Original Paper

DPP-4 Inhibition on Top of Angiotensin Receptor Blockade Offers a New Therapeutic Approach for Diabetic Nephropathy

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Key Words

Diabetic nephropathy • DPP-4 inhibitor • Linagliptin • Renin-angiotensin system

Abstract

Background: The need for an improved treatment for diabetic nephropathy is greatest in patients who do not adequately respond to angiotensin II receptor blockers (ARBs). This study investigated the effect of the novel dipeptidyl peptidase-4 inhibitor linagliptin alone and in combination with the ARB telmisartan on the progression of diabetic nephropathy in diabetic endothelial nitric oxide synthase (eNOS) knockout mice. **Methods:** Sixty male eNOS knockout C57BL/6J mice were divided into four groups after receiving intraperitoneal high-dose streptozotocin: telmisartan (1 mg/kg), linagliptin (3 mg/kg), linagliptin + telmisartan (3 mg/kg + 1 mg/kg) and vehicle. Fourteen mice were used as non-diabetic controls. **Results:** After 12 weeks, urine and blood were obtained and blood pressure measured. Glucose concentrations were increased and similar in all diabetic groups. Telmisartan alone reduced systolic blood pressure by 5.9 mmHg versus diabetic controls (111.2 ± 2.3 mmHg vs 117.1 ± 2.2 mmHg; mean $\pm$ SEM; $P=0.071$). Combined treatment significantly reduced albuminuria compared with diabetic controls (71.7 ± 15.3 μ g/24 h vs 170.8 ± 34.2 μ g/24 h; $P=0.017$), whereas the effects of single treatment with either telmisartan (97.8 ± 26.4 μ g/24 h) or linagliptin (120.8 ± 37.7 μ g/24 h) were not statistically significant. DPP-4 inhibition, alone and in combination, led to significantly lower plasma osteopontin levels compared with telmisartan alone. Histological analysis revealed reduced glomerulosclerosis after Linagliptin alone and in combination with telmisartan in comparison to non treated diabetic animals

($p < 0.01$ and $p < 0.05$). Kidney malonaldehyde immune-reactivity, a marker of oxidative stress, was significantly lower in animals treated with linagliptin. **Conclusions:** DPP-4 inhibition on top of ARB treatment significantly reduced urinary albumin excretion and oxidative stress in diabetic eNOS knockout mice. Linagliptin on top of an angiotensin II receptor blocker may offer a new therapeutic approach for patients with diabetic nephropathy.

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Introduction

As the prevalence of diabetes mellitus and its complications such as diabetic nephropathy rises in western industrial nations, the severe consequences of the disease become increasingly important. Diabetic nephropathy accounts for approximately one third of all patients who require regular dialysis [1]. Although an association between hyperglycaemia and diabetic nephropathy is evident, it is unclear why some patients with diabetes develop overt nephropathy despite optimal treatment. Evidence suggests that a therapeutic approach including blockade of renin-angiotensin system (RAS) is very effective [2-5]. Telmisartan, an effective angiotensin II receptor blocker (ARB) [6, 7] has been shown to dosage-dependently reduce transition rates to overt nephropathy [8] and to significantly increase nitric oxide (NO) activity in the renal endothelium among patients with type 2 diabetes (T2DM) [4, 9]. Hence, the need for an improved treatment for diabetic nephropathy is greatest in patients who do not adequately respond to ARBs.

Dipeptidyl peptidase-4 (DPP-4) is a ubiquitously available serine peptidase that cleaves a number of endocrine peptides. Substrates for DPP-4 include glucagon-like peptide (GLP)-1, B-type natriuretic peptide (BNP) and stromal cell-derived factor-1 α (SDF-1 α). Experimental models have attributed potential effects in kidneys and heart to these peptides [10-12]. The anti-diabetic action of DPP-4 blockade is mainly mediated by enhancement of the incretins gastric inhibitory polypeptide (GIP) and GLP-1 [13], both of which reduce hyperglycaemia [14]. In patients with T2DM, this strategy decreases both pre- and postprandial glucose levels as well as HbA_{1c} [15] without risk of hypoglycaemia [16, 17].

Linagliptin is a competitive DPP-4 inhibitor that is approved for the treatment of patients with T2DM. In non-clinical and clinical studies linagliptin has been shown to be a highly potent and well-tolerated drug [18-20]. In contrast to other approved DPP-4 inhibitors, renal excretion of linagliptin is low [11, 17, 19]. Therefore, linagliptin can be used in renally impaired patients without dose adjustment.

This study investigated the effect of linagliptin, alone and in combination with telmisartan, on the progression of diabetic nephropathy in diabetic endothelial nitric oxide synthase (eNOS) knockout mice, a new model closely resembling human pathology [21-23]. Mice were treated with adequate dosages for 11 weeks and assessed for major renal parameters, blood glucose (BG) and blood pressure (BP).

Materials and Methods

Chemicals

Linagliptin [BI1356; 8-[(3R)-aminopiperidin-1-yl]-7-(but-2-yn-1-yl)-3-methyl-1-[(4-methyl-quinazolin-2-yl)methyl]-3,7-dihydro-1H-purine-2,6-dione] and telmisartan were developed and synthesized by Boehringer Ingelheim. Unless otherwise stated, all other reagents were of analytical grade and were purchased from Sigma-Aldrich (Seelze, Germany), Merck KGaA (Darmstadt, Germany) and Carl Roth GmbH & Co. KG (Karlsruhe, Germany).

Study design

Mice were kept under standardized conditions with respect to temperature and humidity and were housed in a 12 h light/12 h dark cycle in solitary cages with food (commercial standard diet) and water *ad*

Fig. 1. Time course of the experiment.

libitum. Animal housing, care, and applications of experimental procedures complied with the Guide for the Care and Use of Laboratory Animals of the State Government of Brandenburg, Germany.

At the age of 7 weeks, 60 male eNOS (NOS3) knockout C57BL/6J mice (C57BL/6J-Nos3tm1Unc; obtained from The Jackson Laboratory, Bar Harbor, ME, USA) were randomly divided into four treatment groups 1.5 weeks after receiving intraperitoneal streptozotocin (STZ) 100 mg/kg on 2 consecutive days. Treatment groups were as follows: telmisartan (1 mg/kg; n = 17); linagliptin (3 mg/kg; n = 14); linagliptin + telmisartan (3 mg/kg + 1 mg/kg; n = 12) and vehicle (n = 17). Another 14 mice received vehicle after they had been administered citrate buffer without STZ in equal volume (non-diabetic controls). Before application of STZ, baseline measurement of BP and metabolic cages were performed to exclude preexisting differences between the study groups (week 0). All substances were given once daily by oral gavage with equal volumes per body weight. Before and several times during treatment, BG levels were measured to confirm hyperglycaemia (> 250 mg/dl) using a standard glucometer (Contour™, Bayer, Leverkusen, Germany). Animals that had only received citrate buffer were all non-diabetic (< 200 mg/dl). Body weights were quantified daily immediately before gavage. After 11 weeks of treatment, experiments with metabolic cages were performed, urine and blood samples were obtained, and BP was measured. A detailed experiment schedule is outlined in Figure 1.

Biomarkers in plasma were measured using Rules Based Medicine (Cystatin C, Osteopontin) or Millipore platforms (GLP-1 active, tumour necrosis factor- α [TNF- α]). Urinary albumin was measured using a multichannel autoanalyzer (Synchron CX7; Beckmann, Hamburg, Germany). Systolic blood pressure (SBP) was assessed via tail-cuff method.

Histological studies

Renal morphology (interstitial fibrosis, perivascular fibrosis, glomerulosclerosis and media-lumen ratio of blood vessels) was measured as described previously [24]. In brief, glomerular matrix expansion was evaluated on periodic acid-Schiff (PAS)-stained slides by rating the percentage of the PAS-positive areas within the glomerulus using a subjective, semi-quantitative score system (grade I–IV) by two investigators who were blinded to the study groups of the animals. The severity of interstitial fibrosis was evaluated after Sirius Red-staining using computer-aided devices. Thirty microscopic pictures per kidney section were transferred to a PowerMAC via Hitachi CCD camera. After manually setting a threshold using a randomly chosen subset of the pictures, we measured the relationship of Sirius Red-stained area (connective tissue) to total area of the picture using ImageJ, an image-processing software (shareware from the National Institutes of Health [NIH]). Accordingly, microscopic pictures of kidney sections after Elastica-van Gieson staining showed that arterial blood vessels were generated. We measured the area contents of the media and the lumen of intra-renal arteries using the ImageJ (shareware from the NIH); then media/lumen ratio was calculated to serve as marker for arterial wall thickening.

Immunohistochemistry

Deparaffinized kidney sections were quenched, blocked and incubated with primary antibody as follows: rabbit anti-mouse transforming growth factor β 1 (TGF- β 1) (1:50 dilution in 1.5% serum, Acris Antibodies GmbH, Herford, Germany); rabbit anti-mouse plasminogen activator inhibitor-1 (PAI-1) (1:100, Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA); and rabbit anti-SDF-1 (1:50, Boehringer Ingelheim Pharma GmbH & Co. KG). Overnight incubation at 4°C was followed by sequential application of biotinylated goat anti-rabbit immunoglobulin G (1:200 dilution for TGF- β 1 and PAI-1; 1:50 dilution for SDF-1), avidin

Table 1: Baseline measurements (values are given as mean $\pm$ SEM)

	Telmisartan 1 mg/kg	Linagliptin 3 mg/kg	Linagliptin 3 mg/kg + telmisartan 1 mg/kg	Diabetic, vehicle	Non-diabetic
Systolic blood pressure (mmHg)	116.4 $\pm$ 2.4	116.0 $\pm$ 2.2	122.0 $\pm$ 2.4*	118.6 $\pm$ 2.0	122.3 $\pm$ 2.3*

* $P < 0.05$ vs diabetic vehicle.

Table 2: Final systolic blood pressure and urine and plasma parameters (values are given as means $\pm$ SEM)

	Telmisartan 1 mg/kg	Linagliptin 3 mg/kg	Linagliptin 3 mg/kg + telmisartan 1 mg/kg	Diabetic, vehicle	Non-diabetic
Systolic blood pressure (mmHg)	111.2 $\pm$ 2.3	120.2 $\pm$ 1.6	113.8 $\pm$ 3.2	117.1 $\pm$ 2.2	118.8 $\pm$ 2.1
Plasma-blood glucose (mg/dl)	563.6 $\pm$ 23.7*	525.4 $\pm$ 36.3*	546.1 $\pm$ 28.2*	517.7 $\pm$ 28.6*	144.4 $\pm$ 5.7*
Plasma-TNF- α (ng/L)	10.9 $\pm$ 2.4**	11.8 $\pm$ 2.4**	13.5 $\pm$ 2.6**	20.8 $\pm$ 1.5	16.8 $\pm$ 3.1
Urinary albumin excretion (μ g/24 h)	97.8 $\pm$ 26.4	120.8 $\pm$ 37.7	71.7 $\pm$ 15.3**	170.8 $\pm$ 34.2	31.4 $\pm$ 10.6**

* $P < 0.05$ vs non-diabetic; ** $P < 0.05$ vs diabetic vehicle.

and biotinylated horseradish peroxidase and DAB chromogen using an ABC-staining system (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA). MDA-staining of deparaffinized kidney sections was performed using a goat anti-mouse antibody (1:200 dilution in 1.5% serum, Immundiagnostik AG, Bensheim, Germany) and HRP-DAB System (R&D Systems Europe Ltd., Abingdon, UK) following the manufacturer's procedure. Negative controls for immunostaining included omission of the corresponding primary antibody. All sections were lightly counterstained with haematoxylin.

Immunostaining was graded on blinded sections by two independent investigators using a semi-quantitative score. Glomerular expression of TGF β -1, PAI-1 and SDF-1 was carried out in 40–50 glomerular cross-sections taking into account absent, podocytic and mesangial staining. Analysis of MDA stained kidney slices was done by analogy of interstitial fibrosis evaluation using 15 pictures of each animal.

Statistical analyses

All values are given as means $\pm$ standard error of the mean (SEM). Statistical analyses were performed with SPSS 18.0 for Windows (SPSS Inc., Chicago, IL, USA). For comparisons between two groups of interest, the unpaired Student's *t*-test was used if variables were parametric and normally distributed, which was tested by Kolmogorow-Smirnov test. Otherwise, Wilcoxon-Mann-Whitney U test was used. Mortality calculations were performed using Kaplan-Meier analysis and Mantel-Cox log-rank test. Differences were considered significant if $P < 0.05$ and highly significant if $P < 0.01$.

Results

Baseline measurements, blood glucose and mortality

Baseline measurements of SBP before application of STZ revealed no significant differences between the study groups (Table 1). Furthermore, baseline metabolic cage experiments revealed no differences with regard to BG (Table 1), urinary albumin and creatinine excretion and diuresis volume (data not shown). After baseline measurements, BG was significantly higher in all diabetic groups compared with the non-diabetic control groups throughout the entire experiment (Table 1). BG levels were similar across the diabetic groups. Analyses revealed no significant differences in mortality (log rank, $P = 0.25$; data not shown).

Blood pressure and cystatin C

At the end of the experiment, SBP in animals that were treated with telmisartan alone was modestly reduced by 5.9 mmHg versus diabetic control ($P = 0.071$; Table 2), whereas the

Fig. 2. Plasma cystatin C values (final measurement) are given as means $\pm$ SEM. * P < 0.05 vs diabetic vehicles.

Fig. 4. Plasma osteopontin levels (final measurements; values are given as means $\pm$ SEM). * P < 0.05 vs telmisartan; ** P < 0.05 vs diabetic vehicles.

Fig. 3. Changes in albuminuria values (final measurement) are given as means $\pm$ SEM. (a) urinary albumin excretion per 24 h. (b) Urinary albumin/creatinine ratio. (c) Reduction in urinary albumin excretion (%) per 24 h in comparison to diabetic vehicles (their mean value equates to 0). Non-diabetic animals were not included to achieve better clarity. * P < 0.05 vs diabetic vehicles.

combination therapy reduced SBP by 3.3 mmHg versus diabetic control (P = 0.392). None of the differences between treatments reached statistical significance. All diabetic animals had similar cystatin C levels (Figure 2), which were significantly lower compared with the non-diabetic controls (P < 0.05 for each), and demonstrated that kidney dysfunction was similar in all groups.

Table 3: Kidney weight and histology (values are given as means $\pm$ SEM)

	Telmisartan 1 mg/kg	Linagliptin 3 mg/kg	Linagliptin 3 mg/kg + telmisartan 1 mg/kg	Diabetic, vehicle	Non-diabetic
Relative weight right kidney (mg/g)	8.0 $\pm$ 0.5	7.5 $\pm$ 0.4	7.6 $\pm$ 0.3	7.3 $\pm$ 0.4	5.0 $\pm$ 0.2*
Relative weight left kidney (mg/g)	8.2 $\pm$ 0.4	7.5 $\pm$ 0.4	7.7 $\pm$ 0.5	7.6 $\pm$ 0.6	5.1 $\pm$ 0.2*
Kidney interstitial fibrosis (%)	0.7 $\pm$ 0.1	0.6 $\pm$ 0.1	0.7 $\pm$ 0.1	0.8 $\pm$ 0.1	0.5 $\pm$ 0.2
Glomerulosclerosis (score)	2.2 $\pm$ 0.1	2.1 $\pm$ 0.0*	2.1 $\pm$ 0.1*	2.4 $\pm$ 0.1	2.4 $\pm$ 0.1

* $P < 0.05$ vs diabetic vehicle.

Fig. 5. Plasma active glucagon-like peptide-1 levels (final measurements; values are given as means $\pm$ SEM). * $P < 0.05$ vs diabetic vehicles.

Fig. 6. Percentage of MDA positive area in kidney tissue samples (values are given as means $\pm$ SEM). **, *** $P < 0.01$; $P < 0.001$ vs diabetic vehicles.

Urinary biomarkers

Combination treatment with linagliptin and telmisartan significantly reduced 24-h urinary albumin excretion and the albumin/creatinine ratio compared with diabetic controls ($P = 0.017$; Figures 3A and 3B; Table 2). However, the effects of telmisartan or linagliptin alone on albumin endpoints were not statistically significant. The combination treatment significantly reduced albuminuria by 58.0% (Figure 3C). Furthermore, the combined treatment led to an excretion level close to that of the non-diabetic controls.

Plasma biomarkers

Linagliptin alone and in combination led to significantly lower plasma osteopontin levels ($290.8 \pm 17.0 \mu\text{g/L}$ [$n = 13$] and $291.0 \pm 23.5 \mu\text{g/L}$ [$n = 12$], respectively) compared with telmisartan alone ($356.4 \pm 20.1 \mu\text{g/L}$ [$n = 14$]; $P = 0.021$ and $P = 0.044$, respectively). Telmisartan values for osteopontin were similar to diabetic controls ($340.1 \pm 17.9 \mu\text{g/L}$; $n = 14$; Figure 4). Differences between the diabetic controls and the linagliptin groups were not statistically significant ($P = 0.059$ vs linagliptin alone and $P = 0.105$ vs linagliptin + telmisartan). Plasma TNF- α concentrations were significantly lower in all treatment groups compared with the untreated diabetic control group (Table 2). As a matter of proof, plasma levels of active GLP-1 were highly increased in both linagliptin groups compared with the other groups (Figure 5 and Table 2).

Organs and histology

Relative organ weights (i.e. organ weights per body weight) did not differ between the treatment groups (Table 3). However, the relative weights of liver and kidneys were significantly higher in diabetic animals compared with non-diabetic animals. Interstitial fibrosis in the kidneys was highest in diabetic control animals and lowest in non-diabetic control animals, whereas the drug-treated animals ranged in between. Differences, however, were not statistically significant (Table 3). Perivascular fibrosis and media lumen ratio in the kidneys revealed no differences between the study groups (data not shown). Linagliptin, alone and in combination with telmisartan, led to a significantly reduced glomerulosclerosis score compared with diabetic controls ($P = 0.002$ for linagliptin alone and $P = 0.027$ for combination, respectively), whereas the reduction achieved by telmisartan alone was not significantly different ($P = 0.056$; Table 3). Immunohistochemical staining of the kidneys revealed no differences between the study groups with regard to glomerular and interstitial TGF- β 1, PAI-1 and SDF-1 expression (data not shown). Staining for renal malonaldehyde immune-reactivity, a marker of oxidative stress, revealed a significant increase in vehicle treated diabetic animals as compared with non-diabetic controls. DPP-4 inhibition with and without telmisartan prevented this increase and therefore showed significantly lower expression rates as compared with diabetic vehicle (Fig. 6), whereas telmisartan alone had no significant beneficial effects on MDA expression.

Discussion

The overall goal of this study was to investigate the effect of the DPP-4 inhibitor linagliptin, alone and in combination with guideline-based treatment with an ARB (telmisartan), on the progression of diabetic nephropathy in a mouse model closely resembling human diabetic nephropathy in terms of clinical course and pathology. In contrast to telmisartan alone, treatment with linagliptin plus telmisartan significantly reduced urinary albumin excretion in diabetic mice in a blood pressure-independent manner. Additionally, linagliptin treatment (either alone or with telmisartan) reduced plasma levels of the vascular calcification marker osteopontin compared with telmisartan alone. By contrast, osteopontin values in the telmisartan group were similar to diabetic vehicle. Moreover, the tissue immune-reactivity of malondialdehyde, a biomarker of oxidative stress, was normalized by DPP-4 inhibition with linagliptin, whereas angiotensin II receptor blockade alone failed to reduce oxidative stress in diabetic eNOS knockout mice. Glomerulosclerosis was significantly lower in the linagliptin groups in contrast to telmisartan alone.

The chosen animal model is robust and mimics many aspects of human diabetic nephropathy such as loss of glomerular filtration rate (GFR), glomerular and tubulointerstitial pathology, and albuminuria [7, 21-23]. Results for plasma cystatin C showed that the experiment had been finished in an early, still hyperfiltrating stage [25]. From a clinical point of view, the knockout of the eNOS gene resembles pathology in diabetic patients who frequently suffer from endothelial dysfunction, showing functional loss of eNOS activity.

Supporting this clinical observation, genetic studies have shown that polymorphisms of the eNOS (NOS3) gene with a consecutive lower production of the important vascular modulator NO [26-28] were associated with the development of nephropathy in patients with T1DM or T2DM [29, 30]. Moreover, this pathogenetic mechanism is intensively discussed as causal for patients with diabetes who are still developing overt nephropathy despite optimal therapy including ARBs [4, 9, 31, 32]. Moreover, there is evidence that RAS blockade ameliorated renal injury in diabetic wild type mice but not in diabetic eNOS knockout mice, indicating that impaired NO bioavailability was responsible for resistance to RAS blockade [7]. In the present study, treatment with telmisartan alone slightly lowered urinary albumin excretion but failed the statistical significance test ($P = 0.101$), whereas linagliptin plus telmisartan led to significantly lower urinary albumin excretion and albumin/creatinine ratio (Figure 3).

Urinary albumin excretion is a very early stage predictor for the development and progression of diabetic nephropathy, signaling renal damage when other markers such as plasma creatinine and cystatin C levels are unremarkable [8, 33, 34]. Albuminuria is not only associated with renal and cardiovascular morbidity and mortality in patients with diabetes [33, 35, 36] but also can predict cardiovascular and non-cardiovascular events in healthy patients [37, 38]. Hence, the reduction of albuminuria in our experiment is of clinical importance.

It is noteworthy that our results appeared independently of changes in BG and SBP. BG levels were highly increased in diabetic animals, and treatment did not have significant influence on glucose. Given that linagliptin increases endogenous insulin release via augmentation of plasma GLP-1, this observation in STZ-treated mice was not surprising [39]. In any case, treatment with linagliptin led to highly elevated plasma GLP-1 levels compared with all other study groups (Figure 5), confirming that linagliptin was administered in an adequate dosage [40]. The study was designed with the intention that telmisartan would be used in a non-BP-lowering dose to avoid BP-lowering effects that might on their own influence the primary endpoint albuminuria [7]. Telmisartan alone slightly reduced SBP without significance ($P = 0.071$), and combination therapy completely lacked significance ($P = 0.392$) compared with diabetic control animals. Indeed, RAS blockers are usually intended to lower BP in patients and are therefore administered in dosages as high as possible. However, in the current model the increase of RAS blocker dosage does not necessarily reduce SBP, as was recently shown by Kosugi and colleagues [7]. Telmisartan in similar dosages of ours (2 mg/ kg per day and body weight) only slightly reduced SBP, whereas enalapril in dosages of up to 50 mg/ kg showed at most only transient SBP lowering effects. Furthermore, neither albuminuria nor renal injuries were effectively reduced by telmisartan (2 mg/ kg) and ramipril (independently of the chosen dosage), respectively. As these results stand in line with our findings, it appears unlikely that we would have seen any further beneficial telmisartan effects if we had increased the dosage, underlining the therapeutical insufficiency of RAS blockade in this model, independently of SBP control. Moreover, as depicted in table 1, baseline SBP measurements revealed a significant increase in the combination group despite animals had not received any treatment at that time of experiment. We believe that this difference is caused by chance. However, if not and that differences was of impact, we would expect less observed benefits in the combination group as compared to the other groups since SBP is a risk factor of diabetic nephropathy and vasculopathy. Hence, this observation potentially underlines our conclusions.

Histologic analyses support our findings concerning albuminuria. Glomerulosclerosis score as the morphologic surrogate of diabetic kidney damage was significantly reduced in animals that were treated with linagliptin, alone and in combination, compared with diabetic controls. As eNOS knock out and hypertension themselves [41] state a major renal risk factor which is at most only aggravated by hyperglycemia, glomerulosclerosis does not statistically differ between diabetic and non-diabetic control animals. In contrast to heart tissue [10], where the beneficial effects of DPP-4 inhibition could most likely be attributed to enhanced recruitment of CXCR-4+ circulating progenitor cells as a result of reduced cleavage of SDF-1 α , our immunohistologic findings did not support this pathway. However, evidence [42] suggests a link between diabetes, DPP-4 inhibitors and the vascular calcification marker osteopontin [43, 44] which was significantly reduced in animals that had been treated with linagliptin compared with telmisartan alone. We were able to show that long-term treatment with linagliptin reduced plasma levels of osteopontin, compared with treatment with telmisartan alone, where levels were similar to the diabetic control group. Osteopontin is known to be associated with vascular calcification and cardiovascular morbidity in humans [44] and has already been called “the killer of patients with CKD” [43]. These findings support those from short-term treatment in a chronic renal failure model in rats [11].

However, linagliptin-related enhancement of plasma GLP-1 levels probably also accounts for direct renoprotective actions. GLP-1 receptors (GLP-1Rs) are expressed in the kidney, heart and vasculature, and recent studies have shown that GLP-1R agonists have

cardiovascular benefits that are independent of improved glucose homeostasis such as modulation of heart rate, BP, vascular tone and myocardial contractility [45]. Furthermore, Koderá and colleagues [12] have recently demonstrated that the GLP-1R agonist exendin-4 exerts renoprotective effects through its anti-inflammatory action via GLP-1R without lowering BG. In the present study we show that similar effects were achieved by inhibition of DPP-4, which resulted in highly increased plasma GLP-1 levels (Figure 5). Moreover, we have shown that DPP-4 inhibition significantly reduced renal malondialdehyde immune-reactivity in diabetic eNOS knock out mice (Figure 6). Reactive oxygen species degrade polyunsaturated lipids, forming malondialdehyde, a reactive aldehyde, that is a reactive electrophile species that causes toxic stress in cells and forms covalent protein adducts referred to as advanced lipoxidation end-products (ALE) [46]. Malondialdehyde is thus a biomarker of the tissue concentration of reactive oxygen species and its tissue damage and is widely used for the assessment of oxidative stress [47, 48].

Oxidative stress is thought to play a major role in the pathogenesis of diabetic nephropathy [48-50]. In this context, it is of note that the GLP-1 analog liraglutide likewise reduced oxidative stress in a rat model of diabetic nephropathy via a GLP-1 receptor- PKA-mediated inhibition of renal NAD(P)H oxidase (Nox4) [51]. GLP-1 plasma concentrations were substantially increased in our *in vivo* study (Figure 5). Recent studies likewise demonstrated that linagliptin inhibits NAD(P)H oxidase *in vitro* and *in vivo* [52].

Finally, treatment with either linagliptin, telmisartan, or both led to significantly lower plasma TNF- α levels compared with diabetic control animals, supporting the hypothesis of lower inflammatory activity in the treatment groups. In contrast to exendin-4 and GLP-1, linagliptin can be orally administered once daily and is approved for use in patients with declining renal function without the need for dose adjustment, which is a benefit for patients with diabetic nephropathy.

Conclusion

In conclusion, this study showed for the first time that additional treatment on top of ARB with the DPP-4 inhibitor linagliptin may offer a new therapeutic approach for patients with diabetic nephropathy. It would be of interest to analyze potential effects of linagliptin in non-diabetic models of chronic renal failure as well, since the observed effects were independent of the glucose lowering effects of linagliptin. Moreover, our findings should stimulate investigators to perform clinical studies using either suitable biomarker strategies for phase 2 studies [53-56] or hard clinical endpoints typically used in phase 3 registration trials for chronic renal failure such as doubling of serum creatinine, need for renal replacement therapy and total mortality [57] investigation the effects of linagliptin in patients with diabetic nephropathy.

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