermann et al. demonstrated that DN can be prevented by protecting glomerular endothelial cells and podocytes against apoptosis [9]. Interestingly, carnosine showed similar effects as reported for activated protein C in the study of Iserman et al., in that both molecules down-regulated bax and cytochrome c and reduced TUNEL positive cells in diabetic animals.

The increase in glomerular apoptosis, and in parallel podocyte loss, in diabetic kidneys was significantly ameliorated by carnosine treatment. Although this study did not address the exact identity of the glomerular apoptotic cells, it is likely that part of these cells were of podocyte origin, as podocyte loss and glomerular cell apoptosis coincided. In human disease and in experimental models podocyte loss has been linked to the development of proteinuria and glomerulosclerosis [49] and is predictive for the progression of DN [14, 50-52]. In keeping with the central role of podocytes in the glomerular architecture and the beneficial effect of carnosine treatment on podocyte loss, our results may indicate that carnosine could protect from DN. Moreover, the clear pro- and anti-apoptotic effect that we have observed in western blot analysis might not represent changes limited to the glomerulus and cells situated in areas outside of the glomerulus might also be protected by carnosine. Therefore, carnosine could have a broader therapeutic spectrum that goes beyond the salutary effect demonstrated in this study.

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