DPP4 Inhibition Attenuates Filtration Barrier Injury and Oxidant Stress in the Zucker Obese Rat

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Objective: Obesity-related glomerulopathy is characterized initially by glomerular hyperfiltration with hypertrophy and then development of proteinuria. Putative mechanisms include endothelial dysfunction and filtration barrier injury due to oxidant stress and immune activation. There has been recent interest in targeting dipeptidyl peptidase 4 (DPP4) enzyme due to increasing role in non-enzymatic cellular processes.

Methods: The Zucker obese (ZO) rat (aged 8 weeks) fed a normal chow or diet containing the DPP4 inhibitor linagliptin for 8 weeks (83 mg/kg rat chow) was utilized.

Results: Compared to lean controls, there were increases in plasma DPP4 activity along with proteinuria in ZO rats. ZO rats further displayed increases in glomerular size and podocyte foot process effacement. These findings occurred in parallel with decreased endothelial stromal-derived factor-1α (SDF-1α), increased oxidant markers, and tyrosine phosphorylation of nephrin and serine phosphorylation of the mammalian target of rapamycin (mTOR). DPP4 inhibition improved proteinuria along with filtration barrier remodeling, circulating and kidney tissue DPP4 activity, increased active glucagon like peptide-1 (GLP-1) as well as SDF-1α, and improved oxidant markers and the podocyte-specific protein nephrin.

Conclusions: These data support a role for DPP4 in glomerular filtration function and targeting DPP4 with inhibition improves oxidant stress-related glomerulopathy and associated proteinuria.

Introduction

The rates of overweight and obesity have increased over the past three decades (1,2) and coincide with a growing incidence in chronic kidney disease (CKD) (3). In this regard, obesity has emerged as an independent risk factor for CKD (4) that is characterized initially by glomerular hyperfiltration with subsequent development of hypertrophy and proteinuria (5,6). The putative mechanisms for obesity-related hypertrophy and proteinuria include activation of growth pathways and endothelial dysfunction/injury to the filtration barrier. The filtration barrier is composed of three layers; the endothelium, a basement membrane, and visceral epithelial podocytes. Damage to the filtration barrier such as that can occur with oxidant stress and dysfunctional immune activation leads to loss of protein (e.g., proteinuria). However, the mechanisms underlying initiation and progression of obesity-related kidney disease are not well understood.

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Recently, there has been increasing interest in the dipeptidyl peptidase 4 (DPP4) enzyme as a novel target to improve kidney injury (7,8). DPP4 is an exopeptidase expressed at high levels in glomerular epithelial and endothelial cells, kidney proximal tubule cells (PTCs), and immune cells such as T-cells (a.k.a. CD26) (9,10). The DPP4 enzyme degrades substrates related to signal transduction, such as glucagon-like peptide (GLP)-1 and immune function (9,10). Under conditions of obesity and insulin resistance, circulating and tissue DPP4 activity, including that in the kidney, may be increased independent to that of the contribution of diabetes (11-14). Recent work suggests that DPP4 inhibition is protective against ischemic-reperfusion injury in the kidney (15) and reduces inflammation as well as oxidant stress in the kidney, heart and in the endothelium of diabetic animal models (16-18). Moreover, in another model of diabetic nephropathy such as the Zucker diabetic fatty (ZDF) rat, DPP4 inhibition improved selective markers of inflammation and oxidant stress in addition to improvements in glomerulosclerosis and albuminuria (17). However, little is known regarding the role of DPP4 inhibition on obesity-related kidney disease in non-diabetic conditions.

In this regard, the Zucker obese (ZO) rat has been widely used as a model of obesity-related kidney disease or glomerulopathy with progressive increases in glomerular size and proteinuria (19,20). In this model, proteinuria is initially due to underlying endothelial dysfunction and injury to the glomerular filtration barrier with loss of endothelial fenestrae and podocyte foot process effacement (19,20). Filtration barrier injury is thought to be attributable to inflammation and oxidative stress and alteration in growth pathways such as the mammalian target of rapamycin (mTOR) (21). In contrast to diabetic kidney disease, obesity-related kidney disease does not manifest severe diabetic changes such as GBM thickening. To address the impact of DPP4 inhibition on kidney tissue injury in an obesity model we used linagliptin, a DPP4 inhibitor that has greater selectivity than other peptidase inhibitors (22), and it has an in vitro IC50 of approximately 1 nM indicating a high level of potency as a DPP4 inhibitor (23). Due to the tissue-specific effects of linagliptin, we hypothesized that treatment with linagliptin over two months would attenuate development of glomerulopathy and filtration barrier injury in the ZO rat through improvements in oxidative stress.

Methods

Methods details for previously described procedures are presented in the Supporting Information.

Experimental parameters

Male ZO and age-matched Zucker Lean (ZL) rats were purchased from Charles River, Inc and housed in a 12 hour light/dark altered room. Animals were cared for in accordance with National Institutes of Health guidelines. All procedures were approved and performed in accordance with the Institutional Animal Care and Use Committee of the University of Missouri. Linagliptin (BI 1356; (R)-8-(3-aminopiperidin-1-yl)-7-buty-2-ynyl-3-methyl-1-(4-methyl-quinazolin2-ylmethyl)-3,7-dihydropurine-2,6-dione) was administered orally by mixing drug with rat chow (24). The final concentration of linagliptin in chow was 83 mg LGT/kg−1 day−1 and 50-100 mM, respectively (24). Rats were divided into four groups to include ZL control (ZL-C), ZL treated with linagliptin (ZL-L), ZO control (ZO-C) and ZO treated with linagliptin (ZO-L). Rats were weighed immediately prior to the start of the experiment (8 weeks of age) and every week thereafter until the end of the experiment (16 weeks of age) to monitor weight gain.

Proteinuria measurements

Both creatinine and protein concentrations in urine were analyzed on an automated clinical chemistry analyzer (AU680; Olympus America, Centerville, PA) using commercially available assays (25).

Protein expression semiquantitation

Preparation of Whole Tissue Extracts: 30-50 mg of kidney tissue was homogenized in ice-cold buffer containing 1% triton X-100, 100 mM NaCl, 20 mM Tris pH 7.5, 2.0 mM EDTA, 10 mM MgCl2, 10 mM NaF, 40 mM β-glycerol phosphate, 1 M PMSF, 2 mM sodium orthovanadate, protease inhibitor cocktail tablet (Roche Diagnostics, Indianapolis, IN), 10 μg/mL leupeptin, 7 μg/mL pepstatin. Homogenates were treated with 1% SDS (Triton-X insoluble fraction) and boiled and centrifuged to collect the supernatant. Protein concentrations were determined using a BCA protein quantitation kit (Thermo Scientific, Rockford, IL).

Immunoblotting: Proteins were separated by SDS-PAGE and transferred to nitrocellulose membranes and blocked for 1 h at RT. Primary antibodies used in the study were DPP4 (ProteinTech, Chicago IL), p-Tyr1217-nephrin (Abcam, Cambridge MA), nephrin (kind gift from Puneet Garg, Ann Arbor MI), p-Ser2448-mTOR, p-Ser2481-mTOR, and mTOR (Cell Signaling, Danvers, MA). Antibody binding was detected by chemiluminescence and images recorded using a Bio-Rad ChemiDoc XRS image analysis system (Bio-Rad Laboratories, Santa Cruz, CA). Protein band density quantitation was performed using Image Lab software (Bio-Rad Laboratories).

Immunohistochemistry

Kidney cortical tissue was harvested and prepared as previously described (25). Primary antibodies used were p- Ser2448-mTOR (Ab109268) and mTOR (Ab2732), both from Abcam (Cambridge MA), SDF-1α (Novus Biologicals, Littleton CO). Brieﬂy, 4-μm sections were incubated with primary antibodies overnight at room temperature. Then slides were washed and incubated with 1:300 secondary antibodies for 4 hrs and viewed with a biphoton confocal laser-scanning microscope and signal intensities quantified by MetaVue as average gray-scale intensities.

DPP4 activity and active and total GLP-1 assay

As previously described (24), blood was collected in EDTA tubes and plasma was stored at −80°C. Kidney whole tissue extracts were prepared in sucrose buffer as described before (19,25). Sucrose buffer and not SDS containing buffer was used to prevent enzyme dissociation/degradation. Further details on DPP4 activity are provided in the Supporting Information. GLP-1 active and total in plasma was performed by Mesoscale discovery metabolite assay as described earlier (26).

Polyclonal antibody generation versus intact SDF-1α

Antigens were generated by conjugating terminus-specific peptides (KPVSLLHHHC) to keyhole limpet hemocyanin (KLH, Pierce, Rockford, IL). Rabbits were immunized at Pineda Antibody Service, Berlin, Germany and polyclonal antibodies were purified using peptide-affinity chromatography.
Targeting DPP4 in the Obese Kidney

DPP4 inhibition improves obesity-induced glomerular filtration barrier injury in the ZO rat

DPP4 is expressed in glomerular endothelial and epithelial cells in the kidney (10). The endothelium comprises a part of the glomerular filtration barrier and injury over time can lead to proteinuria. Similar to previous work in the ZO (5,17,19), there were significant increases in proteinuria in the ZO compared to ZL rats (P < 0.05) and proteinuria was ameliorated with linagliptin (Figure 2A). On ultrastructural analysis utilizing TEM, ZO rats exhibited loss of endothelial fenestrae along with podocyte effacement and loss of slit pore diaphragm which were restored by linagliptin treatment (Figure 2B). To determine the underlying proteins involved in the integrity of the glomerular filtration barrier, we examined expression of the podocyte-specific protein nephrin. Phosphorylation of nephrin is indicative of podocyte effacement in certain conditions such as passive Heymann nephritis or 27A antibody injection (28). In this regard, the increases in proteinuria and ultrastructural changes to the filtration barrier in ZO rats were accompanied by increases in Tyr1217 phosphorylation of nephrin, and this was attenuated with linagliptin treatment (Figure 2C).

DPP4 inhibition attenuates obesity-related glomerulomegaly in the ZO rat

In parallel with our proteinuria findings, there were increases in glomerular size in ZO compared to ZL rats that were improved with linagliptin in ZO rats (Figure 3A). Along with notable glomerulomegaly in ZO controls on light microscopy there was marked podocyte hypertrophy on ultrastructural TEM analysis (Figure 3B). Compared to ZL control rats, the ZO controls demonstrated marked hypertrophy and increase of variable electron dense intracytoplasmic proteinaceous and glycogen inclusions in the peripherally located podocytes. These findings were attenuated in the linagliptin treated ZO rats (Figure 3B). Cell growth and hypertrophy is dependent on serine (Ser)/threonine (Thr) kinases that regulate cell growth and protein translation such as the mammalian target of rapamycin (mTOR) pathway. In parallel with our light and ultrastructural observations in the ZO rat, there were increases in phosphorylation of mTOR in the

Results

DPP4 inhibition suppresses DPP4 activity and increases SDF-1α in the kidney, as well as increases active SDF-1α and active GLP-1 in the plasma

Very recently, we established that linagliptin (83 mg/kg chow) suppressed DPP4 activity in the plasma by >80% in ZO rats (24). At this dose, we demonstrated improvements in cardiac diastolic function and blood pressure despite minimal changes in body weight or insulin sensitivity. In the current study, kidney cortical tissue DPP4 activity was suppressed by 50% in ZL rats and ~75% in ZO rats although there was no significant increase in the ZO when compared to the ZL rats (Figure 1A). To then determine whether the reduction in renal DPP4 activity was related to protein expression, we examined kidney protein extracts and found no significant change in DPP4 expression with linagliptin treatment (Figure 1B).

In plasma, an important substrate of DPP4, GLP-1, trended towards an increase in the ZO rat as determined by both active and total GLP-1 levels (Figure 1C,D). However, linagliptin treated ZO and ZL rats had a ~sixfold increase in active GLP-1 levels when compared to untreated controls (Figure 1C). DPP4 is an exopeptidase and degrades other substrates with Xaa-Ala/Pro at the N-terminus and therefore has the potential to mediate pleiotropic effects independent of GLP-1. In this regard, another known substrate, SDF-1α, is a chemokine responsible for recruitment of endothelial progenitor cells and endothelial function (27). The expression of SDF-1α was localized to the glomerulus as well as the distal tubule on immunostaining (Figure 1E). There were decreases in glomerular intensities of SDF-1 α in the ZO compared to ZL controls, a difference which was abolished with DPP4 inhibition (Figure 1E). Similar to GLP-1, linagliptin increased plasma SDF-1α (Figure 1F).

Measurement of NADPH oxidase activity

NADPH oxidase activity was determined in plasma membrane fractions as described before (19,25) and details are in the Supporting Information.

Reactive oxygen species (ROS) formation

Accumulation of ROS in whole kidney tissue was measured by chemiluminescence assay as described before (19) and details are in the Supporting Information.

3-Nitrotyrosine

3-nitrotyrosine staining of kidney sections was done as described previously (25) and details are in the Supporting Information.

Quantification of glomeruli size

Five zm sections of paraffin embedded cortical tissue from different treatments groups were stained with Verhoeff van Gieson (VVG) stain. Briefly, images from all cross sections of cortical tissue (minimum 10 glomeruli per animal cut at the vascular pole) were randomly captured, and the glomerular tuft area was quantified with MetaVue. Samples from five rats from each of the four treatment groups were analyzed.

Ultrasound analysis with transmission electron microscopy

Details of kidney tissue preparation, sectioning, staining and viewing are as previously described (19,25). A JOEL 1200-EX transmission electron microscope (TEM) was utilized to review three fields randomly chosen per rat to obtain three 60,000 X images/LV.

Statistical analysis

Results are reported as the mean ± SE. Two-way ANOVA and post hoc t-tests (Holm-Sidak) were performed to examine differences in outcomes between control and linagliptin treated ZL and ZO groups. A P value <0.05 was considered significant.

Results

DPP4 inhibition suppresses DPP4 activity and increases SDF-1α in the kidney, as well as increases active SDF-1α and active GLP-1 in the plasma

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kidney cortex (p-Ser2448-mTOR and p-Ser2481-mTOR; Figure 3C, immunoblots and immunohistochemistry). Moreover, consistent with our structural observations there were significant reductions in glomerular mTOR (p)/activation with linagliptin treatment (Figure 3C lower panel).

DPP4 inhibition improves oxidant stress
A major source of ROS in the kidney is derived from the activation of NADPH oxidase enzyme. We and others have reported increased NADPH oxidase activity and expression of NADPH oxidase subunits the ZO kidney (19). In the current study, there

**Figure 1** DPP4 inhibition in the Zucker obese (ZO) rat improves (A) DPP4 kidney tissue activity but not (B) expression as determined by western blot in the Zucker obese (ZO) rat. DPP4 inhibition also increases substrates (C) circulating active GLP1 and (D) SDF-1α determined by immunostaining with corresponding measures of intensity on the right and does not affect (D) total GLP1 measured on Lumines100. (E) Representative images of SDF-1α immunostaining with corresponding measures of intensity to the right. (F) Circulating levels of serum SDF-1α. Data are represented by mean ± SE. *P < 0.05 when ZO controls (ZO-C) are compared to Zucker lean controls (ZL-C); †P < 0.05 when linagliptin treated Zucker obese rats (ZO-L) are compared to ZO-C. For Western blot, equal loading was ensured via quantitation of Ponceau S. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]
were increases in NADPH oxidase activation (Figure 4A) as well as ROS production (Figure 4B) in ZO when compared to ZL rats; findings that were improved with linagliptin treatment in the ZO. We noted a paradoxical increase in NADPH oxidase activity in linagliptin treated ZL rats; however, this did not contribute to ROS accumulation. 3-nitrotirosine (3-NT) is a marker for peroxynitrite formation and largely thought to be a marker for NO-dependent nitrative stress. Similar to our NADPH oxidase and ROS observations, renal 3-NT was also increased in the ZO compared to ZL and this was attenuated with linagliptin (Figure 4C).

Discussion

The current observation that DPP4 inhibition improved obesity-related glomerulopathy, including glomerular filtration barrier injury and proteinuria in the ZO rat, suggest that targeting DPP4 may have a beneficial effect on the initial stages of obesity-related kidney disease. In this regard, obesity-related kidney disease parallels the initial stages of diabetic kidney disease and our finding that the ZO rat developed glomerular hypertrophy, loss of filtration barrier integrity with loss of endothelial fenestrae and segmental podocyte effacement, and proteinuria support what has previously been observed in the ZO as well as the Zucker diabetic rat (5,17,19,20). In contrast to diabetic kidney disease, obesity-related kidney disease may have less severe glomerular injury with more subtle kidney lesions such as focal and segmental podocyte effacement. Although reduction in blood pressure may be partially responsible for the observed reductions in glomerular injury, there is evidence in this study for non-hemodynamic benefits of DPP4 inhibition (14). Indeed, worsening of glomerular structural integrity occurred contemporaneously with reductions in SDF-1α, increases in the redox-sensitive growth kinase mTOR, and increases in NADPH oxidase, ROS production and 3-NT content suggest the glomerular filtration barrier is particularly susceptible to redox homeostasis. The observation that DPP4 inhibition normalized SDF-1α, the podocyte-specific nephrin, and markers of oxidant stress in the ZO kidney support a unique role for DPP4 inhibition on glomerular integrity in obesity-related kidney disease.

DPP4 expression is localized to the podocytes, endothelial cells and the proximal tubule brush border in the kidney (10,14). Similarly, GLP-1 receptors are expressed on podocytes and endothelial cells (29,30). The observation that DPP4 inhibition reduced circulating active GLP-1 level in the ZO rat along with marked inhibition of DPP4 enzyme activity in kidney tissue lysates support a role for DPP4 actions in the kidney and on glomerular function. Although DPP4-dependent effects do contribute to glycemic control, DPP4 is known to have a number of substrates not related to GLP-1 regulation (30-31). In this regard, recent data suggest that the DPP4 enzyme regulates other substrates such as neuropeptide Y and SDF-1α (30-32). SDF-1α is constitutively expressed in stromal cells, dendritic cells as well as endothelial cells and is responsible for the recruitment of endothelial progenitor cells that differentiate into mature endothelial cells and promote vascular repair and improve endothelial function (31,32). Accordingly, the finding that DPP4 inhibition increased glomerular expression of SDF-1α suggests a role for DPP4 in regulating SDF-1α in the glomerular endothelium, beyond its actions on GLP-1. In addition, we observed an increase in total SDF-1α levels in the serum of both ZL and ZO rats treated with linagliptin suggesting that DPP4 inhibition increases systemic as well as kidney active SDF-1α levels.

The glomerular filtration barrier is composed of endothelium, basement membrane and podocytes and injury results in loss of endothelial fenestrae and podocyte foot process effacement. The mechanisms proposed for loss of endothelial fenestrae include sFlt1 overexpression or decreased VEGF expression. Podocytes cover the outside of the basement membrane and form interdigitating foot processes that serve as a final barrier against urinary protein loss. Nephrin is an integral membrane protein in the podocyte that interacts with other structural proteins such as podocin and CD2AP in forming the slit pore diaphragm. Modulation of the slit pore...
diaphragm underlies podocyte effacement which in turn precipitates proteinuria. Podocyte effacement can result from loss of nephrin as occurs in congenital nephrotic syndrome of the Finnish type or decrease in phosphorylation of nephrin seen in minimal change disease (28). However, nephrin phosphorylation is increased in passive Heymann nephritis or 27A antibody injection models of podocyte effacement (28). Hyperphosphorylation of nephrin could disrupt nephrin-podocin interaction or it could lead to unstable nephrin protein and loss from the slit pore diaphragm resulting in podocyte effacement. In addition, nephrin is critical to signaling and lamellipodial modulation via tyrosine phosphorylation at the COOH terminus and hyperphosphorylation may be a dynamic feature in the adaptation to effacement. In this regard, there have been limited data on the function of DPP4 on the podocyte, yet one study suggests DPP4 is present in both podocytes and the endothelium (10).

Our ultrastructural observations of foot process effacement were also accompanied by podocyte hypertrophy as well as mesangial...

Figure 3 DPP4 inhibition improves obesity-related glomerulomegaly in the Zucker obese (ZO) rat. (A) Representative light micrographs of Verhoeff-van gieson stained kidney cortical sections, which stains collagen pink, with corresponding measures of glomerular area to the right. (B) Ultrastructural analysis using transmission electron microscopy demonstrates marked epithelial podocyte cell hypertrophy and increase of variable electron dense intracytoplasmic proteinaceous and glycogen inclusions (arrow) in the body of podocytes in the ZO control (ZO-C). These findings were absent in linagliptin treated ZO rats (ZO-L) that were similar to Zucker lean controls (ZL-C). Magnification ×800; bar = 2 μm. (C) Western blot analysis of mTOR growth kinases with quantitative analysis to the right. (D) Representative images of Ser2448 phosphorylated mTOR immunostaining with corresponding measures to the right. Data are represented by mean ± SE. *P < 0.05 when ZO controls (ZO-C) are compared to Zucker lean controls (ZL-C); †P < 0.05 when linagliptin treated Zucker obese rats (ZO-L) are compared to ZO-C. For Western blot, equal loading was ensured via quantitation of Ponceau S. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]
cell expansion, findings typically associated with obesity (5,6). There has been much interest in growth kinases in the kidney in recent years and specifically the mTOR (21,33). mTOR is an evolutionarily conserved nutrient sensing kinase that is highly regulated by insulin, carbohydrates and other nutrients (33) that is recently shown to induce mesangial expansion (34). In this regard, mTOR-dependent mesangial expansion occurs through effects on protein transcription and translation through the repressor protein 4E-binding protein (4EBP) and the 70-kD ribosomal S6 kinases (p70S6K) (33-35). The observation that increased phosphorylation of mTOR in the ZO renal cortical tissue was accompanied by glomerular expansion and podocyte hypertrophy, support this notion. Moreover, DPP4 inhibition decreased phosphorylation of mTOR in the glomeruli suggesting that DPP4 may regulate mTOR signaling in the kidney in cell types other than mesangial cells. In this respect, the reduction in glomerular size is likely due to the effects of DPP4 inhibition on mTOR/S6K signaling related growth.

There is accumulating data suggesting that mTOR is redox-sensitive and the pathways that govern hypertrophy and hyperfiltration in obesity-related glomerulopathy are susceptible to oxidant injury (5,6,35,36). In this regard, ROS are generated by endothelial and epithelial cells within the kidney and mediate a wide range of cellular functions. In the early stages of obesity-induced hyperfiltration, ROS contribute to endothelial dysfunction through reductions in bioavailable nitric oxide, impaired endothelial growth or repair, and increased activation of adhesion and inflammatory cytokines that occur independent of elevations in blood pressure or hyperglycemia (37,38). The effects on impaired endothelial function subsequently contribute to podocyte injury and inflammation in the mesangium, mediated by TNF-α and MCP-1 pathways (20,37,38). Our observations that NADPH oxidase activity and ROS production were increased in the ZO kidney are consistent with this notion. Moreover, the increased glomerular intensities of 3-NT are consistent with the notion of an early stage of obesity-induced glomerular injury. DPP4 inhibition has been shown to reduce circulating serum malondialdehyde in a model of renal ischemia-reperfusion injury and kidney tissue malondialdehyde levels in an endothelial nitric oxide synthase (eNOS) knockout mouse (15,39). A recent report demonstrated that deletion of DPP4 in isolated cardiomyocytes protected cells from H2O2-induced oxidant injury (40). Thus, our data support an anti-oxidative role for DPP4 inhibition mediated by a reduction in NADPH oxidase-dependent ROS generation resulting in improved lipid peroxidation and marked reductions in glomerular 3-NT content. Although we have observed increased NADPH oxidase by linagliptin in control ZL rats, this was not accompanied by increased accumulation of ROS or peroxynitrite suggesting either more efficient removal of superoxide through up regulation of antioxidant pathways or that NADPH oxidase is increased as a compensatory mechanism in response to decreased ROS.

In summary, the findings of this investigation suggest for the first time a role for DPP4 in glomerular filtration function and that targeted inhibition of DPP4 improves obesity-related glomerulopathy and filtration barrier injury due to oxidant stress. Our finding that DPP4 inhibition improved oxidant stress in this early stage of kidney injury is particularly noteworthy. Although a reduction in blood pressure may be partially responsible for kidney improvement, our
data suggest contribution of non-hemodynamic factors that are regulated by DPP4 (14).

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