DPP4 inhibition improves functional outcome after renal ischemia-reperfusion injury

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DPP4 inhibitors are currently used in the treatment of type 2 diabetes patients to improve glucose tolerance by increasing the half-life of glucose-dependent insulinotropic peptide (GIP) and glucagon-like peptide 1 (GLP-1). Most commonly used inhibitors are vildagliptin (Galvus, Novartis), sitagliptin (Januvia, MSD), and saxagliptin (Onglyza, Bristol-Myers Squibb/AstraZeneca). Several other compounds became available more recently (e.g., linagliptin, Tradjenta, Boehringer Ingelheim/Eli Lilly) or are still in (pre)clinical development (4).

Inhibition of DPP4 was shown to attenuate the development of ischemia-reperfusion injury (IRI) in the lung during transplantation (16, 17, 38–40) and also to protect the heart in models of myocardial infarction (14, 18, 35). Although the exact mechanism behind the observed tissue-protective effects is not known, the prolonged half-life of peptide substrates, which are normally truncated by DPP4, is likely to contribute.

Notwithstanding the clinical importance of renal IRI in the setting of acute kidney injury and renal transplantation, and the very high expression of DPP4 in the proximal tubule, the effects of DPP4 inhibition are not fully understood in the renal setting. The effect of chronic DPP4 inhibition on kidney function after IRI was previously evaluated by Vaghasiya et al. (33) in diabetic animals. The present study was set up (Fig. 1) to assess the potentially protective effect of a single dose of the clinically relevant DPP4 inhibitor vildagliptin on the outcome of IRI-induced acute kidney failure in the normoglycemic Wistar-Han rat in a model of 30-min unilateral renal ischemia followed by contralateral nephrectomy.

MATERIALS AND METHODS

Animals and experimental design. All procedures were carried out in accordance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals (no. 85–23, 1985) following approval by the Antwerp University Ethical Committee. Male Wistar-Han rats (n = 84, 6 wk old; Iffa Credo, Brussels, Belgium) were randomly assigned to three treatment groups: saline-, 1 mg/kg vildagliptin (VG; VG1)-, or 10 mg/kg VG-treated (VG10) animals (n = 28). The study setup is presented in Fig. 1. At experiment initiation, animals had been kept in a fasting state for ~12 h. Animals were intravenously injected with saline or VG (custom synthesized, GL-Synthesis, Worcester, MA) 15 min before surgery. Rats were anesthetized with pentobarbital sodium. DPP4 inhibition was administered through a single bolus. The left renal pedicle of rats in the ischemia group was clamped for 30 min with a microvascular clamp, followed by a right nephrectomy. Body temperature was kept constant (35.5 ± 0.5°C). Eight animals per experimental group were euthanized at 2, 12, or 48 h after reperfusion. Four sham-operated animals per experimental group were euthanized 48 h after sham operation. Serum samples were taken from the tail vein before saline or VG administration, from the retroorbital sinus after the ischemic operation, and from the abdominal aorta at death. Right and left kidneys were

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Sham-operated animals (white) were euthanized 48 h after sham operation. Automated meter with glucose strips (Menarini, Florence, Italy) was used to measure blood glucose. To assess changes in blood glucose, which could affect the outcome of renal IRI, glucose was measured in whole blood using a GlucoMen Lx Plus automated meter with glucose strips (Menarini, Florence, Italy). Therefore, the percentage of in vivo DPP4 activity was estimated according to the method described by Mattheussen et al. (23).

**Blood glucose.** To assess changes in blood glucose which could affect the outcome of renal IRI, glucose was measured in whole blood in a separate ischemic experiment using a GlucoMen Lx Plus automated meter with glucose strips (Menarini, Florence, Italy).

**Renal function.** Serum creatinine levels were measured using an automatic carbohydrate reader (Vitros CREA Slides for Vitros Chemistry Systems 350, Ortho Clinical Diagnostics, Bucks, UK) at the University Hospital of Antwerp (UZA Edegem, Belgium). In a separate experiment, systolic blood pressure was noninvasively measured 2, 12, and 48 h after injection of saline or VG10 (n = 10) using a volume pressure-recording sensor and an occlusion tail cuff (CODA 6 System, Kent Scientific, Torrington, CT). Glomerular filtration was assessed using inulin. Inulin concentrations in the serum were measured at 30, 60, 90, 120, and 240 min after injection of inulin (Inutest 25%, Fresenius Pharma, Graz, Austria) with or without VG10 (n = 10), using a d-Glucose/d-Fructose-kit (Boehringer Ingelheim, Ingelheim-am-Rhein, Germany) on a Cobas Mira Biochemistry analyzer (Roche, Basel, Switzerland) to determine inulin clearance. Creatinine concentrations were also measured in this experiment to exclude any other interference of vildagliptin with creatinine excretion. The latter experiment was performed twice, so that each individual animal was alternately treated with VG10 and saline.

**Tubular morphology, regeneration, and proliferation.** Proximal tubules in the outer stripe of the outer medulla (OSOM) of periodic acid-Schiff-, proliferating cell nuclear antigen (PAS-PCNA)-stained sections were assigned a score: 1) tubules with a normal appearance, 2) tubules with signs of sublethal injury, 3) tubules with signs of acute tubular necrosis, and 4) tubules with signs of regeneration. Proliferation of tubular epithelium was evaluated by counting the average number of PCNA+ cells/mm² in the OSOM at ×500 magnification, using digital image-processing software (AxioVision, Carl Zeiss).

**Apoptosis.** DNA breaks were detected by TUNEL assay using the ApoTag Peroxidase In Situ Apoptosis Detection Kit (Chemicon, Temecula, CA) according to the manufacturer’s instructions. Sections were counterstained with hematoxylin and apoptotic bodies were counted in 20 fields of the OSOM at ×400 magnification.

**Oxidative stress.** At 48 h of reperfusion, the in vivo lipid peroxidation status was determined by measuring malondialdehyde (MDA) in the serum using a fully validated HPLC method from Hermans et al. (12). To evaluate protein expression of heme oxygenase-1 (HO-1), kidney sections were stained using a primary rabbit polyclonal antibody (H4535, Sigma-Aldrich, St. Louis, MO) and evaluated using a semiautomatic scoring system.

**Infiltration of immune cells.** To estimate the presence of polymorphonuclear cells, standard hematoxylin-eosin staining was performed and cells were counted based on the morphology of the nucleus in 20 evenly distributed fields (at ×320 magnification). Infiltrating mono-
cytes/macrophages were evaluated on ED-1-stained sections (antibody MCA341R, Serotec, Oxford, UK); infiltrating CD3⁺ T cells were evaluated in the inner stripe of the outer medulla (ISOM; at body MCA341R, Serotec, Oxford, UK); infiltrating CD3⁺ cytos/macrophages were evaluated on ED-1-stained sections (anti-

Fig. 3. Serum creatinine levels (mg/dl). DPP4 inhibition resulted in a significant dose-dependent reduction of renal dysfunction after 12 h of reperfusion. *P < 0.05 vs. saline-treated animals. *P < 0.05 vs. VG1-treated animals.

RESULTS

DPP4 activity. At the start of reperfusion (time 0 = 45 min after saline or vildagliptin injection), DPP4 activity was measured in both serum and the contralateral kidney to evaluate the efficiency of the DPP4 inhibitor after intravenous injection of VG1 and VG10. An almost complete DPP4 inhibition was observed in serum of both VG1- and VG10-treated animals with <10% residual DPP4 activity (Fig. 2A). A dose-dependent inhibition of DPP4 activity could be observed in the contralateral kidney (Fig. 2B).

Blood glucose. Glycemia of VG10-treated and saline-treated animals was measured at different time points. Blood glucose did not differ significantly in control animals vs. VG10-treated animals at the beginning of ischemia (113.0 ± 11.3 vs. 110.0 ± 10.6 mg/dl), the beginning of reperfusion (128.5 ± 6.4 vs. 133.0 ± 26.9 mg/dl), and after 2 h (113.0 ± 0.0 vs. 120.3 ± 11.2 mg/dl), 12 h (113.0 ± 14.1 vs. 119.7 ± 2.1 mg/dl), and 48 h of reperfusion (116.5 ± 7.8 vs. 118.3 ± 3.2 mg/dl).

Renal function. DPP4 inhibition clearly prevented renal dysfunction in a dose-dependent way as reflected by lower serum creatinine levels in both VG1- and VG10-treated animals compared with saline-treated animals. The effects were most pronounced 12 h after the start of reperfusion (1.31 ± 0.32 and 0.70 ± 0.19 vs. 1.91 ± 0.28 mg/dl; P < 0.05) (Fig. 3).

Tubular morphology and regeneration. DPP4 inhibition attenuated histological damage after renal IRI as shown by significantly reduced necrosis. After 2 h of reperfusion, tubular necrosis was nearly absent in VG1- and VG10-treated animals

Fig. 4. Tubular morphology of S3 proximal tubular cells at 2, 12, and 48 h of reperfu-
sion. DPP4 inhibition attenuated the histological damage after renal ischemia-reperfusion injury (IRI) as shown by significant less necrosis after 2 and 12 h of reperfusion in vildagliptin-treated animals compared with saline-treated animals. *P < 0.05 vs. saline-treated animals. *P < 0.05 vs. VG1-treated animals.
while clearly present in saline-treated animals (0.3 and 0.0 vs. 5.3%, \( P < 0.05 \)). After 12 h of reperfusion, tubular necrosis was still significantly reduced in vildagliptin-treated animals compared with saline-treated animals (48.2 and 62.1 vs. 77.5%, \( P < 0.05 \)) (Fig. 4). Surprisingly, tubular necrosis was higher in VG10-treated animals compared with VG1-treated animals. As this figure leaves the presumption that DPP4 inhibition has no effect on regeneration, this was confirmed by counting the number of PCNA+ cells, where indeed no significant differences were observed among saline-, VG1-, and VG10-treated animals at 48 h of reperfusion (1,777 ± 340, 1,748 ± 161, and 1,810 ± 338 PCNA+ cells/mm², respectively) (Fig. 5).

**Apoptosis.** IRI is associated with ischemia-induced apoptosis, which might contribute to renal dysfunction. To evaluate the effect of DPP4 inhibition on the development of apoptosis in ischemic kidneys, the mRNA expression of proapoptotic Bax and antiapoptotic Bcl-2 was investigated by quantitative real-time RT-PCR. The number of apoptotic bodies was analyzed using a TUNEL assay in the saline- and VG10-treated group. As presented in Fig. 6A, the administration of VG10 significantly reduced the Bax/Bcl-2 mRNA expression ratio at a reperfusion time of 48 h, suggesting a potential antiapoptotic effect of DPP4 inhibition. This was confirmed by a threefold decreased number of apoptotic bodies in the OSOM of VG10 vs. saline-treated animals after 48 h of reperfusion (414.1 ± 231.7 vs. 1,212.0 ± 650.8 apoptotic bodies/mm²; \( P < 0.05 \)) (Fig. 6B).

**Oxidative stress.** Oxidative damage due to lipid peroxidation was measured as MDA levels in rat serum. DPP4 inhibition significantly reduced MDA levels in both VG1- and VG10-treated animals compared with saline-treated animals in both ischemic (0.54 ± 0.17 and 0.56 ± 0.21 vs. 1.00 ± 0.35 μM; \( P < 0.05 \)) and sham-operated rats (0.48 ± 0.09 and 0.59 ± 0.12 vs. 0.84 ± 0.16 μM; \( P < 0.05 \)) at 48 h of reperfusion (Fig. 7). Also, expression of HO-1, a marker of oxidative stress, was assessed in the total kidney on both mRNA (Fig. 8A) and protein levels (Fig. 8B). The amount of HO-1 mRNA was, although not significant, decreased in vildagliptin-treated animals compared with saline-treated animals after 48 h of reperfusion. A decrease in protein expression was observed in the vildagliptin-treated groups at 12 and 48 h of reperfusion, which, however, was not significant compared with saline-treated animals due to high variation (Fig. 8B).

**mRNA expression of immunological markers.** mRNA expression of a selection of immunological parameters including CXC chemokine ligand CXCL10 was evaluated in kidney

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### Figure 5

**Fig. 5.** Average number of PAS-PCNA+ cells/mm² in the outer strip of the outer medulla (OSOM). No differences were observed among treatment groups.

### Figure 6

**Fig. 6.** A: ratio of mRNA expression of Bax and Bcl-2. Values are average ± SE. *\( P < 0.05 \) vs. saline- and VG1-treated animals. B: no. of apoptotic bodies per mm² shown by terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) staining. *\( P < 0.05 \) vs. saline-treated animals. The Bax/Bcl-2 mRNA ratio was significantly decreased in VG10-treated animals after 48 h of reperfusion. A 3-fold increased number of apoptotic bodies were observed in the OSOM of saline-treated animals compared with VG10-treated animals. These results indicate a reduction of apoptosis in tubular cells after DPP4 inhibition.
homogenates. DPP4 inhibition resulted in an early and significant decrease in CXCL10 mRNA expression in the vildagliptin-treated groups compared with saline-treated animals at 2 h of reperfusion (Fig. 9A). An anti-inflammatory trend was observed in a comparison of relative expressions of IL-10, IL-16, and TNF-α mRNA expression (Fig. 9).

Infiltration of immune cells. The infiltration of monocytes, neutrophils, and T cells was also assessed through staining of tissue sections. No significant differences were found in a comparison of ischemic animals to sham-operated animals (data not shown).

Hemodynamic effects. A potential influence of DPP4 inhibition on renal hemodynamics/glomerular filtration could also have contributed to lower serum creatinine levels. Therefore, the effect of a single administration of DPP4 inhibitor (VG10) on systolic blood pressure and inulin clearance was determined. No differences in systolic blood pressure were observed 2, 12, or 48 h after a single saline or VG10 injection. Serum inulin concentration measurement revealed no influence of vildagliptin on inulin clearance (1.18 ± 0.03 vs. 1.19 ± 0.05 ml·min⁻¹·100 g body wt⁻¹ after VG10 and saline treatment, respectively). Also, serum creatinine levels were not influenced by administration of vildagliptin.

DISCUSSION

Acute DPP4 inhibition results in a significant, dose-dependent protective effect on kidney function after IRI, as shown by reduced serum creatinine levels. This effect is accompanied by morphological protection indicated by a decrease in tubular necrosis and inhibition of the apoptotic pathway in renal proximal epithelial cells. Although a reduction of necrosis was observed in both treated groups, the mechanism by which less necrosis occurred in the VG1-treated group compared with the VG10-treated group remains to be clarified. The decrease in the Bax/Bcl-2 mRNA expression ratio and a reduced number of TUNEL-stained apoptotic bodies in the OSOM, which interestingly only occurs at 48 h of reperfusion, further emphasizes this protection. Although this temporal relationship is unclear, our results are in line with those of Vaghasiya et al. (33), who reported a decrease in DNA fragmentation and apoptosis in chronic sitagliptin-treated type 2 diabetic rats (33).
Melin et al. (24) reported that the (morphological) outcome of renal IRI is worse in hyperglycemic conditions. However, since vildagliptin did not influence blood sugar in fasting animals undergoing ischemia, differences in blood sugar levels could not have affected kidney function/morphology.

Furthermore, in the current study mRNA expression of the proinflammatory marker CXCL10 was significantly decreased in vildagliptin-treated animals, which is favorable since CXCL10 expression correlates with graft rejection/tissue injury following kidney transplantation (11, 15).

HO-1 is a marker of oxidative stress produced by interstitial inflammatory cells after IRI in quantities reflecting the extent of the causal insult (8). The present study supports a decreasing tendency of HO-1 expression (mRNA and protein) as a result of vildagliptin treatment. As the quantity of macrophages and other infiltrating immune cells did not change as a result of vildagliptin treatment, the inflammatory profile and expression of cytokines may have been altered in those cells. A reduced lipid peroxidation status, reflecting a reduction of oxidative stress, through vildagliptin treatment was also noticed in the serum of ischemic as well as sham-operated animals.

In our study, intravenous administration of vildagliptin results in a very efficient inhibition in the circulation. DPP4 activity in serum greatly influences the half-life of circulating DPP4 substrates. Prolongation of their half-life might have protected the kidney from IRI. Some DPP4 substrates which could potentially influence the outcome of IRI will be discussed below.

GLP-1 is one of the best-known and -studied of the DDP4 substrates. The cardioprotective effects of GLP-1 have been extensively studied in ischemic heart models and involve an upregulation of cardioprotective genes, resulting in a reduction of the infarct size (1, 36). Its importance in renal IRI was recently demonstrated by Vaghasiya et al. (34), who reported a protective effect of the GLP-1 agonist exenatide on the outcome of IRI in diabetic rats. GLP-1 has been assigned an antihypertensive effect due to its diuretic and natriuretic actions. The mechanisms behind these actions were only just

Fig. 9. mRNA expression of CXCL10 (A), IL-16 (B), IL-10 (C), and TNF-α (D). Values are average ± SE. DPP4 inhibition resulted in a significant decrease of CXCL10 mRNA expression in the vildagliptin-treated groups compared with saline-treated animals at 2 h of reperfusion. *P < 0.05 vs. saline-treated animals.
lately shown to be an increased glomerular filtration rate (GFR) and a downregulation of sodium/hydrogen antiporter 3 (NHE3) in the proximal tubule (3, 10). The same downregulation of NHE3 is seen when a DPP4 inhibitor is administered (9) and is associated with an attenuation of blood pressure rise in young hypertensive rats (27). NHE3, generally downregulated during IRI (6), reabsorbs filtered sodium out of the proximal tubuli, which is energetically very demanding; DPP4 inhibition might lessen the severity of the ischemic insult by moving NHE3 to the intermicrovillar domain, thus reducing the metabolic needs of the kidney. However, while DPP4 inhibition in animal models results in increased diuresis and natriuresis (27, 33), there does not seem to be an effect on the GFR (27), which is in line with our study where neither systolic blood pressure nor the GFR differed significantly in vildagliptin-treated animals vs. saline-treated animals.

Ischemic injury of the kidney was reported to result in a fast upregulation of mRNA expression of SDF-1α as well as its receptor (32). Although the chemotactic signal of the SDF-α/CXCR4 axis is considered essential for migration of CXCR4+ progenitor cells toward the kidney during reperfusion (32), it has been shown that modulating the SDF-1α/CXCR4 axis does not result in alteration of trafficking of mesenchymal cells toward the injured kidney (31). Nevertheless, SDF-1α plays a crucial role in the functional and morphological protection of ischemically injured organs [the heart (13, 37) and kidney (30)] as well as in angiogenesis (28). Prolongation of the half-life of endogenous SDF-1α may contribute to the protective effect of vildagliptin in our study.

Vasoactive intestinal peptide (VIP), a circulating neuropeptide with receptors (PAC1, VPAC1, and VPAC2) in the kidney (2), was suggested to have a tissue-protective function in ischemic models, through its anti-inflammatory and antioxidative properties (17). The modulatory function of the VPAC1 receptor on sodium reabsorption was previously reported in proximal tubular epithelial cells (29). The anti-inflammatory effects of vildagliptin treatment observed in the present study are in line with previous publications concerning the anti-inflammatory effect of VIP on monocytes (reduction of TNF-α and increase of IL-10 expression) (5). Furthermore, the antioxidative effects (reduced MDA and HO-1 levels) of vildagliptin treatment point to a possible role of VIP in the induced protection.

The report by Vaghasiya et al. (33) on IRI outcome in diabetic rats after sitagliptin administration supports that the functional protection by vildagliptin is related to DPP4 inhibition. The present study is the first to assess the effect of a single dose of DPP4 inhibitor in the setting of renal IRI in nondiabetic rats and supports further studies toward the potential use of DPP4 inhibitors in pathologies other than type 2 diabetes (20).

Inhibition of DPP4 with vildagliptin results in a functional protection of the kidney against IRI, reflected by a dose-dependent decrease in serum creatinine levels and a reduction of tubular necrosis. This protection by vildagliptin is associated with immunological and antioxidative changes, but the exact mechanism behind this effect, as well as the potential application in a transplantation setting, remains to be elucidated.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS


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