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Glucose-independent renoprotective mechanisms of the tissue dipeptidyl peptidase-4 inhibitor, saxagliptin, in Dahl salt-sensitive hypertensive rats

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ABSTRACT

Although previous studies have shown an important role of renal dipeptidyl peptidase-4 (DPP-4) inhibition in ameliorating kidney injury in hypertensive rats, the renal distribution of DPP-4 and mechanisms of renoprotective action of DPP-4 inhibition remain unclear. In this study, we examined the effects of the DPP-4 inhibitor saxagliptin on DPP-4 activity in renal cells (using *in situ* DPP-4 staining) and on renal gene expression related to inflammation and fibrosis in the renal injury in hypertensive Dahl salt-sensitive (Dahl-S) rats. Male rats fed a high-salt (8% NaCl) diet received vehicle (water) or saxagliptin (12.7 mg/kg/day) for 4 weeks. Blood pressure (BP), serum glucose and 24-h urinary albumin and sodium excretions were measured, and renal histopathology was performed. High salt-diet increased BP and urinary albumin excretion, consequently resulting in glomerular sclerosis and tubulointerstitial fibrosis. Although saxagliptin did not affect BP and blood glucose levels, it significantly ameliorated urinary albumin excretion. *In situ* staining showed DPP-4 activity in glomerular and tubular cells. Saxagliptin significantly suppressed DPP-4 activity in renal tissue extracts and in glomerular and tubular cells. Saxagliptin also significantly attenuated the increase in inflammation and fibrosis-related gene expressions in the kidney. Our results demonstrate that saxagliptin inhibited the development of renal injury independent of its glucose-lowering effect. Glomerular and tubular DPP-4 inhibition by saxagliptin was associated with improvements in albuminuria and the suppression of inflammation and fibrosis-related genes. Thus, local glomerular and tubular DPP-4 inhibition by saxagliptin may play an important role in its renoprotective effects in Dahl-S rats.

1. Introduction

Dipeptidyl peptidase-4 (DPP-4) inhibitors lower blood glucose by preventing the degradation of incretin hormone GLP-1 and gastrointestinal peptide (GIP), and are used widely for the treatment of type 2 diabetes mellitus (Russell-Jones and Gough, 2012). In addition to an incretin action of DPP-4 inhibitors, DPP-4 inhibitors could induce the pleiotropic actions of DPP-4 such as anti-inflammatory, anti-fibrotic and antioxidant effects (Panchapakesan and Pollock, 2015).

DPP-4, a serine protease, exists in membrane-bound and soluble forms; the soluble form in the circulation is thought to arise from shedding of the membrane-bound form which degrades GLP-1, thus mainly contributing to the control of postprandial blood glucose levels (Baggio and Drucker, 2007; Panchapakesan and Pollock, 2015). The membrane-bound form of DPP-4 is expressed on the surface of many cell types, including kidney tubular cells, endothelial cells and T cells, and may be responsible for the pleiotropic actions of DPP-4. In addition, DPP-4 is known to be responsible for cleavage of various substrates such as brain natriuretic peptide (BNP), neuropeptide Y (NPY) and stromal cell-derived factor-1 (SDF-1), which could affect the additional actions of DPP-4 inhibition beyond hypoglycemic activity (Muskiet et al., 2014).

DPP-4 is expressed ubiquitously in various tissues, predominantly in the kidney (Mentlein, 1999). The membrane bound form of DPP-4 is expressed on proximal tubular cells, mesangial cells and podocytes in the kidney (Kettmann et al., 1992). In the kidney, DPP-4 cleaves several peptides and hormones which regulate natriuresis, inflammation, vascular effects and sympathetic

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activity (Muskiet et al., 2014; Avogaro and Fadini, 2014). Recent animal studies have demonstrated that DPP-4 inhibitors prevent renal damage independent of glucose-lowering action (Kanasaki et al., 2014; Panchapakesan and Pollock, 2015; Tanaka et al., 2014). Thus, DPP-4 plays an important role in the progression of diabetic nephropathy.

The SAVOR-TIMI53 trial (16,492 patients with type 2 diabetes) demonstrated that saxagliptin significantly improves the albumin/creatinine ratio compared with placebo (Scirica et al., 2013). We recently reported the renoprotective effects of saxagliptin, a potent DPP-4 inhibitor, in hypertensive Dahl salt-sensitive (Dahl-S) rats, and indicated the important role of renal tissue DPP-4, but not circulating, in the progression of hypertension-induced renal injury (Sakai et al., 2015). However, the specific renal cells targeted by the action of DPP-4 inhibition and the mechanism of suppression of renal injury remain unclear. In the present study, we evaluated DPP-4 activity in renal cells, glomeruli and tubules using an *in situ* staining method, and assessed the mechanism of renoprotective action of saxagliptin in Dahl-S rats. Here, we report evidence that saxagliptin suppresses both glomerular and tubular DPP-4 activities which are associated with a reduction in inflammation and fibrotic markers independent of glycemic control in rat hypertensive renal injury.

2. Materials and methods

2.1. Animals

All animals received humane care in compliance with the "Guiding Principles for the Care and Use of Laboratory Animals" formulated by the Japanese Pharmacological Society, and all animal experiments were approved by the Committee for Animal Experiments of Kyowa Hakko Kirin Co., Ltd. Male Dahl-S rats were obtained from Japan SLC Inc. (Japan Shizuoka Laboratory Animal Center, Inc., Japan).

2.2. Experimental procedure

The Dahl rat model is a well-established model of salt-induced hypertension and kidney injury. In this study, male 6-week-old Dahl-S rats were fed a high-salt diet (8% NaCl) to induce salt-sensitive hypertension. Two weeks after the start of the high-salt diet, Dahl-S rats were divided into two groups and given (1) water (control, n=10) and (2) saxagliptin (12.7 mg/kg/day, n=10) with drinking water for 4 weeks. Dahl-S rats fed a normal salt diet (0.19% NaCl) served as the normal group (ND: normal salt diet-fed Dahl-S rats, n=5) and were given tap water *ad libitum*.

Saxagliptin monohydrate (saxagliptin) was obtained from Bristol Myers Squibb (Pennington, NJ, USA) and was dissolved in distilled water; the dose of saxagliptin 0.04 mg/ml in drinking water was almost equal to that of 12.7 mg/kg/day. Urine samples were collected at 4 weeks after the start of dosing, and urinary protein, albumin and sodium concentrations were measured. Systolic blood pressure (SBP) values were measured using the tail-cuff method at 4 weeks after treatment. After 4 weeks of treatment, rats were anesthetized with isoflurane, and then blood and kidney samples were collected. Blood samples were used for the measurement of serum glucose, serum creatinine and plasma DPP-4 activity, and kidney samples were used for the measurement of DPP-4 activity, *in situ* DPP-4 staining and histological examination.

2.3. Measurement of plasma and renal DPP-4 activities

DPP-4 activity in plasma and renal extracts was measured as described previously (Sakai et al., 2015). In brief, the sample was mixed with 50 μmol/l Gly-Pro-7-AMIDO-4-METHYLCOUMARIN (Gly-Pro-AMC) in 25 mmol/l of HEPES, 140 mmol/l of NaCl, 80 mmol/l of $MgCl_2/6H_2O$, 1 w/v% BSA, pH 7.8 for 20 min at room temperature. The fluorescence intensity was measured at an excitation wavelength of 460 nm and an emission wavelength of 390 nm. DPP-4 activity was expressed as the AMC amount generated after 20 min of incubation. The protein concentration of the supernatant from the renal extract was measured using a BCA protein assay kit (PIERCE, Rockford, IL, USA).

2.4. In situ staining of DPP-4 activity

Frozen kidney sections (10 μm) were fixed in a mixture of formalin, phosphate buffered saline and acetone (1:35:15) and washed with water. The sections were incubated with a coloring solution (1.76 mol/l glycyl-prolyl-4-methoxy-β-naphthylamide, 2.52 mol/l Fast Blue B, 3.71 vol% N-N dimethyl formamide, 95.7 mmol/l phosphate buffer) and then washed with water and dried. The slides were mounted with water and scanned with the Aperio ScanScope® XT (Aperio Technologies) using a 20 × objective. Images were analyzed with the Aperio analysis algorithm (Color deconvolution ver. 9). *In situ* DPP-4 activity staining index (H-score) was calculated semiquantitatively as follows: the staining intensity of DPP-4 positive area was graded into –, 0; 1+, 1; 2+, 2 and 3+, 3 and the percentage of each positive area was calculated using Aperio Image Scope software. H-score was calculated by multiplying the staining intensity (0 – 4) by the percentage of each positive area. One hundred glomeruli and total area of tubule per section were examined.

2.5. Measurement of renal mRNAs

Total RNA from renal tissue was extracted with RNeasy Fibrous Tissue Mini Kit (Qiagen). cDNA was synthesized using SuperScript VILO cDNA Synthesis Kit (Life Technologies). mRNA expression of the following genes was evaluated by quantitative real-time polymerase chain reaction (PCR) on a StepOnePlus™ Real-Time PCR system (Applied Biosystems) using TaqMan® Gene Expression Master Mix (Applied Biosystems): MCP-1: Monocyte Chemoattractant Protein-1 (Rn00580555_m1), TNF-α: Tumor Necrosis Factor-α (Rn01525859_g1), IL-6: Interleukin-6 (Rn99999011_m1), PAI-1: Plasminogen Activator Inhibitor-1 (Rn01481341_m1), TGF-β: Transforming Growth Factor-β (Rn00572010_m1), Col I: type I Collagen (Rn01463849_g1), Col III: type III Collagen (Rn01437686_m1), αSMA: α-smooth muscle actin (Rn01759928_g1). mRNA quantities of target gene were normalized against r18S: 18 S ribosomal RNA (Mm03928990_g1) as a control gene.

2.6. Histological examination

Kidneys were fixed in 10 vol% neutral buffered formalin solution and embedded in paraffin, and the paraffin sections were then stained with periodic acid-Schiff (PAS) and hematoxylin and eosin (H&E) for light microscopic observation. Specimens were observed under a light microscope, and histopathological scoring was performed as described below.

Glomerular injury scores were calculated semiquantitatively as described previously (Nagase et al., 2006) by examining 100 glomeruli per section. Vascular injury score was calculated using a previously described method (Ishimitsu et al., 1994). Tubulointerstitial injury was defined as basophilic tubule, hyaline cast, interstitial inflammation and/or tubular dilatation. Each section was scored on a scale of 0–4, according to the following criteria: 0, normal; 1, 1% to 25%; 2, 26% to 50%; 3, 51% to 75%; 4, 76% to 100% of the tubulointerstitial injured area (Shibata et al., 2006).