DPP4 Inhibition in Human Kidney Proximal Tubular Cells - Renoprotection in Diabetic Nephropathy?

Usha Panchapakesan*, Simon Gross, Murali Gangadharan Komala, Kate Pegg and Carol A Pollock

Renal Research Group, Kolling Institute of Medical Research, Royal North Shore Hospital, University of Sydney, Australia

Abstract

Novel diabetic drugs that target the incretin system e.g. dipeptidyl peptidase 4 (DPP4) inhibitors have pleiotropic effects other than inhibiting the cleavage of glucagon like peptide 1 (GLP-1). As DPP4 is located at the brush border of the kidney proximal tubular cells (PTC), we hypothesise that it is involved in the processing of luminal peptides influencing the kidney tubulointerstitium in diabetes. Using HK2 cells (human kidney proximal tubular cell line) in order to study the GLP-1 independent effects of DPP4 inhibition on the PTC cells were exposed to high glucose conditions, high mobility protein 1 (HMGB1) and meprin beta. Relevant markers like DPP4, fibronectin, collagen IV, transforming growth factor beta (TGFβ), activator protein 1 (AP-1) and nuclear factor-kappa B (NF-κB) were measured. We show that linagliptin (DPP4 inhibitor) interferes with the activation of TGFβ and downstream reduction in fibronectin but not collagen IV expression. Linagliptin reduced HMGB1 but not high glucose induced NF-κB binding. Linagliptin also reduced high glucose induced AP-1 binding. Meprin beta peptides induce AP-1 binding and this was unchanged with linagliptin. Our data provides new insight into the GLP-1 independent role of DPP4 inhibition in kidney PTC exposed to stimuli relevant in diabetic renal complications. Identifying the functionally relevant renal substrates of DPP4 will help us understand and anticipate long term effects on the kidney in patients with diabetes.

Keywords: DPP4; Kidney; Proximal tubule; Diabetic nephropathy; Transforming growth factor beta

Introduction

The incretin system has been the target of newer glucose lowering medications used in the treatment of Type 2 diabetes mellitus. Specific drugs include the glucagon-like peptide 1 (GLP-1) analogues and dipeptidyl peptidase 4 (DPP4) inhibitors. The incretin hormones consist of GLP-1 and gastrointestinal peptide which are released from the gut in response to a meal. GLP-1 promotes insulin release and inhibits glucagon secretion in a glucose dependent manner which results in the regulation of postprandial glucose excursions. Endogenous GLP-1 hormones have a very short half life as they are rapidly degraded by DPP4 where GLP-1 (7-36) amide is cleaved to GLP-1 (9-36) amide, which is the major circulating form. Hence DPP4 inhibitors exert their hypoglycaemic action by raising endogenous levels of GLP-1.

While the main role of DPP4, also known CD26 (EC 3.4.14.5), is to terminate the action of GLP-1(7-36), it is not exclusively so. DPP4 is a serine exopeptidase belonging to the S9B protein family, members of which cleave X-proline dipeptides from the N-terminus of polypeptides, such as chemokines, neuropeptides, and peptide hormones [1]. It is a 110-kDa type 11 integral membrane glycoprotein and is expressed ubiquitously in most organs and cell types. Importantly, DPP4 is therefore able to cleave a host of other peptides and exert pleiotropic effects independent of GLP-1. Additionally, DPP4 exists as 2 forms (soluble and membrane bound) both of which are capable of proteolytic activity. The soluble form in the circulation is thought to arise from shedding of the membrane forms and is responsible for the glucose lowering effect of the DPP4 inhibitors in clinical use. In contrast, the membrane bound form of DPP4 expressed on the surface of many cell types including kidney tubular cells, endothelial cells and T cells [2] is of major interest with respect to the pleiotropic actions of DPP4.

DPP4 is located on the apical/brush border surface of the kidney proximal tubular cell (PTC) [3], is found in the urine [4] has a high level of expression and activity in the kidney. The proteolytic functions of DPP4 in the kidney have been described using liquid chromatography mass spectrometry based peptidomics, where kidney tissue from DPP4+/− (wild type) and DPP4−/− (knockout) mice were compared and this revealed 10 peptides regulated by DPP4 in vivo [5]. Further studies with brush border membranes showed that aminopeptidase activity is required to generate DPP4 substrates [2]. This suggests that DPP4 is involved in the extracellular catabolism of proteins in the kidney, specifically the degradation/catabolism of proline containing peptides [6].

High mobility group box 1 (HMGB1) and meprin beta have been implicated in the pathophysiology of diabetic nephropathy and interestingly have been identified as DPP4 substrates [5,7]. In patients with diabetes, HMGB1 is a proinflammatory cytokine and serum levels have been shown to positively correlate with micro and macroalbuminuria [8]. Meprin β gene polymorphisms has been associated with susceptibility to nephropathy in Pima Indians (a group with high incidence of type 2 diabetes and accelerated rate of renal failure) [9]. The level of protein expression and localization of meprin β has also been associated with several types of renal pathology such as renal ischemia-reperfusion injury [10], acute renal failure [11] and in diabetic kidneys of db/db mice [12]. The physiological relevance of meprin B (homodimers of β subunits) has been further investigated by Herzog et al., where purified recombinant meprin β was able to process IL-1beta precursor to a biologically active form [13]. This suggests that meprin B has a role in activation of this proinflammatory cytokine. Inhibition of meprin either at a gene or protein level has been shown...
to afford renal protection in various settings. For example, wild type mice were markedly more susceptible to renal injury after ischemia/ reperfusion compared to meprin β knockout mice with higher levels of tumour necrosis factor alpha, transforming growth factor beta (TGFβ), inducible nitric oxide and heat shock protein 27 [14], which we have previously shown to be unregulated in tubular cells exposed to high glucose [15]. In keeping with this, studies using actinonin (a meprin inhibitor) showed that meprin inhibition was protective in renal hypoxic injury in vitro and in vivo [16]. Apart from the transcription, expression and localisation of meprin as described above, meprin activity was found to be increased in streptozotocin induced diabetic rats [17]. The functional relevance of DPP4 cleaving meprin β from 21-41 (substrate) to 25-41 (product) and what effect it has on the activity is not known and will be investigated in our studies.

We hypothesise that as DPP4 is present on the brush border of the kidney FTC, inhibition of membrane DPP4 is likely to alter the degradation/regulation of peptides in the lumen and thus influence the kidney tubulointerstitium in patients with diabetes mellitus. We chose to conduct our experiments in vitro using HK2 cells (human kidney proximal tubular cell line) in order to investigate the GLP-1 independent effects of DPP4 inhibition on the proximal tubular cell, which is difficult to evaluate in vivo. GLP-1 is secreted by intestinal ileal L cells. Although human kidney proximal tubular cells express the GLP-1 receptor, they do not express GLP-1. GLP-1R has been demonstrated at the mRNA level in porcine proximal tubular cell [18] and in human proximal tubular cells (our own unpublished data). With respect to animal models, GLP-1 receptor knockout mice are available however it has been shown that some of the actions of GLP-1 (7-36 amide) and its metabolite (9-36 amide) are independent of the GLP-1 receptor [19]. Hence using a GLP-1 receptor knockout model to investigate GLP-1 independent effects of DPP4 inhibition will not fully delineate whether the effects seen are a result of DPP4 inhibitors raising levels of GLP-1 or independent of this. Hence in vitro studies like ours are necessary to study the direct effects of DPPV inhibition.

Given that DPP4 cleaves a variety of substrates, we expose our cells not only to high glucose conditions but also to substrates known to be cleaved by DPP4 and considered to be relevant in diabetic nephropathy, for example HMGB1 and meprin beta. An important aspect of our hypothesis is that signalling pathways which are intracellularly regulated by high glucose will not be affected by DPP4 inhibitors, as DPP4 cleaves extracellular peptides.

Materials and Methods

Cell culture

HK2 cells, a primary human proximal tubular cell line (American Type Culture Collection), were grown in Keratinocyte Serum Free Media (KSF) supplemented with bovine pituitary extract 20-30 µg/ml and epidermal growth factor 0.1-0.2 µg/ml (Gibco, NY, USA). Cell culture media was changed every 48 hours. These cells were grown at 37°C in a humidified 5% CO2 incubator and were subcultured at 50-80% confluence using 0.05% trypsin 0.02% EDTA (Gibco, NY, USA). The DPP4 inhibitor used in our studies is linagliptin (generously provided by Boehringer Ingelheim, Germany) is an oral DPP4 inhibitor used clinically to treat patients with Type 2 diabetes mellitus. The endpoint markers in our study include the TGFβ/profibrotic and inflammatory pathways, both of which are well evidenced in the pathophysiology of diabetic nephropathy.

The IC50 of linagliptin is 1 nM and the final concentration on our cell culture system was 30 nM (as per advice from Boehringer Ingelheim). When 80% confluent, cells were exposed to 5 mM glucose, 30 mM D-glucose; 30 mM D-glucose plus 30 nM linagliptin for up to 72 h then harvested. For the TGFβ experiments, a final concentration of 0.5 nM recombinant human TGFβ (R&D systems, MN, USA) was used instead of glucose. Recombinant high mobility group box protein 1 (ProteinOne, MD, USA) was used at a final concentration of 50 ng/ml. Custom synthesised meprin beta 21-41 and 25-41 were purchased from Auspep Pty. Ltd. and cells were exposed to 1000 ng/ml final concentration after initial dose finding experiments. For the thrombospondin-1 (TSP-1) experiments, recombinant human TSP-1 (R&D Systems) was used at a concentration of 10ng/ml after initial dose finding experiments.

Flow cytometry

The expression of DPP4 on the surface of HK2 cells was determined with flow cytometry. Cells were exposed to 5 mM and 30 mM glucose for up to 72 hours then harvested and washed with phosphate buffered saline/1% bovine serum albumin. Equal numbers of cells were incubated with human CD26 (DPP4) – phycoerythrin labeled antibody and isotype control (Miltenyi Biotec Inc, CA, USA). The percentage of cells positive for CD26 (DPP4) was determined using a FACSCalibur standard four colour cytometer (Becton Dickinson, CA, USA).

Western blot analysis

Cells collected were 95% confluent and the cell pellet was resuspended in cell lysis buffer containing 50 mM Tris-HCl, 150 mM NaCl, 5 mM EDTA (pH 7.4), 0.5% Triton-X100, and protease inhibitors (Roche Diagnostics, Mannheim, Germany). Cell lysate was spun at 13000 rpm at 4°C for 5 minutes and stored at -20°C. Protein quantification (Bio-Rad, CA, USA) was carried out to determine the protein concentration of the cell lysate. 50-80 µg total cell protein was mixed with 6x Laemmli sample buffer containing mercaptoethanol and heated at 95°C for 10 minutes. Samples were then analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) using a 10% gel and electroblotted to Hybond Nitrocellulose membranes (Amersham Pharmacia Biotech, Bucks, UK). Membranes were blocked in Tris-buffered saline containing 0.2% Tween-20 (TTBS) in 5% skim milk for 2-3 hours and then incubated overnight at 4°C with the following primary antibodies collagen IV 1:5000 (ab5851, Abcam Ltd, Cambridge), fibronectin 1:1000 (Santa Cruz, CA, USA) and phosphorylated smad2 Ser465/467 (psmad2) 1:500 (Cell Signaling Technology) in TTBS (Tris Buffered saline with 0.2% Tween) containing 5% skim milk. Membranes were washed with TTBS and incubated with horseradish peroxidase conjugated secondary antibody. Proteins were visualized using the enhanced chemiluminescence (ECL) detection system (Amersham Pharmacia Biotech, Bucks, UK). All membranes were reprobed with β actin 1:1000 (Santa Cruz, CA, USA) and results were corrected for actin as a loading control and analyzed using Image J software (Java based software program, NIH).

Nuclear extraction and Electrophoretic Mobility Shift Assay (EMSA)

Nuclear extracts were prepared using NucBuster™ Protein Extraction Kit (Novagen, Darmstadt, Germany) as per manufacturer’s instructions. The DIG Gel Shift Kit (Roche Applied Science, Indianapolis, US), was used in the EMSA. In brief, 25 µg of nuclear extract were incubated with 1 µg poly [d (I-C)] as the non specific competitor, 1 µg poly-L-lysine a binding buffer (100mM Hepes, pH 7.6, 5 mM EDTA, 50 mM (NH4)2SO4, 5mM DTT, Tween 20, 1%...
HK2 cells were treated with 5 mM glucose (control media), 30 mM glucose with and without 30 nM linagliptin for 48 hours. At 48 hours supernatants were collected, spun and stored at -20°C until TGFβ1 levels were determined with an immunoassay system (Promega, WI, USA) as per manufacturer’s instructions and read using a microplate reader at 450 nm. This system is linear between 15.6-1000 pg/ml. Samples were acid treated then neutralized to convert the latent form to the bioactive form of TGFβ1 in order to measure total TGFβ1. Active TGFβ1 was assayed after the supernatants were concentrated using a centrifugal filter unit (Millipore). The assay used was designed to measure biologically active TGFβ1. Cell lysate protein concentration was determined using Bio-Rad Protein assay and TGFβ1 levels were corrected for protein content per well.

RNA extraction, reverse transcription and real-time PCR

RNA was extracted using a RNeasy mini kit (Qiagen, Valencia, CA) according to manufacturer’s instructions. RNA was then treated with DNase and RT-PCR was performed with SuperScriptIII One-Step RT-PCR System and Platinum Taq DNA polymerase (Invitrogen). cDNA was generated by reverse transcribing 1 µg of total RNA in a 20 µl reaction volume of 20 µl using VILO cDNA synthesis kits (Invitrogen). Quantitative real-time PCR was performed using an ABI Prism 7900 HT Sequence Detection System (Applied Biosystems, Foster City, CA) with SYBR Green PCR mastermix. Reactions were performed at least in triplicate or as detailed in the text with n reflecting the number of separate experiments. Statistical comparisons between groups were made by analysis of variance (ANOVA) or unpaired t-tests where appropriate. Analyses were performed using the software package, Statview version 5.0 (Abacus Concepts Inc., Berkley, CA, USA). P values <0.05 considered significant.

Results

High glucose reduces surface DPP4 expression

When HK2 cells were exposed to high glucose for up to 72 hours, there was a small but significant down regulation in the expression of surface DPP4 to 88.4 ± 1.3% (n=4, p value <0.05) when measured using flow cytometry at 48 hours however this was not sustained at 72 hours (Figure 1).

Linagliptin reduces high glucose induced fibronectin expression

HK2 cells were exposed to high glucose and linagliptin for 24 hours. Here we confirm previous findings that high glucose increases fibronectin expression as well as demonstrate that linagliptin reduces high glucose induced fibronectin expression from 132.6 ± 8.3% of control to 92.2 ± 17.8% of control (n=4, p value <0.05; Figure 2a).

We further show that linagliptin alone does not have any effect on basal expression of fibronectin (Figure 2b). In this set of data, TGFβ (0.5 ng/ml) is used as a positive control.

Linagliptin does not reduce high glucose induced collagen IV expression

Unlike its effect on high glucose induced fibronectin expression, linagliptin did not reduce the up regulation of collagen IV (CIV) after expression 24 hours (n=3; Figure 3).

Linagliptin reduces high glucose induced active TGFβ1 and downstream p smad2 but not total TGFβ1 secretion

HK2 cells exposed to high glucose for 48 hours increased both total (latent and active) and active TGFβ1 to 157.2 ± 8.0% and 397.6 ± 29.3% of control respectively, (n=3-4, p value <0.05). Interestingly linagliptin reduced high glucose induced collagen IV expression to 92.2 ± 17.8% of control (n=4, p value <0.05; Figure 2a).

We further show that linagliptin alone does not have any effect on the conversion of latent to active TGFβ1. This is shown in Figures 4a-4c.

Figure 1: The regulation of surface DPP4 High glucose (30 mM) exposure for 48 h down regulates DPP4 expression on HK2 cells however there was no change at 24 h and 72 h. HK2 cells were labeled with DPP4-PE antibody and the percentage of DPP4 positive cells were measured with flow cytometry. N=3, *p<0.05
The effect of linagliptin on the conversion of TGFβ1 is not TSP-1 mediated

The purpose of these experiments was to investigate the mechanism by which linagliptin interrupts the conversion of latent to active TGFβ1. We used high glucose to stimulate the production of total TGFβ1 and hence downstream fibronectin transcription. We demonstrate that linagliptin reduced high glucose induced fibronection transcription from 1.24 fold to 0.97 fold of control values (n=6, p value <0.05). TSP-1 did not have any further effect on fibronectin in the presence of high glucose. The addition of linagliptin in the presence of TSP-1 and high glucose reduced FN expression to 0.97 fold (n=5, p value <0.05; Figure 5).

Linagliptin reduces high glucose induced AP-1 binding

HK2 cells exposed to high glucose for 72 hours increased AP-1 binding to 225.7 ± 5.6% of control values. Linagliptin reduced this to 139.6 ± 17.9 % of control values (n=3, p value <0.05 Figure 6).

Linagliptin reduces HMGB1 but not high glucose induced NF-κB binding

Our results demonstrate that the effect of linagliptin on NF-κB binding is dependent on the type of stimulus used in the system. High glucose increased NF-κB binding as expected to 130.8 ± 11.6% of control values (n=5) and this was not altered with the addition of linagliptin as shown in Figure 7a. High glucose increased secreted HMGB1 to 190.6 ± 38.0% (n=3; Figure 7b). When HK2 cells were pre-treated with linagliptin for 24h prior to stimulation with recombinant HMGB1, NF-κB binding was reduced to 38.0 ± 5.9 % ( n=3, p value <0.05; Figure 7c. To assess whether linagliptin is inhibiting the cleavage of recombinant HMGB1 in our system, we used the same HMGB1 antibody directed to the terminal end of HMGB1 (Ab 67281) as per Marchetti et al. [7]
meprin beta peptides induce AP-1 binding and this is unchanged with linagliptin

Meprin beta 21-41 (DPP4 substrate) and 25-41 (product) were added to HK2 cells for 24 hours and both peptides were able to increase AP-1 binding. The addition of linagliptin (to inhibit the cleavage of meprin 21-41 to 25-41) did not differ significantly compared to meprin 21-41 alone (Figure 8).

**Discussion**

In our studies, although high glucose reduced surface expression of DPP4 at 48 hours, this was not sustained at 72 hours. Other groups have postulated that shedding of DPP4 from the cell membrane surface occurs as a response to injury. It is known that other factors such as insulin and tumour necrosis factor α as well as certain cancers also promote shedding of surface DPP4 [20,21].

Targeting the TGFβ pathway is a major therapeutic goal in ameliorating fibrosis in diabetic nephropathy. There are very few studies investigating the direct non GLP-1 effect of DPP4 inhibition on the TGFβ pathway. Inhibitors of DPP4 like activity were able to abrogate the TGFβ1-induced stimulation of collagen synthesis, matrix deposition, and TGFβ1 (1) and fibronectin [22] in skin fibroblasts. In the kidney, TGFβ is secreted in a latent form and requires a complex interplay of soluble signalling molecules in the activation process which releases it from the latency associated peptide (LAP). Once released from the LAP, the unbound TGFβ can then bind to its receptor to initiate cell signalling via smad2/3 pathway. Several other molecules such as plasminogen, thrombospondin-1 and mannose 6 phosphate receptor (M6PR) participate in this activation process. We have shown that high glucose increases both total and active TGFβ however linagliptin only reduced active but not total TGFβ levels in the supernatant. This implies that there is an interruption in the conversion of total to active TGFβ1. The reduction in active TGFβ1 levels translated to a downstream effect of reduced phosphorylated smad2 expression and the extracellular matrix protein fibronectin, both at a transcriptional and protein level. This suggests that linagliptin is reducing fibronectone through a TGFβ dependent mechanism. As we are using an in vitro cell culture system devoid of GLP-1 and plasminogen, the mechanism by which linagliptin interferes with the conversion of TGFβ1 is unlikely to involve these molecules. Several groups have shown that thrombospondin (TSP-1) is
involved in the renal activation of TGFβ1 [23,24] hence the next logical step was to investigate the effect of linagliptin on TSP-1. Given that the suppressive effects of linagliptin were not overcome by an excess of TSP-1 in our experiments, this would suggest that linagliptin is not interfering in the activation of TGFβ1 through TSP-1.

Collagen IV (CIV) accumulation in the basement membrane of PTCs is one of the earliest changes in the diabetic kidney. In our studies, high glucose induced CIV expression was not reduced by linagliptin. This was an expected finding as high glucose can regulate CIV through the polyol pathway and directly stimulate gene expression [25,26] independently of TGFβ1. The polyol pathway is activated in cells such as PTCs where glucose entry is independent of insulin. So, this finding is in keeping with our hypothesis that DPP4 inhibition is more likely to alter extracellular mediators but not the intracellular regulation induced by hyperglycaemia.

The role of DPP4 in modifying inflammation seems variable. On the one hand there is a negative correlation between the expression and activity of DPP4 with neurogenic inflammation [27], airway inflammation [28] and arthritis [29]. However there are also reports that the DPP4 inhibition exerts antiatherosclerotic effects and reduces inflammation via inhibition of monocyte activation/chemotaxis in male LDLR (-/-) mice [30]; reduce local inflammation in adipose tissue and pancreatic islets of obese mice [31] and reduced inflammation as well as accelerated epithelialisation of wounds in diabetic ob/ob mice [32]. It is important to note that DPP4 inhibition raises GLP-1 levels in vivo models so it is difficult to differentiate whether the effects seen are as a result of raised GLP-1 levels or direct effects of DPP4 inhibition independent of GLP-1.

In our studies we saw quite divergent effects of linagliptin on 2 major transcription factors relevant to diabetic nephropathy. Transcription factors are proteins that bind to promoter regions of target genes and regulate their transcription. Both NF-κB and AP-1 are key transcription factors mediating the fibrotic and inflammatory pathways in HK2 cells exposed to high glucose [33,34]. There was marked suppression of high glucose induced AP-1 binding, the mechanism of which is unclear. Linagliptin did not alter high glucose induced NF-κB binding but had significant effects when HMGB1 was used as a stimulus. Furthermore, we showed that mechanistically this was not related to cleaving HMGB1. The difference in NF-κB binding seen is likely due to glucose entering the kidney PTC cells via the sodium glucose cotransporter 2 and causing intracellular effects whereas HMGB1 is an extracellular ligand of membrane bound toll like receptor 2 and 4 as well as the receptor for advanced glycation end products (RAGE) and so is exposed to DPP4.
As discussed earlier, meprin beta is implicated in the development of diabetic nephropathy. Given that proteomics studies have identified meprin beta 21-41 as a substrate of DPP4 (cleaved to meprin beta 25-41), it would be logical to investigate the functional relevance of linagliptin on meprin beta in vitro. Both peptides caused an increase in AP-1 binding and the addition of linagliptin did not significantly alter this (in contrast to high glucose induced AP-1 binding). As both the substrate and product cause AP-1 binding, it would be expected that inhibiting the cleavage of meprin 21-41 would not have significant differences in our system.

DPP4 is a multifunctional protein and its function varies according to its location/cell type and availability or concentration of substrates [35]. For example, the physiological and clinical relevance of non GLP-1 substrates is highlighted by the commonly seen adverse effects of DPP4 inhibitors such as rhinitis and sinus congestion which is thought to occur because the loss of DPP4 activity contributes to the neurogenic inflammation induced by substance P in the nasal mucosa [27]. Although peptidomics have aided the identification of DPP4 substrates in the kidney, further studies delineating the functional relevance of this is lacking.

The varied biological role of DPP4 in many physiological processes is to be expected and our results presented here support this notion. Although linagliptin interrupts the TGFβ1 pathway, its effect on the substrate and product cause AP-1 binding, it would be expected that inhibiting the cleavage of meprin 21-41 would not have significant differences in our system.

References


