L-carnosine inhibits high-glucose-mediated matrix accumulation in human mesangial cells by interfering with TGF-β production and signalling

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Abstract

Background. Transforming growth factor beta is recognized as a major cytokine in extracellular matrix (ECM) pathobiology as occurs in diabetic nephropathy. While experimental studies have advanced a protective role of carnosine for diabetic complications, a link between carnosine, TGF-β and matrix accumulation remains to be elucidated. In the present study, we tested the hypothesis that L-carnosine inhibits TGF-β production and signalling, thereby reducing hyperglycaemia-associated ECM accumulation.

Methods. Human mesangial cells (MC) were cultured in high-glucose (HG, 25 mM D-glucose) medium alone or in HG medium to which 20 mM L-carnosine was added. Collagen VI (Col6) and fibronectin (FN) deposition and messenger RNA expression were studied. In addition, TGF-β production and activation of Smad1/5/8 (ALK1) and Smad2/3 (ALK5) pathways were assessed.

Results. Under HG conditions, deposition of Col6 and FN were increased 1.4- and 1.6-fold. This was significantly inhibited on the protein and messenger RNA level by L-carnosine. TGF-β production increased under HG conditions but was completely normalized by addition of L-carnosine. Addition of exogenous TGF-β could not overcome the effect of L-carnosine on Col6 and FN expression, indicating additionally interference with TGF-β downstream signalling. Along the same line, L-carnosine reduced TGF-β-mediated Smad2 phosphorylation, suggesting an inhibitory effect on ALK5 signalling. ALK1 signalling remained unchanged. Under HG conditions, pharmacologic inhibition of ALK5 prevented Col6 accumulation but did not change FN deposition.

Conclusions. L-carnosine can modulate matrix accumulation in two ways. Firstly, inhibition of TGF-β production might result in an overall inhibition of matrix accumulation and secondly, L-carnosine inhibits TGF-β-induced matrix accumulation, most likely via inhibition of the ALK5 pathway.

Keywords: carnosine; diabetic nephropathy; fibrosis; mesangial matrix; TGF-β

Background

Diabetic nephropathy (DN) is a major microvascular complication of diabetes with similar typical renal lesions in Type 1 and 2 diabetes mellitus patients. Pathophysiologic changes in DN include hyperfiltration and microalbuminuria followed by deterioration of renal functions [1]. Morphologically, DN is characterized by excessive extracellular matrix (ECM) formation in the mesangium and thickening of glomerular and tubular basement membranes, ultimately progressing to glomerulosclerosis and tubulo-interstitial fibrosis. A key molecule in DN pathogenesis is the pro-fibrotic cytokine TGF-β1. Elevated glucose concentrations are known to stimulate TGF-β1 production within the glomerulus [2], consequently resulting in elevated urinary TGF-β1 levels [3]. A number of upstream mediators, e.g. protein kinase C, reactive oxygen species (ROS), hexosamines and transcription factors (activator protein 1) are involved in this process [4]. Over the past years, a number of risk factors for the development of DN have been identified. Yet, how these factors influence DN pathogenesis in mechanistic terms is not well defined. Recently, we demonstrated that a polymorphism in the serum carnosinase (CNDP-1) gene is associated with susceptibility to develop DN [5]. Serum carnosinase is the rate-limiting enzyme for the hydrolysis of carnosine into β-alanine and histidine, suggesting that carnosine might be protective for complications associated with hyperglycaemia. Indeed, several studies have indicated that carnosine has the propensity to function as a natural ACE inhibitor, as a scavenger for ROS and as an inhibitor of advanced glycation end-product (AGE) formation [5]. Moreover, animal studies have also shown that...
overexpression of CNDP-1 in db/db mice results in an earlier onset of diabetes, while in nontransgenic db/db mice that were supplemented with l-carnosine, diabetes manifested significantly later and milder [6]. The influence of l-carnosine on hyperglycaemia-induced ECM formation is thus far not well studied. In the present study, we therefore tested the hypothesis that carnosine inhibits TGF-β production and signalling, thereby reducing ECM production under hyperglycaemic conditions.

Methods

Cell culture

Human SV40-transformed mesangial cells (MC) [7] were grown for 14 days in Dulbecco’s modified Eagle’s medium (PAA, Coelbe, Germany) containing 10% fetal calf serum (Greiner, Frickenhausen, Germany) and 10 U/mL penicillin/streptomycin (Sigma, St Louis, MO) in normal culture flasks (Greiner, Frickenhausen, Germany) or on uncoated coverslips. The cells were either cultured in the presence of 25 mmol/L D-glucose (Sigma) or in medium containing 5 mmol/L D-glucose. To each condition, 20 μL of the culture supernatant was transferred electrophoretically to a polyvinylidenfluorid membrane and subjected to 10% sodium dodecyl sulphate–polyacrylamide gel electrophoresis. Proteins were transferred electrophoretically to a polyvinylidenfluorid membrane (Roche, Mannheim, Germany) by semidy blotting. Hereafter, the membranes were blocked for 1 h at room temperature in Tris-buffered saline-Tween 20 (0.3%; Sigma, Steinheim, Germany) containing 10% milk powder. Subsequent the membranes were incubated for 48 h with antibodies directed against pSmad1/5/8 and pSmad2 (Santa Cruz Biotechnology). After incubation with appropriate hors eradish peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology), antibody binding was visualized by enhanced chemiluminescence (PerkinElmer, Boston, MA). Intensity of the bands was measured by densitometry using the ImageJ 1.36b software. For loading control, membranes were stripped and re-probed with antibodies directed against glyceraldehyde 3-phosphate dehydrogenase (GAPDH).

Reverse transcription–polymerase chain reaction

Total RNA was isolated from cultured MC using Trizol®-Reagent (Invitrogen, Carlsbad, CA). Hereafter, DNase treatment was carried out, using RNase free DNase I (Ambion, Woodward, Austin, TX). RNA concentration and quality were assessed by RNA 6000 nano assays on a Bioanalyzer 2100 system (Agilent, Waldbronn, Germany). A sample of 0.5 μg of RNA was converted into complementary DNA (cDNA) using T7-(dT) 24 primers and the SuperScript Choice system for cDNA synthesis (Life Technologies, Inc., Rockville, MD). Specific DNA standards were generated by polymerase chain reaction (PCR) amplification of cDNA, purification of the amplified products and quantification by spectrophotometry. Quantitative PCR (qPCR) of cDNA specimen and DNA standards were conducted in a total volume of 25 μL, containing 2 μL FastStart DNA Master SYBR Green, 10 pM of forward (fwd) and reverse (rev) primers and 2 mM MgCl2. Primer sequences were as follows: Col6 (fwd) GCTGCAAGGAGAATCTACGGAG and (rev) GCAGGTTGAATCTTGAGACCTC; FN (fwd) CCTCAATGTTCCGAGGTTCA and (rev) AAATCCGATGGTGCTGACCA; Id1 (fwd) CGTCGTCTGCTACGACATGAA and (rev) TTGCTGGACCTCTT; PAI-1 (fwd) CATGTGCATTTGCGCCCTT and (rev) GCCTGGTGATGTGCTTT; TIMP-1 (fwd) AATTCGCGACCTGATCA and (rev) GAAAGATGGGAGTGGGAGAA; TIMP-2 (fwd) ATCCAGCGCAAGTGCTGAG and (rev) ATCCCTGACCTGACCTCCCT; MMP-2 (fwd) TACTGGAATCTACTCAGCA: and (rev) CTTCAGGTAATGCGACCTCTG and MMP-9 (fwd) CGGAGCTGACCTGACCTGATGG and (rev) GAGGTGCCGAGGATCCATACGT.

The amplification profile consisted of 10 min at 95°C followed by 45 cycles of amplification, each cycle consisting of denaturation at 95°C for 10 s, annealing for 10 s at 60°C and extension for 9 s at 72°C. Standard curves were generated in all experiments. PCR efficiency was assessed from the slopes of the standard curves and was found to be between 90 and 100%. Linearity of the assay could be demonstrated by serial dilution of all standards and cDNA. All samples were normalized for an equal expression of GAPDH.

Statistical analyses

All values are shown as mean ± SD if not indicated otherwise. Data were analysed by one-way analysis of variance followed Bonferroni post hoc test to assess differences between groups. A P-value < 0.05 was considered to be significant.

Results

High-glucose-induced FN and Col6 accumulation is inhibited by l-carnosine

MC were cultured for 14 days under normal (M) or high-glucose (HG) conditions and the influence of l-carnosine (C) on the expression of collagen VI (Col6) and fibronectin (FN) was studied. Compared to MC cultured in normal medium, the expression of both matrix components increased significantly when 25 mmol/L glucose was added to the medium, as measured by quantification of immunofluorescence intensities, indicating a 1.4-fold increase for Col6 and a 1.6-fold increase for FN. Addition of l-carnosine to these cells (HG + C) remarkably inhibited matrix accumulation for both components (P < 0.01; Figure 1A and B). Cell number

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did not differ between groups (M: 88 ± 7 cells/ROI, HG: 86 ± 11 cells/ROI, HG + C: 89 ± 7 cells/ROI; not significant for all groups). Changes in matrix accumulation were reflected by messenger RNA expression for both genes, that is Col6 expression increased 1.6-fold and FN expression 1.5-fold under HG conditions, while this was completely normalized by L-carnosine (Figure 1C). No change in matrix production was observed when L-carnosine was added to normal medium. In addition, β-alanine and histidine either alone or in combination did not inhibit HG-induced Col6 or FN accumulation (data not shown).

**HG-induced TGF-β production is inhibited by L-carnosine**

We next tested if TGF-β production was influenced by L-carnosine. TGF-β production increased from 0.88 ± 0.10 to 1.52 ± 0.28 ng/mL per 10⁶ MC cultured in HG medium and this was significantly inhibited by L-carnosine (0.92 ± 0.24 ng/mL per 10⁶ cells, P < 0.01; Figure 2A).

When MC were cultured for 14 days in the presence of TGF-β, matrix production was increased to a similar extent as was observed under HG conditions (1.52-times for Col6 and 1.54-fold for FN). Interestingly, this was also inhibited by L-carnosine (0.97 ± 0.08 and 0.87 ± 0.11, Figure 2B), suggesting that L-carnosine not only interferes with HG induced TGF-β production but possibly also with TGF-β signalling.

**Influence of L-carnosine on ALK signalling**

To address how L-carnosine inhibits TGF-β-induced matrix production, we investigated by western blot the activation of Smad1/5/8 (representing the ALK1 pathway) and Smad2 (representing the ALK5 pathway). While activation of Smad1/5/8 was not observed after TGF-β stimulation, Smad2 C-terminal phosphorylation was 2-fold increased after stimulation when normalized for equal GAPDH expression. When the cells were cultured in the presence of L-carnosine, Smad2 activation by TGF-β was slightly but significantly decreased (Figure 3).

To further substantiate that the inhibition of pSmad2 was of functional relevance, Id1 and PAI-1 gene expression were analysed by quantitative PCR. The expression of these genes is associated with activation of the ALK1 and the ALK5 pathway, respectively. Similar to what was observed for Smad activation, the expression of Id1 was not changed, while the expression of PAI-1 increased 2-fold. In cells that were cultured in the presence of L-carnosine, TGF-β-induced PAI-1 expression was significantly reduced (Figure 4A).

We also tested if L-carnosine influenced PAI-1 expression when MC were cultured under hyperglycaemic conditions. Whereas no change in Id1 expression was observed under the different culture conditions, expression of PAI-1 was significantly increased when MC were cultured in HG medium compared to normal medium.
l-carnosine inhibits matrix by TGF-β signalling

Discussion

In the present study, we tested the hypothesis that carnosine inhibits TGF-β production and signalling, thereby reducing ECM accumulation induced by hyperglycaemia. The main findings of our study are as follows. Firstly, hyperglycaemia increases the expression of the matrix components Col6 and FN as well as the production of TGF-β. Both of which are inhibited by l-carnosine. Secondly, l-carnosine inhibits TGF-β-induced matrix accumulation, most likely via inhibition of the ALK5 pathway. Thirdly, hyperglycaemia reduces the expression of MMP9. This is not influenced by l-carnosine. There was no influence of hyperglycaemia on the expression of MMP2, TIMP1 or TIMP2.

The activation of matrix-producing cells with consecutive matrix overproduction is generally regarded as a central event in renal fibrogenesis. In this scenario, glomerular MC, interstitial fibroblasts and tubular epithelial cells are the key players that mediate the transition of normal tissue into tissue with fibrotic scarring. It is now widely accepted that in this process, the TGF-β system is of eminent importance [9].

Most chronic nephropathies share pathogenic mechanisms that contribute to disease progression, independent of the original cause or disease. Among these, diabetic nephropathy is the foremost cause for end-stage renal failure in the western world. As a consequence of the hyperglycaemic environment in diabetic patients, MC are likely to become activated, either directly by high glucose or indirectly by an increased amount of AGEs or ROS [10]. Yet, only approximately one-third of diabetic patients are susceptible to develop DN, which suggests that the consequence of MC activation, i.e. TGF-β production and ECM accumulation, in non-susceptible patients might be countered locally, thereby interrupting in the sequel that leads to tissue fibrosis.

Recently, we [5] and others [11] have demonstrated that diabetic patients homozygous for the CNDP1 Carnosinase-Mannheim allele are at a lower risk to develop DN compared to diabetic patients with other CNDP1 genotypes. These patients have a low CNDP1 activity, most likely due to a poor secretion of carnosinase [12]. Based on the results presented in this study, it is conceivable that in such patients, local concentrations of carnosine, the natural substrate of carnosinase, may slow down or prevent the progression of glomerulosclerosis. It seems that carnosine affects the TGF-β system in two ways, i.e. by inhibiting TGF-β production and by inhibiting TGF-β signalling.

Because TGF-β-mediated ECM accumulation is partly Smad independent [13, 14], this might explain why the
ALK5 inhibitor did not completely parallel the findings obtained with l-carnosine. Tsuchida et al. have suggested that Smad4 is essential for basal and TGF-β-induced Col1A1 expression and contributes to the early but not sustained TGF-β-induced PAI-1 expression in MC.

Although we did not study the expression of Col1A1, our data for Col6 are compatible with this observation. Similar to our own data, it has also been reported that TGF-β-induced FN expression is partly Smad independent [15]. Recently, Bhattacharyya et al. [16] have shown that TGF-β upregulates the expression of the transcription factor early growth response-1 (Egr-1) in a mitogen-activated protein kinase kinase 1-specific fashion. Egr-1 can directly transactivate the fibronectin gene as demonstrated by Liu et al. [17]. Hence, inhibition of ALK5 by SB431542 may not be as effective as the inhibition of TGF-β production by l-carnosine on ECM accumulation.

Goumans et al. [18] have reported that in endothelial cells, two distinct Type 1 receptors/Smad signaling pathways with opposing effects are present. Activation of the ALK1 pathway is associated with phosphorylation of Smad1/5/8, while activation of the ALK5 pathway results...
in phosphorylation of Smad2. Signaling via ALK1 induces Id1 expression and promotes cell migration and proliferation. In contrast, signaling through ALK5 induces PAI-1 expression and inhibits cell migration and proliferation [18]. The role of PAI-1 in conjunction with TGF-β on tissue fibrosis has clearly been demonstrated [19]. An increased PAI-1 expression in DN has also been documented by Lee et al. [20]. The inhibitory effect of l-carnosine in our study might be partially explained by a decrease in TGF-β production and by a reduced activation of ALK5 signaling.

Matrix accumulation can occur as a consequence of an increased de novo matrix synthesis or as a consequence of a decreased matrix turnover. MMP2 and MMP9 are the major proteases mediating matrix degradation. A number of studies suggested the involvement of aberrant MMP expression in progression of DN or renal interstitial fibrosis [21, 22]. Decreased MMP2 expression has been reported in rodent models of diabetes [23, 24], as well as in MC cultured under HG conditions [25]. Yet, in renal tissue of Type 1 diabetic patients, MMP2 enzyme activity and expression seems to be elevated [26]. Systemic concentrations and activity levels of MMP-9 are also increased in patients with Type 2 diabetes. This is not concordant to the findings in the streptozotocin-induced diabetes animal model and to the findings of mouse MC cultured in HG conditions [22] where a decreased MMP9 expression and activity have been observed. The present data are partly in line with the latter findings that the expression of MMP9 is decreased in human MC cultured under HG. However, no difference was found for MMP2 and the inhibitors TIMP1 and 2. Since l-carnosine neither affects the expression of MMPs nor of TIMP, our data suggest that the beneficial effect of l-carnosine on HG-induced matrix accumulation does not involve an altered matrix turnover.

In conclusion, we show that l-carnosine can effectively inhibit hyperglycaemia-induced ECM accumulation by inhibiting TGF-β production and signalling. Carnosine supplementation or carnosinase inhibition might therefore be of therapeutic relevance in diabetic patients with an increased risk for developing DN.

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References

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