Blood pressure and glucose independent renoprotective effects of dipeptidyl peptidase-4 inhibition in a mouse model of type-2 diabetic nephropathy

Yuliya Sharkovskaya, Christoph Reichetzeder, Markus Alter, Oleg Tsuprykov, Sebastian Bachmann, Thomas Secher, Thomas Klein, and Berthold Hocher

Background: Despite the beneficial effects of type 4 dipeptidyl peptidase (DPP-4) inhibitors on glucose levels, its effects on diabetic nephropathy remain unclear.

Method: This study examined the long-term renoprotective effects of DPP-4 inhibitor linagliptin in db/db mice, a model of type 2 diabetes. Results were compared with the known beneficial effects of renin–angiotensin system blockade by enalapril. Ten-week-old male diabetic db/db mice were treated for 3 months with either vehicle (n = 10), 3 mg linagliptin/kg per day (n = 8), or 20 mg enalapril/kg per day (n = 10). Heterozygous db/m mice treated with vehicle served as healthy controls (n = 8).

Results: Neither linagliptin nor enalapril had significant effects on the parameters of glucose metabolism or blood pressure in diabetic db/db mice. However, linagliptin treatment reduced albuminuria and attenuated kidney injury. In addition, expression of podocyte marker podocalyxin was normalized. We also analysed DPP-4 expression by immunofluorescence in human kidney biopsies and detected upregulation of DPP-4 in the glomeruli of patients with diabetic nephropathy, suggesting that our findings might be of relevance for human kidney disease as well.

Conclusion: Treatment with DPP-4 inhibitor linagliptin delays the progression of diabetic nephropathy damage in a glucose-independent and blood-pressure-independent manner. The observed effects may be because of the attenuation of podocyte injury and inhibition of myofibroblast transformation.

Keywords: diabetic nephropathy, DPP-4 inhibitors, linagliptin

Abbreviations: BP, blood pressure; DPP-4, type 4 dipeptidyl peptidase; GIP, glucose-dependent insulinotropic polypeptide; GLP-1, glucagon-like peptide-1; HbA1C, type 1A glycated haemoglobin; PYY, peptide YY; SDF1α, stromal-cell-derived factor 1α, T2DM, type 2 diabetes mellitus

INTRODUCTION

Secondary complications associated with type 2 diabetes mellitus (T2DM) are frequent, severe, and progressive [1]. Diabetic complications, which include diabetic nephropathy, coronary heart disease, stroke, peripheral arterial disease, neuropathy, retinopathy as well as heart failure and periodontal disease, have a significant impact on disease morbidity and mortality. They are responsible for a reduction in the mean life-expectancy of 12 years in men and 19 years in women, place a heavy burden on the healthcare resources, and diminish the patient's quality of life. Of these, diabetic nephropathy is perhaps the complication with the single greatest socioeconomic impact [1]. At present, diabetic nephropathy is the leading cause of end-stage renal disease and affects approximately one-third of those with diabetes [2]. The pathogenesis of diabetic nephropathy is incompletely understood but includes glycosylation of circulating and intrarenal proteins, hypertension, hyperfiltration, and microalbuminuria, followed by a progressive decline of renal function associated with cellular and extracellular matrix accumulation in the glomerular and tubule-interstitial elements [3].

Despite providing effective reductions in the levels of type 1A glycated haemoglobin (HbA1C), current therapeutic options for the treatment of T2DM appear to offer little benefit in terms of protection from end-organ damage. Some recent analyses of carefully devised and monitored studies recording data on cardiovascular events have even questioned the cardiovascular safety of established anti-diabetic drugs [4,5]. There is a recognized clinical need for novel therapeutic strategies that can impact on the development of diabetic nephropathy [6]. Recently introduced type 4 dipeptidyl peptidase (DPP-4) inhibitors that target the endogenous enzyme responsible for the degradation of glucagon-like peptide-1 (GLP-1) and glucose-dependent insulinotropic polypeptide (GIP) [7,8] represent the addition of an alternate means of managing T2DM [9].

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The beneficial effects of DPP-4 inhibitors in the kidney are presumed to be mostly mediated by increased GLP-1 action [10–12]. GLP-1 has vasodilatory, anti-inflammatory and antifibrotic properties, reduces water and salt intake in rats, healthy men, and obese individuals [13]. However, the full pharmacodynamic profile of DPP-4 inhibitors has yet to be defined. The DPP-4 enzyme is known to have other protein substrates such as high-mobility group protein-B1, meprin B, brain and atrial natriuretic peptides (BNP/ANP), neuropeptide Y (NPY), peptide YY (PYY), and stromal-cell-derived factor 1α (SDF-1α) that could provide additional renal and cardiovascular effects [11,13]. The GLP-1-independent effects of DPP-4 inhibitor albiglutin on renal outcome were established in GLP-1 receptor–/- ‘knockout’ mice, in which GLP-1R-agonist-mediated effects were dependent on the presence of the GLP-1 receptor [14]. There is anticipation that DPP-4 inhibition will offer benefits beyond those seen with existing therapies. Preclinical and clinical studies suggest that DPP-4 inhibitors may be effective in slowing progression of diabetic kidney disease [15–19]. In addition, recent data raise the possibility of DPP-4 inhibition to prevent glomerulosclerosis [20] and even reverse it in the rodent models of human type 1 diabetes [21].

A major limitation of the most recent incretin-based studies is the inability to establish renoprotective effects of DPP-4 inhibitors without affecting blood glucose levels and blood pressure (BP). Therefore, in the present study, we aimed to further investigate the short-term and long-term effects of a DPP-4 inhibitor linagliptin on the development and progression of diabetic nephropathy in db/db mice. This study was designed to isolate these effects from any benefit that may be derived from linagliptin’s glucose-lowering action and the results were compared with known beneficial effects of renin–angiotensin system (RAS) blockade by enalapril. We also analysed DPP-4 expression in human kidney tissue in order to correlate our observations with those that have been observed in the clinical setting.

METHODS

Animal experiments were conducted in accordance with the Guide for the Care and Use of Laboratory Animals (US Department of Health and Human Services).

Human renal samples were provided by the department of pathology at the Charité – University Medicine Berlin. Normal renal tissue samples were obtained from normal and diabetic age-matched C57BL6 (Bar Harbor, Maine, USA). In a second short-term study, animals (control) were obtained from Jackson Laboratories, IBL 27724) and total GIP (Millipore EZRGIP-55K), as well as active GLP-2 and total and active SDF1α by means of inhouse established assays was performed.

In the long-term study, diabetic db/db mice were randomized by body weight, serum glucose, and urine albumin into three groups: vehicle (n = 10), linagliptin 3 mg/kg per day (n = 8), or enalapril 20 mg/kg per day (n = 10). Heterozygous control db/m mice were treated with vehicle alone and served as the control group (n = 8). The study duration was 12 weeks.

Serum was analysed for glucose, triglycerides, insulin, cystatin C, and glycosylated haemoglobin (HbA1C). In house, clinical chemistry analysis was performed on a Beckman CX-4. Insulin and cystatin C were analysed by MyriadRBMS. Urine was analysed for glucose, creatinine, albumin and cystatin C. Body weight was recorded weekly for dose–volume adjustment. Food and water consumption were recorded weekly. Serum and urine analysis were sampled at baseline and monthly thereafter. On day 8 and day 70, animals were moved to a clean cage and fasted for 15 h prior to performing the oral glucose tolerance test (OGTT) test. OGTT was performed using 2 g/kg p.o. glucose load. Blood samples were assayed for glucose at –60, 0, 30, 60, 90, and 120 min relative to glucose load. BP was measured by the standard tail-cuff method 6 days before the beginning and at the end of the experiment [23]. At the end of the experiment (week 12), the animals were sacrificed under isoflurane anaesthesia and kidneys were harvested for further studies. BP was measured via tail cuff.

Histological studies

Following sacrifice, the kidneys of mice were quickly removed, sliced, and then immersed (fixed) in 4% paraformaldehyde and phosphate-buffered saline (PBS).

After fixation, sampled tissues were thoroughly washed in PBS, dehydrated in graded ethanol, cleared in xylene, and embedded in paraffin. Serial sections cut at 3 μm were mounted on silane-coated glass, and analysed for interstitial fibrosis by Sirius Red (PSR) and glomerulosclerosis by periodic acid Schiff (PAS) staining. Quantitative histomorphometry to determine interstitial fibrosis and glomerulosclerosis was assessed using the computer-aided image analysis system Image J and rated as described previously [24]. In brief, glomerulosclerosis was defined by the presence of PAS-positive material within the glomeruli. The level of glomerulosclerosis was assessed using a semiquantitative scoring method; two investigators scored the results in a blinded fashion. The severity of interstitial fibrosis was evaluated after Sirius red-staining using computer-aided histomorphometry devices. At least 30 microscopic images per kidney section were transferred to a PowerMAC from a Hitachi CCD camera. A random subset
of images was used to manually set the threshold for detecting only positive Sirius red-stained area after which the relationship between positive Sirius red-stained area (connective tissue) and the total positive area of the picture was assessed using ImageJ, image-processing software (freeware from the National Institute of Health, U.S. Department of Health and Human Services).

Patients

Human renal samples were provided by the department of pathology at the Charité – University Medicine Berlin. The study included renal biopsies of five patients who were affected by T2DM and microalbuminuria. As control, four normal renal tissue samples were obtained from normal margins after resection of renal cell carcinomas. Every patient had given a prior written consent for their biopsies to be used for research purposes.

Immunohistochemistry

Paraffin tissue sections from mouse and human kidneys were incubated with the following primary antibodies: goat antiamouse DPPIV/CD26 (1:1000; R&D System, Minneapolis, Minnesota, USA); goat antimouse Podocalyxin (1:1000; R&D System); rabbit polyclonal to GLP1R (1:100; Abcam, Cambridge, UK); anti-synaptopodin mouse monoclonal, G1D4 (1:500, Progen, Heidelberg, Germany); monoclonal anti-α-smooth muscle antibody (1:500; Sigma-Aldrich, Hamburg, Germany); and rabbit polyclonal to collagen I (1:500; Abcam). Paraffin tissue sections were incubated with blocking medium (30 min), followed by primary antibody diluted in blocking medium (1 h). Where samples underwent multiple staining, antibodies were applied sequentially, with each application separated by a washing step. Fluorescent Cy2-conjugated, Cy3-conjugated antibodies (DIANOVA GmbH, Hamburg, Germany) and biotinylated donkey antigoat secondary antibody (Jackson Immunoresearch) were applied for detection. The antigen–antibody complexes were visualized with 3-amino-9-ethylcarbazole (AEC, DAKO) chromogen solution after incubation with Vectastain Elite ABC Kit (Avidin/Biotin/Horseradish Peroxidase-System; Vector Laboratories, Burlingame, California, USA) for 30 min at room temperature. Sections were evaluated in a Leica DMRB (Leica, Burlingame, California, USA) for 40X objective in a ScanScope AT slide scanner (Aperio). Ground fluorescence levels were determined over cell nuclei and subtracted from the signal. Quantitative analysis was done by image analysis as described for interstitial fibrosis and glomerulosclerosis measurements above.

RESULTS

In-situ hybridization

Frozen kidney tissue was mounted with tissue-tek OCT compound (Sakura) on a CM 1850 cryostat (Leica) at −20°C. Sections were cut 12 μm thick and collected on Super Frost Plus slides (Thermo Scientific), briefly air dried and stored at −80°C until used. Frozen sections were fixed in 10% neutral buffered formalin (4% formaldehyde) for 1 hour at 4°C and dehydrated through graded alcohol. Subsequently, in situ hybridization with the RNAscope 2.0 HD Brown assay kit (Advanced Cell Diagnostics) was performed according to the manufacturer’s instructions using a mouse GLP-1R probe. A probe against the bacterial gene dapB, with no known expression in eukaryotes, was used as negative control. All probes were provided by the manufacturer. After in situ hybridization, the slides were counterstained in Gill’s hematoxylin (Sigma) and coverslipped with pertex (Histolab). The slides were scanned under a 40X objective in a ScanScope AT slide scanner (Aperio).

Statistical analysis

Differences between treatment groups were compared using analysis of variance (ANOVA) or Kruskal–Wallis tests (for normally or nonnormally distributed data) as appropriate. The Student’s t-test or Mann–Whitney U tests were used to detect significant differences between two groups of interest. Results are expressed as means ± standard errors of the mean; differences were considered significant when estimates for the probability error (P) were less than 0.05. Survival analysis was carried out using Kaplan–Meier analysis and the log-rank test.

Localization of type 4 dipeptidyl peptidase in normal mouse glomerulus

First, we characterized the expression of DPP4 in healthy mice kidneys. We observed the strong expression of DPP-4 in the glomeruli of mice, on the brush borders of the proximal tubule cells, and in the epithelia of thin descending limb of Henle’s loop (Fig. 1a). Then, we examined which cells express DPP-4 in the glomerulus. DPP-4 was colocalized completely with synaptopodin, a podocyte foot process marker, but not with caveolin-1, a marker for endothelial cells, suggesting that DPP-4 is located in podocytes (Fig. 1b). Immunogold electron microscopy was used to investigate the fine structural distribution of DPP-4 in healthy mouse glomeruli. The anti-DPP4 antibody produced strong labelling of podocyte foot process, slit diaphragm, and single labelling of glomerular endothelium (Fig. 1c).

In-vivo study, 3 months treatment

Animal characteristics

At 15 weeks of age, body weights in diabetic db/db mice were significantly higher than in heterozygous control mice (db/+). These mice remained significantly greater throughout the dosing period. Daily food intake was largely similar and there was no statistically significant difference in weight gain between the diabetic db/db groups (Fig. 2a).
Fasting glucose was measured weekly in all groups and was similar in all groups at each timepoint. Diabetic db/db mice showed elevated fasting levels of glucose, insulin, glucose tolerance after an OGTT, and HbA1C throughout the entire period of the experiment. Neither linagliptin nor enalapril had significant effects on the levels of glucose and insulin, or HbA1C. Linagliptin did not modify glucose disposal following OGTT in diabetic db/db mice and the glucose tolerance was even higher in db/db mice treated with enalapril compared with vehicle db/db mice (Table 1, Fig. 2b).

Baseline serum triglycerides were significantly higher in diabetic animals (71.5 ± 2.8 vs. 52.5 ± 3.9 mg/dl) and increased to 165 ± 34 mg/dl by the study end in vehicle-treated animals. Neither enalapril nor linagliptin did not show significant improvement in triglyceride levels.

Kidney function
During the experimental period, diabetic db/db mice displayed clear evidence of renal tubular dysfunction, characterized by a markedly elevated urinary cystatin-C-to-creatinine ratio (CCR). Administration of linagliptin or enalapril significantly ameliorated the increase of CCR in diabetic db/db mice after 12 weeks of treatment. The reduction of CCR in linagliptin-treated mice was comparable with the enalapril-treated group, although enalapril had a tendency...
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achieved by linagliptin treatment in order to investigate the involvement of putative DPP-4 substrates. As expected, the 81% DPP-4 inhibition by linagliptin resulted in a marked (7.5-fold) increase of active GLP-1 and a downregulation of total GLP-1, but interestingly a much higher increase of active GIP levels (up to 1312 + 359.1 pg/ml) representing a 22.7-fold increase was observed compared with control. Despite a slight increase of total SDF1 alpha, intact SDF1 alpha and GLP-2 were not detectable (Table 3).

Immunohistochemical localization of type 4 dipeptidyl peptidase and glucagon-like peptide-1 receptor mRNA in the glomeruli of db/db mice

Immunofluorescence of the DPP-4 showed a strong expression in the podocytes of db/db mice and healthy control db/m mice. Quantitative assessment of DPP-4 expression in kidney sections showed no significant differences between the groups (data not shown).

Because of the recent publications on the limitations of commercially available antibodies for GLP-1R, we detected GLP-1R mRNA in kidney sections by in-situ hybridization as illustrated in Fig. 6. The specificity of the signal was validated on rat-brain sections with clear GLP-1R expression in the dorsomedial hypothalamus and arcuate nucleus together with a low background. The data from mouse kidney indicates that GLP-1R is expressed in afferent and efferent renal arterioles, as well as renal arteries. No signal could be detected in glomeruli, tubules, or collecting ducts. Semi-quantitative in-situ hybridization was performed in order to compare the expression level of GLP-1 receptor in kidneys of db/db and db/m mice. No statistical significant differences were observed although a tendency towards a higher expression in db/db vs. db/m control mice was apparent (data not shown).

Expression of type 4 dipeptidyl peptidase in diseased human kidney

Immunofluorescence staining of the renal biopsy samples is shown in Fig. 7. DPP-4 immunoreactivity was identified in the glomeruli of patients with diabetic nephropathy, but not in healthy kidneys. DPP-4 co-localized with synaptopodin in diabetic nephropathy.

DISCUSSION

This study demonstrates the renoprotective effects of linagliptin on diabetic-nephropathy-associated kidney damage in an experimental mouse model of T2DM. The protective effects occurred without affecting blood glucose levels and BP. Our investigations were conducted in genetically diabetic C57BL6 db/db developing hyperphagia and obesity with progressive hyperglycemia, hyperinsulinemia, and insulin resistance [25]. We chose this model because the renal injury profile involving glomerular hypertrophy, podocyte loss, glomerulosclerosis, and renal fibrosis is similar to that seen during the development of human diabetic nephropathy [26,27]. The upregulation of DPP-4 activity in the kidney and urine of patients with human glomerular diseases reported here and previously [28,29] suggests clinical relevance of our observations. This also fits...
nicely with the finding of a recent meta-analysis of human data. It showed a reduction in the UACR on top of that seen following guideline-based treatment with an angiotensin receptor blocker in patients with overt diabetic nephropathy [22]. Our data suggest that the renoprotective action of DPP-4 inhibition might be caused by the attenuation of podocyte injury and inhibition of myofibroblast transformation.

In the present study, \( db/db \) mice developed diabetes, as evidenced by hyperglycemia, increased HbA1C and impaired OGTT, and these changes were associated with early signs of diabetic nephropathy: podocyte injury, glomerular mesangial matrix expansion, and accelerated renal fibrosis. Amongst several animal models of T2DM, the \( db/db \) mouse appears to most closely resemble the progressive nature of mesangial matrix expansion in human diabetic nephropathy [26]. Interestingly, in the present study long-term exposure to linagliptin was associated with an attenuated level of mesangial matrix expansion: glomerulosclerosis and renal fibrosis in diabetic \( db/db \) mice. This association may explain, in part, the involvement of the DPP-4 enzyme in the pathogenesis of diabetic nephropathy that has been noted previously [15,16,20,21]. Further progression of diabetic nephropathy is characterized by a recruitment of inflammatory cells and an accumulation of extracellular matrix proteins in the renal interstitium [30]. The penultimate steps in the pathological continuum involve the development of renal fibrosis and tubular atrophy, which finally lead to kidney failure. In the present study, the computer-aided image analysis of histological sections and immunohistochemical observations provided evidence for an antifibrotic effect of linagliptin by reducing the extent of interstitial fibrosis and normalizing the expression of type I collagen and \( \alpha \)-smooth muscle actin in the kidney tissue of diabetic \( db/db \) mice.

Disruption of the podocyte interdigitation affects the functioning of the glomerular filtration slits and, as tissue

### TABLE 1. Effects of chronic treatment with linagliptin and enalapril on plasma glucose, insulin, HbA1C, and blood pressure in diabetic \( db/db \) and nondiabetic \( db/m \) mice

<table>
<thead>
<tr>
<th></th>
<th>Plasma glucose (mg/dl)</th>
<th>Plasma insulin (( \mu )IU/ml)</th>
<th>HbA1C (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Baseline</td>
<td>Day 84</td>
<td>Baseline</td>
</tr>
<tr>
<td>( db/db ) Vehicle</td>
<td>671 ± 35</td>
<td>843 ± 98</td>
<td>15.1 ± 3.2</td>
</tr>
<tr>
<td>( db/db ) Linagliptin</td>
<td>664 ± 23</td>
<td>692 ± 42</td>
<td>20.6 ± 1.4</td>
</tr>
<tr>
<td>( db/db ) Enalapril</td>
<td>658 ± 27</td>
<td>832 ± 23</td>
<td>16.0 ± 3.3</td>
</tr>
<tr>
<td>( db/m ) Vehicle</td>
<td>159 ± 4</td>
<td>218 ± 12*</td>
<td>7.7 ± 0.7*</td>
</tr>
</tbody>
</table>

Male \( db/db \) mice (age 10 weeks), \( n = 8–10 \) per group were treated daily with vehicle, linagliptin 3 mg/kg/day, or enalapril 20 mg/kg/day for 86 days. Baseline plasma was taken at day –6. \( db/m \) mice were used as healthy controls. Plasma parameters represent mean ± SEM. HbA1C, type 1A glycated haemoglobin.

\*P < 0.05 vs. vehicle treated \( db/db \) mice.
damage progresses, it precedes the appearance of albuminuria in both humans and diabetic db/db mice [30,31]. Podocyte cell-surface proteins such as nephrin, podocalyxin, and P-cadherin become affected. The highly expressed integral sialoprotein on the apical surface of the podocyte, podocalyxin, contributes to proper foot process and slit diaphragm formation, and plays a critical role in maintaining the ultrastructure of glomerular podocytes [32]. Expression of podocalyxin is decreased in the glomeruli of streptozotocin diabetic rats [33,34], and appears to be reduced in the kidney biopsy samples taken from patients with diabetic nephropathy [35]. Our observations of enhanced podocalyxin expression in linagliptin-treated db/db mice therefore appear to support the possibility that DPP-4 inhibition somehow protects podocytes from diabetes-induced renal injury.

Hyperglycemia-induced activation of angiotensin II promotes renal vascular vasoconstriction, inflammation, apoptosis, glomerular cell proliferation, tubular atrophy, and accumulation of extracellular matrix material by stimulating transforming growth factor-1 (TGF-1) [36,37]. Of note in the present study was that the renoprotective actions of linagliptin were comparable to those of enalapril, despite the fact that DPP-4 inhibition does not act on the RAS.

As glucose was unaffected in our tests, an alternate mechanism must explain the renoprotective effects seen in linagliptin-treated db/db mice. Table 2 summarizes the effects of linagliptin and enalapril treatment on SBP in diabetic db/db and nondiabetic db/m mice. Data are means ± SEM.

### Table 2. The effects of linagliptin and enalapril treatment on SBP in diabetic db/db and nondiabetic db/m mice

<table>
<thead>
<tr>
<th>Mice</th>
<th>Baseline (mmHg)</th>
<th>40 Days of treatment (mmHg)</th>
<th>80 Days of treatment (mmHg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>db/db</td>
<td>105 ± 4</td>
<td>104 ± 3</td>
<td>106 ± 2</td>
</tr>
<tr>
<td>db/db + Linagliptin</td>
<td>103 ± 3</td>
<td>106 ± 2</td>
<td>106 ± 3</td>
</tr>
<tr>
<td>db/db + Enalapril</td>
<td>107 ± 5</td>
<td>104 ± 3</td>
<td>105 ± 3</td>
</tr>
<tr>
<td>db/m</td>
<td>102 ± 4</td>
<td>105 ± 3</td>
<td>105 ± 4</td>
</tr>
</tbody>
</table>

Data are given as mean ± SEM.
with linagliptin. One possibility would be the enhanced activation of GLP-1R resulting from DPP-4 inhibition by linagliptin. High GLP-1R mRNA expression has been reported in the glomerulus and proximal convoluted tubule of micro-dissected rat nephron segments [38]. An autoradiographic and in-situ hybridization analysis in both mice and rats localized the renal GLP-1R within the glomerular capillary and arterial walls, and its activation increased the renal blood flow [39–41]. Moreover, recent studies have revealed that markedly reducing the expression of GLP-1 receptors in the glomeruli and activating the GLP-1R pathway with a GLP-1R agonist confers protection against oxidative stress, glomerular endothelial dysfunction, apoptotic and profibrotic actions in both type 1 and type 2 diabetic rodents [42–44]. Alternate GLP-1 mechanisms may also be involved, including the inhibition of angiotensin II signalling [45,46]. Mima et al. [42] demonstrated how hyperglycemia can activate PKCβ isoforms, which enhance the toxic actions of angiotensin II and inhibit GLP-1’s protective effects by reducing the expression of GLP-1 receptors in the glomerular endothelial cells.

To gain further understanding of the role GLP-1R may play in diabetic nephropathy, we used in-situ hybridization to study GLP-1R mRNAs in the kidney of diabetic db/db mice, addressing any concerns over low specificity and sensitivity of the existing GLP-1R antibodies [47]. Our results
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Figure 4 (Continued)

indicate the tendency of upregulation of GLP-1R in diabetic db/db mice.

On the other hand, it is possible that the beneficial effects associated with DPP-4 inhibition may not solely be a consequence of enhanced GLP-1 action. DPP-4 is also responsible for the cleavage of various substrates such as GIP, BNP/ANP, NPY, PYY, or SDF-1 alpha with their own renal and cardiovascular effects [11]. Our data show that linagliptin treatment after 7 days led to a more pronounced increase (in total as well as fold increase) of intact GIP than intact GLP-1 compared with vehicle db/db mice. Compared to GLP-1, the renal effects of GIP have been less investigated. In addition to the glucose-lowering effects, GIP regulates fat metabolism and enhances glucocorticoid secretion [48]. Interestingly, GIPRtransgenic mice, expressing the mutated human glucose-dependent insulino-

tropic polypeptide receptor (GIPR) and exhibiting severe hyperglycemia, develop progressive diabetes-associated kidney lesions with many parallels to the human disease, that is, renal, glomerular, and podocyte hypertrophy, thickening of the glomerular basement membrane, reduction of the glomerular density of podocytes, progressive glomerulosclerosis albuminuria, and tubulointerstitial changes [49]. Therefore, further analysis of the kidney is thought to be necessary to determine the physiological significance of the changes in GIP.

The membrane-bound form of DPP-4 is highly expressed on the surface of many cell types, including brush border microvilli of the proximal tubules and glomerular podocytes in rodents [50,51]. Enhanced expression of DPP-4 has been observed in the intestine, liver, and kidney of rats treated with high-fat diets and streptozotocin [52]. This implies a role of DPP-4 in the development of T2DM. Moreover, DPP-4-deficient rats are resistant to developing diabetes, but predisposed to dyslipidaemia and reduction of glomerular filtration rate in streptozotocin-induced type 1 diabetes [53]. Our immunohistochemical and EM results confirm a strong expression of DPP-4 in podocytes, proximal tubules, and thin descending limb of Henle derived from mouse kidney. We did not observe any significant differences in the renal expression of DPP-4 between linagliptin-treated and diabetic control animals.

One possible explanation for these findings is that hyperglycemia-induced cell damage resulted in a release of DPP-4 into the circulation. Previously published data indicating that DPP-4 activity is reduced in streptozotocin-induced diabetic rats, even though kidney DPP-4 mRNA and urinary DPP-4 excretion is increased, are in line with our current findings [53].

The mechanisms by which DPP-4 exerts renal pleiotropic actions have not been fully determined. How inhibition of DPP-4 ameliorated albuminuria [19], prevented glomerulosclerosis [20], and even reversed it in rodent models of human type 1 diabetes was recently reported [21]. Amelioration of diabetic albuminuria was found in patients with T2DM after treatment with DPP-4 inhibitors [22,54].

The observed reduction in albumin excretion in these studies is thought to reflect beneficial effects of DPP-4 inhibition on podocytes in the context in which podocyte loss is one of the first events leading to proteinuria [11,13]. This concept has been supported by our findings, which showed a significant increase in the expression of podocalyxin in linagliptin-treated mice compared with diabetic control animals.

Increased DPP-4 activity in the kidney and urine are well recognized hallmarks for human glomerular diseases [28,29,55]. In this study, we investigated the expression and localization of DPP-4 in the human glomerulus to further clarify the relevance of our findings in human disease. We found that DPP-4 immunoreactivity is detected in the glomeruli of patients with diabetic nephropathy, but not in healthy kidneys. Our results could indicate that DPP-4 upregulation represents a common mechanism in the development of albuminuria, glomerulosclerosis, and renal dysfunction.

Study limitations

There are several limitations to the present work that may restrict interpretation of our findings. First, we did not show any significant effects of linagliptin on blood glucose levels or HbA1C in long-term treatment which is different to clinical studies. However, the lack of a blood-glucose-lowering effects of linagliptin is consistent with the previous reports in db/db mice that showed no beneficial effects of DPP-4 inhibitors in later stages of the disease (older than 16 weeks of age) [56,57]. In earlier stages of diabetes (animals at 4 weeks of age), Chen et al. [58] demonstrated marked improvements in glucose homeostasis in db/db mice. This is also consistent to our results in the short-term study. Therefore, this study was undertaken to investigate the long-term effects of linagliptin independent of its glucose-lowering action. The dose of enalapril we used was intended to not impact on SBP.
Furthermore, our animal model did not develop severe albuminuria, a characteristic of the clinical progression of diabetic nephropathy. However, there have been reports that albuminuria is not particularly progressive in the db/db mouse model on a C57BL6 background [59]. Further in-vivo experiments in GLP-1R knock-out mice might provide additional understanding of the direct effects of the GLP-1R and DPP-4 on the progression of diabetic nephropathy.

Further limitations of our study were that only histopathological and immunohistological parameters. Methods applied are well approved, have been published before, and are detailed.

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**TABLE 3. Effect of linagliptin on DPP-4 substrates in db/db mice**

<table>
<thead>
<tr>
<th></th>
<th>Vehicle</th>
<th>Linagliptin</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose (mmol/l), fed</td>
<td>15 ± 1.7</td>
<td>11.2 ± 0.6</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>DPP-4 activity (AU)</td>
<td>12950 ± 6403</td>
<td>24136 ± 1214 (81% inhibition)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Active GLP-1 (pg/ml)</td>
<td>2.4 ± 0.4</td>
<td>18.0 ± 2.2 (7.5-fold)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Total GLP-1 (pg/ml)</td>
<td>38.3 ± 6.7</td>
<td>16.8 ± 1.6 (56% inhibition)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Active GIP (pg/ml)</td>
<td>53.2 ± 16.7</td>
<td>1312 ± 359.1 (~25-fold increase)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Active GLP-2 (pg/ml)</td>
<td>n.d</td>
<td>40.7*</td>
<td>n.a</td>
</tr>
<tr>
<td>Active SDF-1a (pg/ml)</td>
<td>n.d</td>
<td>n.d</td>
<td>n.a</td>
</tr>
<tr>
<td>Total SDF-1a (pg/ml)</td>
<td>2279 ± 148</td>
<td>2499 ± 144</td>
<td>n.s.</td>
</tr>
</tbody>
</table>

Male db/db mice (age 5 weeks), n = 8 per group were treated daily with linagliptin or vehicle for 7 days. Plasma parameters represent mean ± SEM. AU, arbitrary unit; DPP-4, type 4 dipeptidyl peptidase; GLP-1, glucagon-like peptide-1; GLP-2, glucagon-like peptide-2; n.a, not applicable; n.d, not detectable; n.s., not significant; SDF-1α, stromal-cell-derived factor 1α. *Only one out of eight values was detectable, unpaired t-test was performed.
described in the ‘Materials and Methods’ section. GLP-1 (9–36), the degradation product of active GLP-1, may have cardiovascular and in particular direct renal effects [60,61]. This was, however, not investigated in the current study and should be done in the future.

Conclusion and clinical implications

The results of the present work support the previous evidence for the DPP-4 inhibitor linagliptin to have beneficial effects on diabetic-nephropathy-associated kidney damage. Our findings suggest that this is achieved via a mechanism that is independent of the blood-glucose-lowering effects. Our observations suggest that the renoprotective action of linagliptin might be caused by attenuation of podocyte injury and inhibition of myofibroblast transformation. However, the exact mechanism of renoprotection is not fully understood. We presume involvement of pleiotropic effects of DPP-4 inhibition as well as mediated actions via several DPP-4 substrates, most likely GLP-1 and also GIP. To finally prove this hypothesis, it is necessary to work with diseased knockout mice lacking the respective DPP-4 substrate receptors. Furthermore, samples taken from human kidneys indicate that diabetic nephropathy can be characterized by an upregulation of glomerular DPP-4. Taken together, these experimental data corroborate the concept that it is possible to attenuate the processes responsible for renal damage. It has yet to be established whether these effects can be achieved in a clinical setting and whether they would deliver long-term improvements in clinical outcomes.

FIGURE 6 In-situ hybridization of kidney sections in healthy mice. Glucagon-like peptide-1 (GLP-1)R expression was observed in renal arterial vessels. No expression could be detected in glomeruli, tubules, or collecting ducts in healthy mice.

FIGURE 7 Expression of DPP-4 in diseased human kidney biopsy sample. Double labeling for DPP-4 (red) and synaptopodin (green) DPP-4 immunoreactivity is detected in glomeruli of a 67 years old male patient having type 2 diabetes since 6 years patient. (d, e, f), but not in healthy kidney from a male 49 years old person (a, b, c). DPP-4 co-localizes with synaptopodin in DN (f) (Immunofluorescence staining; original magnification 400x).
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B.H. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis. Author’s contribution was as follows: Y.S.: wrote paper, performed histological analysis; C.R.: statistical analysis; M.A.: statistical analysis; T.P.: animal experiment; O.T.: data analysis; T.K.: designed study and wrote paper; S.B.: image analysis; B.H.: wrote paper, designed study, did data analysis.

Conflicts of interest

B.H. received a research grant from Boehringer Ingelheim for performing this study. T.K. is a research employee of Boehringer Ingelheim.

REFERENCES

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Dipeptidyl peptidase-4 inhibition

Reviewers’ Summary Evaluations

Referee 1

The authors performed experiments to examine the renoprotective effect of a DPP-4 inhibitor in type 2 diabetic mice (C57BL6 background). Although these animals did not show obvious albuminuria, treatment with a DPP-4 inhibitor, linagliptin, elicited a beneficial effect on renal tissue damage through the attenuation of podocyte injury and inhibition of myofibroblast transformation in a glucose- and blood pressure-independent manner. Thus, these data suggest a possible new strategy to treat the patients with type 2 diabetic nephropathy. Future studies will be needed to investigate whether DPP-4 inhibitor reduces albuminuria via protection of glomerular podocyte in these patients.

Referee 2

By using a glycemic neutral DPP-4 inhibitor in nonhypertensive type-II diabetic rats, the authors have described a reduction of albuminuria and kidney injury in comparison with untreated animals. Again, these data point to the need of addressing new antidabetic strategies different than mere the glycemic control. In this regard, the associated alteration of the lipid component may have something to do. Unfortunately, the authors were unable to show whether these actions were insulin- or GLP-1R-dependent. Further investigations focusing on the renal role of GLP-1 isoforms before [i.e., GLP-1 (7–36)] and after [i.e., GLP-1 (9–36)] DPP-4 activity may suggest more specific therapeutical strategies against diabetic nephropathy.