The role of dipeptidyl peptidase 4 (DPP4) in the preservation of renal function: DPP4 involvement in hemoglobin expression

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Abstract

In a previous study, we demonstrated that dipeptidyl peptidase 4 (DPP4)-deficient rats were susceptible to reduced glomerular filtration rate as a result of streptozotocin (STZ)-induced diabetes. Therefore, we proposed that DPP4 might be responsible for the preservation of renal function. In this study, to verify the role of DPP4 in the preservation of renal function, we performed a microarray analysis of the kidneys of WT and DPP4-deficient rats after STZ treatment, and gene expression analysis using rat kidneys, human embryonic kidney 293 (HEK293) cells, and human renal cancer cells (Caki-1). The microarray analysis indicated that the expression levels of the transporter activity, heme-binding, and pheromone binding-related genes changed significantly. The results of gene expression analysis indicated that there were no significant differences in the expression levels of hemoglobin mRNA between the DPP4-deficient and WT rats; however, the expression levels of hemoglobin mRNA in the kidneys of DPP4-deficient rats tended to decrease when compared with those of both the non-STZ-treated and STZ-treated WT rats. The expression levels of hemoglobin in HEK293 and Caki-1 cells were significantly decreased when DPP4 was knocked down by siRNA, were significantly increased by the addition of soluble human DPP4, and were also significantly increased by the addition of the DPP4 inhibitor, sitagliptin. The expression level of DPP4 was also significantly increased by the addition of sitagliptin in both cell types. Our findings indicate that DPP4 regulates the expression of the hemoglobin genes, and might play a role in the preservation of renal function; however, the underlying mechanism of this preservation remains to be elucidated.

Key Words
- DPP4 deficient
- streptozotocin
- diabetes
- renal function
- hemoglobin

Introduction

Diabetes is a major worldwide public health problem (Zimmet et al. 2001) that causes complications, such as retinopathy, nephropathy, neuropathy, dyslipidemia, and cardiovascular disease (Crofford 1995, Cannon 2008, Srinivasan et al. 2008). Recently, incretin mimetics, such as glucagon-like peptide 1 (GLP1) agonists and dipeptidyl peptidase 4 (DPP4) inhibitors, have been used as anti-diabetic agents. DPP4 is present on the surface of various cell types, including kidney and liver cells, and in a soluble form in plasma (Mentlein 1999). DPP4 is a serine protease...
that preferentially cleaves Xaa-Pro and Xaa-Ala dipeptides from the N-termini of polypeptides, such as glucose-dependent insulinotropic polypeptide and GLP1, which are members of the incretin system, and stimulate pancreatic insulin secretion, insulin biosynthesis, and proliferation of pancreatic β-cells, and inhibit food intake (Holst & Gromada 2004, Holst 2006). Therefore, GLP1 agonists and DPP4 inhibitors are considered to be useful as anti-diabetic agents that result in a low incidence of hypoglycemia and minimal weight gain (Nielsen 2005, Green et al. 2006, Hinnen et al. 2006, Kendall et al. 2006). DPP4 inhibitors have been shown to protect against myocardial injury, cardiac dysfunction (Chang et al. 2013), and asthma (Stephan et al. 2013), and also to reduce colon carcinogenesis (Femia et al. 2013) in animal models. However, the advantages and disadvantages of DPP4 inhibitors and the role of DPP4 in various tissues are not fully understood.

We previously showed that DPP4-deficient rats developed renal dysfunction when compared with WT rats following streptozotocin (STZ) treatment to induce diabetes. Creatinine clearance in DPP4-deficient rats after 42 days of STZ treatment was significantly lower than that in WT rats (Kirino et al. 2009). No significant difference in body weight or food intake was found between the WT and DPP4-deficient rats treated with or without STZ. F344/DucrlCrlj (DPP4-deficient) rats from Charles River Japan (Osaka, Japan), express DPP4 mRNA but have reduced levels of the active protein because an abnormal isoform is translated. Thus, F344/DucrlCrlj rats lack DPP4 enzyme activity (Watanabe et al. 1987). Therefore, it has been suggested that DPP4 is related to the preservation of renal function. The goal of this study was to verify the role of DPP4 in the preservation of renal function by analyzing the genes expressed in the kidney tissues of WT and DPP4-deficient rats and from renal cell lines.

Materials and methods

Animals and tissue preparation

Four groups were used in this experiment, as previously reported (Kirino et al. 2009). Briefly, 8-week-old male F344/Jcl (WT) and F344/DucrlCrlj (DPP4-deficient) rats (140–160 g) were purchased from Clea Japan (Tokyo, Japan) and Charles River Japan respectively. At 9 weeks of age, the rats were randomly divided into the following four groups: non-STZ-treated, WT (n = 6); STZ-treated, WT (n = 10); non-STZ-treated, DPP4-deficient (n = 6); and STZ-treated, DPP4-deficient (n = 10) rats. In the STZ-treated groups, experimental diabetes was induced by a single i.p. injection of STZ (30 mg/kg; Wako Pure Chemical, Osaka, Japan) in 0.05 M citrate buffer (pH 4.5) after an overnight fast. The rats were fed again following injections. The non-STZ-treated rats received an equivalent amount of citrate buffer. At 42 days after the STZ or vehicle treatment, the rats were placed under anesthesia with urethane (5 g/kg, i.p.; Sigma–Aldrich) and killed. The kidneys of each rat were collected, and the collected tissues were immediately cut into slices <5-mm thick, which were then stored in RNAlater RNA Stabilization Reagent (Qiagen) at −20 °C. All animal care protocols and experiments were conducted in accordance to the guidelines of the animal use and care committee of the University of Tokushima.

Microarray analysis of gene expression

Total RNA was isolated from the kidneys of the rats using the RNeasy Lipid Tissue Mini Kit (Qiagen), according to the manufacturer’s methods. The relative purity of the mRNA was examined using an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). First-strand cDNA was synthesized from 300 ng of total RNA using the GeneChip Whole Transcript (WT) cDNA Synthesis and Amplification Kit (Affymetrix, Santa Clara, CA, USA) according to the manufacturer’s methods. The resultant cDNA was fragmented and end-labeled with the GeneChip WT terminal Labeling Kit (Affymetrix). Then, 5.5 µg of the fragmented and labeled DNA target were hybridized to the Affymetrix GeneChip Rat Gene 1.0 ST Array at 45 °C for 17 h in a GeneChip Hybridization Oven 640 (Affymetrix), according to the manufacturer’s recommendations. The abovementioned microarray chip contains 27,342 probe sets for known and unknown genes. The hybridized arrays were washed and stained in a GeneChip Fluidics Station 450 and scanned in a GeneChip Scanner 3000 7G (Affymetrix). CEL files were generated for each array. In each comparison experiment, the intensity data for the two chips were normalized by dye swap and flag treatments, and genes that demonstrated significantly different expression levels (P < 0.05) between the WT and DPP4-deficient rats were categorized by gene ontology (GO) using the GeneSpring GX 10.0 Software (Silicon Genetics, Redwood City, CA, USA).

Quantitative PCR (qPCR)

cDNA was synthesized using 2 μg of total RNA and SuperScript III Reverse Transcriptase (Invitrogen)
according to the manufacturer’s instructions. qPCR was carried out using SYBR Premix Ex Taq (Takara, Tokyo, Japan) in the AB 7500 real-time PCR system (Applied Biosystems) according to the following thermal cycling profile: initial denaturation at 95 °C for 10 s followed by 40 cycles of amplification (denaturation at 95 °C for 10 s, annealing at 60 °C for 10 s, and extension at 72 °C for 34 s). Data were analyzed based on standard curve methods; i.e. we quantified the expression levels of various genes and normalized the expression level of each gene to that of GAPDH. The primer sets used are listed in Supplementary Table 1, see section on supplementary data given at the end of this article.

**Cell culture**

Human embryonic kidney 293 (HEK293) cells and human renal cancer cells (Caki-1) were obtained from the Health Science Research Resources Bank (cell no. JCRB9068 and JCRB0801, respectively, Osaka, Japan), and cultured in DMEM (Sigma–Aldrich) supplemented with 10% fetal bovine serum, 50 U/ml penicillin, and 50 mg/ml streptomycin at 37 °C in a humidified atmosphere of 5% CO₂ in air. Recombinant soluble human DPP4 (ATGen, Seongnam, South Korea) was added to the culture medium at a final concentration of 500 ng/ml. After 12, 24, and 48 h, the cells were harvested and used for qPCR. Total RNA was isolated from the cells using the RNeasy Plus Mini Kit (Qiagen), according to the manufacturer’s instructions. Sitagliptin phosphate monohydrate (Sigma–Aldrich) dissolved in water was added to the culture medium at final concentrations of 10, 100, 1000, and 5000 μM. Forty-eight hours after the addition of sitagliptin, the cells were harvested and used for qPCR and the DPP4 activity assay. Proliferation was assessed using an 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay. After 5 min of pre-incubation at room temperature, the reaction was initiated by the addition of 40 μl of assay buffer containing 0.1 mmol/l of the substrate Gly-Pro-AMC. After incubation for 20 min, fluorescence was determined using a spectrofluorometer (Infinite M200 PRO, Tecan Japan Co. Ltd (Kawasaki, Japan); excitation: 380 nm/emission: 460 nm). The standard curve of free AMC was generated using 0–50 μmol/l solutions of AMC (Sigma–Aldrich). DPP4 activity was expressed as the amount of cleaved AMC.

**Knockdown of DPP4**

HEK293 and Caki-1 cells were plated into 60-mm dishes and cultured at 37 °C, before being transfected with siRNA against DPP4 (siDPP4-1, SI00030212; siDPP4-2, SI00030219; siDPP4-3, SI00030226; and siDPP4-5, SI03099642; Qiagen) or control siRNA (Silencer Negative Control #1 siRNA; Ambion, Austin, TX, USA) at final concentrations of 5 nM, using the Lipofectamine RNAiMAX transfection reagent (Invitrogen) according to the manufacturer’s instructions. After 48 h, the cells were harvested and used for qPCR.

**DPP4 activity assay**

To measure DPP4 activity, cells were harvested, washed with PBS, and solubilized in four volumes of homogenization buffer (50 mmol/l Tris–HCl (pH 7.5), 250 mmol/l sucrose, 5 mmol/l MgCl₂, 2 mmol/l ATP, 1 mmol/l dithiothreitol (DTT), and 0.5 mmol/l EDTA) containing 0.025% (w/v) digitonin. The cells were due to disruption by ultrasonication (20 pulses of 1 s). The cells were centrifuged, and the resultant supernatant was sampled. Protein concentrations were determined using the Bradford assay. DPP4 activity was determined from the cleavage rate of 7-amino-4-methylcoumarin (AMC) from the synthetic substrate H-glycyl-prolyl-AMC (Gly-Pro-AMC; Sigma–Aldrich), as previously described (Kirino et al. 2009). Briefly, 5 μl of sample was mixed with 35 μl of assay buffer (25 mmol/l HEPES, 140 mmol/l NaCl, 80 mmol/l MgCl₂, and 1% (w/v) BSA (pH 7.8)). After 5 min of pre-incubation at room temperature, the reaction was initiated by the addition of 40 μl of assay buffer containing 0.1 mmol/l of the substrate Gly-Pro-AMC. After incubation for 20 min, fluorescence was determined using a spectrofluorometer (Infinite M200 PRO, Tecan Japan Co. Ltd (Kawasaki, Japan); excitation: 380 nm/emission: 460 nm). The standard curve of free AMC was generated using 0–50 μmol/l solutions of AMC (Sigma–Aldrich). DPP4 activity was expressed as the amount of cleaved AMC.

**Immunoblotting**

The cells were washed with PBS, solubilized in four volumes of lysis buffer (50 mM Tris–HCl, pH 7.4, 1% Nonident P-40, 0.25% SDS, 150 mM NaCl, and 1 mM EDTA) with protease inhibitors, and ultrasonicated. The cells were centrifuged (15 000 g, 4 °C, 5 min) and the supernatant was used for further analysis. The protein concentrations were determined using the Bradford assay. The proteins were separated by SDS–PAGE (10%), and electrotransferred onto PVDF membrane (Hybond-P; GE Healthcare Bio-Sciences, Buckinghamshire, England). The membrane was then blocked with TBS-T buffer (20 mM Tris–HCl, pH 7.6, 137 mM NaCl, and 0.1% Tween-20) with 5% Block Ace (DS Pharma Biomedical Co. Ltd, Osaka, Japan).
To detect DPP4, rabbit anti-human DPP4 (Santa Cruz Biotechnology, Inc.) and HRP-conjugated rabbit IgG antibodies were used as the primary and secondary antibodies respectively. The proteins were visualized using an ECL detection system (GE Healthcare Bio-Sciences) and the signals were detected using a luminescent image analyzer, LAS-3000 mini (Fujifilm Corp., Tokyo, Japan).

Statistical analyses

Data were compared using the unpaired t-test or one-way ANOVA followed by post hoc comparisons using Bonferroni’s multiple comparison test. Statistical analyses were conducted using SPSS version 20.0 (SPSS, Inc.).

Results

Microarray and GO analysis in the rat kidneys

Microarray analysis using one randomly selected representative animal from each group was conducted to examine the differences between the gene expression in the kidneys of DPP4-deficient rats and WT rats after STZ treatment. In total, 71 genes were up- or down-regulated by at least twofold in the DPP4-deficient rats compared with the WT rats (Supplementary Tables 2 and 3, see section on supplementary data given at the end of this article). GO analysis was performed to categorize the genes according to biological process, molecular function, and cellular component. Our statistical filtering of the array analysis was based on at least a twofold change ($P < 0.05$) in expression. GO analysis revealed that transporter activity, heme binding, and pheromone binding-related genes demonstrated significant changes in their expression (Table 1). Among those genes, hemoglobin genes ($Hbb$ and $MGC72973$) were downregulated and the solute carrier family genes ($Slc5a3$, $Slc12a1$, and $Slco4a1$) were upregulated in the DPP4-deficient rats compared with the WT rats.

qPCR analysis of rat kidneys

To verify the results of the microarray analysis, qPCR was conducted for the 11 genes ($Hbb$, $MGC72973$, $Slc5a3$, $Slc12a1$, $Slco4a1$, $Obp3$, $Cyp2c (Cyp2c11)$, $Cyp2c7 (Cyp2c39)$, $Ptgs1$, $Cyp24a1$, and $Cyp2c13$; Table 1) that were indicated in the GO analysis, using the kidney tissues of six non-STZ-treated WT, ten STZ-treated WT, six non-STZ-treated DPP4-deficient, and ten STZ-treated DPP4-deficient rats. Although there were no significant differences in the mRNA expression levels of $Hbb$ between the DPP4-deficient and WT rats, the mRNA expression levels of $Hbb$ in both the WT and DPP4-deficient rats treated with STZ were significantly decreased when compared with the WT rats not treated with STZ, and the mRNA expression levels of $Hbb$ in the DPP4-deficient rats tended to decrease when compared with those in both the non-STZ-treated and STZ-treated WT rats (Fig. 1). Next, we carried out qPCR for other hemoglobin genes ($Hba1$ and $Hba2$). Although there were no significant differences in the mRNA expression levels of $Hba1$ between DPP4-deficient and WT rats, the mRNA expression levels of $Hba1$ tended to decrease when compared with those in both the non-STZ-treated and STZ-treated WT rats (Fig. 1).

Table 1  Gene ontology analysis of the genes whose expression differed by a factor of greater than twofold between the kidneys of DPP4-deficient and WT rats 42 days after treatment with STZ

<table>
<thead>
<tr>
<th>Gene symbol</th>
<th>Gene name</th>
<th>Fold</th>
</tr>
</thead>
<tbody>
<tr>
<td>$Hbb$</td>
<td>Hemoglobin, beta</td>
<td>2.28 down</td>
</tr>
<tr>
<td>$MGC72973$</td>
<td>Beta-glo</td>
<td>2.94 down</td>
</tr>
<tr>
<td>$Slc5a3$</td>
<td>Solute carrier family 5 (inositol transporters), member 3</td>
<td>2.19 up</td>
</tr>
<tr>
<td>$Slc12a1$</td>
<td>Solute carrier family 12, member 1</td>
<td>2.10 up</td>
</tr>
<tr>
<td>$Slco4a1$</td>
<td>Solute carrier organic anion transporter family, member 4a1</td>
<td>2.06 up</td>
</tr>
<tr>
<td>$Obp3$</td>
<td>Alpha-2u globulin PGCL4</td>
<td>3.69 down</td>
</tr>
<tr>
<td>$Cyp2c$</td>
<td>Cytochrome P450, subfamily IIC</td>
<td>3.43 down</td>
</tr>
<tr>
<td>$Hbb$</td>
<td>Hemoglobin, beta</td>
<td>2.28 down</td>
</tr>
<tr>
<td>$MGC72973$</td>
<td>Beta-glo</td>
<td>2.94 down</td>
</tr>
<tr>
<td>$Cyp2c7$</td>
<td>Cytochrome P450, family 2, subfamily $c$, polypeptide 7</td>
<td>3.91 down</td>
</tr>
<tr>
<td>$Ptgs1$</td>
<td>Prostaglandin-endoperoxide synthase 1</td>
<td>2.62 up</td>
</tr>
<tr>
<td>$Cyp24a1$</td>
<td>Cytochrome P450, subfamily 24</td>
<td>2.50 up</td>
</tr>
<tr>
<td>$Cyp2c13$</td>
<td>Cytochrome P450 2c13</td>
<td>2.45 down</td>
</tr>
<tr>
<td>$Obp3$</td>
<td>Alpha-2u globulin PGCL4</td>
<td>3.69 down</td>
</tr>
</tbody>
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knockdown against human DPP4, we tested DPP4 mRNA expression using four pre-designed siRNAs (siDPP4-1, siDPP4-2, siDPP4-3, and siDPP4-5). Among the pre-designed siRNAs, siDPP4-1 displayed the greatest knockdown of DPP4 mRNA expression in the HEK293 and Caki-1 cells, as determined by RT-PCR (Figs 2a and 3a). Therefore, we used siDPP4-1 as the DPP4-specific siRNA. We achieved >70% knockdown of DPP4 mRNA expression in each of the cell types, as determined by qPCR (Figs 2b and 3b). The expression levels of HBA1, HBA2, HBG1, and HBG2 were significantly decreased by DPP4 siRNA knockdown in HEK293 cells (Fig. 2), and the expression levels of HBA2 and HBB were significantly decreased by DPP4 siRNA knockdown in Caki-1 cells (Fig. 3). The HBA1 gene could not be analyzed because the

Figure 1
Changes in Hbb and Hba1 mRNA expression levels in WT and DPP4-deficient rats at 42 days after the i.p. injection of STZ (30 mg/kg) or the vehicle. The mRNA expression levels were measured by qPCR. All mRNA signals were normalized to the signal for Gapdh. Data are shown as the mean ± s.e. Non-STZ-treated, WT (n = 6); STZ-treated, WT (n = 10); non-STZ-treated, DPP4-deficient (n = 6); and STZ-treated, DPP4-deficient (n = 10). *P < 0.05 and **P < 0.001; one-way ANOVA followed by post hoc comparisons using Bonferroni’s multiple comparison test.

Effect of DPP4 on mRNA expression of hemoglobin genes in HEK293 and Caki-1 cells

The results of the microarray and qPCR analyses indicated that DPP4 contributes to the regulation of hemoglobin gene expression. We then studied the effect of DPP4 siRNA knockdown and the effect of adding soluble human DPP4 on the expression levels of hemoglobin genes in HEK293 cells and human renal cancer cells (Caki-1). The hemoglobin molecule consists of two of each of two different types of polypeptide chains. Fetal hemoglobin (Hb F; α2γ2) consists of two α-chains and two γ-chains. There are two identical α-globin genes, designated HBA1 (α1) and HBA2 (α2), and two identical γ-globin genes, designated HBG1 (Δγ) and HBG2 (γγ). In postnatal hemoglobin (Hb A; α2β2), the γ chains are gradually replaced by β chains, which are encoded by the β-globin gene, designated HBB (β) (Nussbaum et al. 2007). Therefore, we studied the expression of HBA1, HBA2, HBG1, and HBG2 genes in HEK293 cells, and the expression of HBA1, HBA2, and HBB genes in Caki-1 cells. To identify the effect of siRNA knockdown against human DPP4, we tested DPP4 mRNA expression using four pre-designed siRNAs (siDPP4-1, siDPP4-2, siDPP4-3, and siDPP4-5). Among the pre-designed siRNAs, siDPP4-1 displayed the greatest knockdown of DPP4 mRNA expression in the HEK293 and Caki-1 cells, as determined by RT-PCR (Figs 2a and 3a). Therefore, we used siDPP4-1 as the DPP4-specific siRNA. We achieved >70% knockdown of DPP4 mRNA expression in each of the cell types, as determined by qPCR (Figs 2b and 3b). The expression levels of HBA1, HBA2, HBG1, and HBG2 were significantly decreased by DPP4 siRNA knockdown in HEK293 cells (Fig. 2), and the expression levels of HBA2 and HBB were significantly decreased by DPP4 siRNA knockdown in Caki-1 cells (Fig. 3). The HBA1 gene could not be analyzed because the
expression level was negligible. Next, we studied the effect of addition of soluble human DPP4 on hemoglobin gene expression. The expression levels of \(\text{HBA1}, \text{HBA2}, \text{HBG1},\) and \(\text{HBG2}\) were significantly increased 48 h after the addition of sitagliptin at a final concentration of 1000 \(\mu\text{M}\) in the HEK293 cells (Fig. 6). In the Caki-1 cells, the expression level of \(\text{HBA2}\) was significantly increased and that of \(\text{HBB}\) also tended to increase 48 h after the addition of sitagliptin (Fig. 7).

**Effect of DPP4 inhibitor on mRNA expression levels of hemoglobin genes in HEK293 and Caki-1 cells**

Next, we studied the effect of a DPP4 inhibitor on the expression of hemoglobin genes in HEK293 and Caki-1 cells. In this study, sitagliptin was used as the DPP4 inhibitor. Sitagliptin is a highly selective DPP4 inhibitor, and it is used as a monotherapy for glycemic control in patients with type 2 diabetes. The expression levels of \(\text{HBA1}, \text{HBA2}, \text{HBG1},\) and \(\text{HBG2}\) were significantly increased 48 h after the addition of sitagliptin at a final concentration of 1000 \(\mu\text{M}\) in the HEK293 cells (Fig. 6). In the Caki-1 cells, the expression level of \(\text{HBA2}\) was significantly increased and that of \(\text{HBB}\) also tended to increase 48 h after the addition of sitagliptin (Fig. 7).

**Figure 3**
Changes in DPP4 (B), HBA2 (C), and HBB (D) mRNA expression levels in Caki-1 cells from the DPP4 siRNA knockdown using DPP4 siRNA1 (DPP4 KD) and control siRNA (Control). The levels of mRNA expression were measured by qPCR and compared with those of the untreated cells. mRNA signals were normalized to the signal for GAPDH. The experiments were repeated three times, and the data are shown as the mean \(\pm\) S.D. (A) RT-PCR analysis of Dpp4 mRNA expression by DPP4 knockdown using four pre-designed siRNAs and control siRNA. ***\(P<0.001\); one-way ANOVA followed by post hoc comparisons using Bonferroni’s multiple comparison test.

**Figure 4**
Changes in HBA1, HBA2, HBG1, and HBG2 mRNA expression levels at 12, 24, and 48 h after the addition of soluble human DPP4 (final concentration of 500 ng/ml) in HEK293 cells. The levels of mRNA expression were measured by qPCR and compared with those of the control group. mRNA signals were normalized to the signal for GAPDH. The experiments were repeated three times, and the data are shown as the mean \(\pm\) S.D. *\(P<0.05\), **\(P<0.01\), and ***\(P<0.001\); Student’s t-test.
The expression level of DPP4 mRNA was significantly increased 48 h after the addition of sitagliptin in both the HEK293 and Caki-1 cells (Figs 6b and 7b). The protein expression level of DPP4 was also increased (Figs 6c and 7c). This concentration of sitagliptin inhibited almost 100% of DPP4 activity (Figs 6a and 7a) and did not affect the cell viability of the HEK293 and Caki-1 cells when measured using the MTT assay (Supplementary Fig. 1, see section on supplementary data given at the end of this article).

Discussion

GO analysis of the microarray results from the analysis of the rat kidneys indicated that hemoglobin genes and the alpha-2u globulin PGCL4 gene (Obp3) were downregulated, and that the solute carrier family genes were upregulated in the DPP4-deficient rats when compared with the WT rats after STZ treatment. Previous reports have described reduced expression of alpha-2u globulin in the liver (Murty et al. 1986) and kidneys (Sharma & Tikoo 2013) of STZ-induced diabetic rats and in the renal cortex of a Db/Db mouse model of type 2 diabetes (Tilton et al. 2007). It is also known that a number of chemicals induce alpha-2u globulin nephropathy (Swenberg et al. 1989, Borghoff et al. 1990). Our results indicate that the expression of Obp3 was decreased by a feedback response with the accumulation of alpha-2u globulin in the kidney, because DPP4-deficient rats displayed renal dysfunction after STZ treatment, while WT rats did not. Solute carriers are membrane transport proteins that control the uptake and efflux of solutes (Schlessinger et al. 2010). Because the control of the solute uptake and efflux decreased due to renal dysfunction in the STZ-induced DPP4-deficient rats,
DPP4 contributes to the regulation of hemoglobin gene expression. However, inhibition of DPP4 activity by the addition of the DPP4 inhibitor sitagliptin, resulted in significantly increased expression levels of hemoglobin genes in both cell types. Because the DPP4 expression levels were increased by the addition of the DPP4 inhibitor, it is considered that DPP4 was upregulated in response to the DPP4 inhibitor via an autoregulatory feedback mechanism linked to the increase in hemoglobin expression. Hence, it is suggested that in addition to the function of DPP4 in proteolytic activity, DPP4 also functions to contribute to the regulation of hemoglobin gene expression. DPP4 is a complex enzyme that is present on the surface of various types of cells, including kidney, liver, pancreas, and plasma cells, and is also present in a soluble form in the circulation (Mentlein 1999). DPP4 has been recognized to play an important role in the cleavage and inactivation of biologically active peptides (De Meester et al. 2000). In addition, DPP4, also known as CD26, is expressed on the CD4+ helper/memory T cell populations, as well as in a membrane-bound form, and can deliver a potent co-stimulatory T-cell activation signal. CD26 is the receptor for adenosine deaminase, an important cell surface immunoregulatory mechanism (Morimoto & Schlossman 1998). Recently, it has been reported that DPP4 has been identified as a functional receptor for human coronavirus–Erasmus Medical Center (hCoV–EMC; Raj et al. 2013), and was delineated as the molecular basis of interaction between Middle East respiratory syndrome CoV (MERS–CoV) and its receptor CD26 (Lu et al. 2013). Antibodies directed against DPP4 inhibited hCoV–EMC infection of primary human bronchial epithelial cells and Huh-7 cells; however, hCoV–EMC infection could not be blocked by the DPP4 inhibitors. Therefore, Raj et al. (2013) have proposed that the abundance of DPP4 receptors present on epithelial and endothelial tissues may be related to infection. In addition, Ikeda et al. (2013) have reported that the addition of soluble CD26 resulted in increased tumor necrosis factor alpha and interleukin 6 mRNA and protein expression, enhanced MAPK1/2 levels in the cytosol, and enhanced c-Fos, NFκB p50, NFκB p65, and CUX1 levels in the nuclei of THP1 cells and human monocytes. Our findings also indicated that soluble DPP4 enhanced the expression levels of hemoglobin genes. We consider that DPP4 does not affect transcription factors or epigenetics, because there is no nuclear transport signal in DPP4, and DPP4 does not localize in the nuclei of kidney cells (data not shown). Soluble DPP4, rather than the membrane-bound form, may indirectly contribute to the regulation

Figure 7
Changes in DPP4 activity (A), DPP4 mRNA expression level (B), western immunoblots of DPP4 (C), and HBA2 (D), and HBB (E) mRNA expression levels 48 h after the addition of the DPP4 inhibitor, sitagliptin (final concentration of 1000 μM), in Caki-1 cells. The levels of mRNA expression were measured by qPCR and compared with those for the control group. mRNA signals were normalized to the signal for Gapdh. The experiments were repeated three times, and the data are shown as the mean ± S.D. *P<0.05, **P<0.01, and ***P<0.001; Student’s t-test.

the solute carrier family genes may have been upregulated to make up for this dysfunction. Among the genes that showed mRNA expression differences in microarray analysis of the kidneys between DPP4-deficient and WT rats after STZ treatment, only hemoglobin genes showed a significant mRNA expression difference among the four groups. The mRNA expression levels of hemoglobin genes in DPP4-deficient rats treated with STZ were significantly decreased relative to the levels in the control WT rats. Because the Hbb mRNA expression level in WT rats treated with STZ was significantly decreased when compared with those in the WT control rats, STZ treatment may decrease hemoglobin mRNA expression. It is suggested that the decrease in the hemoglobin mRNA level of DPP4-deficient rats treated with STZ was intensified by synergistic or additive effects of DPP4 deficiency and STZ treatment.

The mRNA expression levels of hemoglobin genes were downregulated by DPP4 siRNA knockdown, and upregulated by the addition of soluble DPP4 in HEK293 and Caki-1 cells. Therefore, it was suggested that DPP4
of hemoglobin gene expression in the cytosol. Further studies are required for the detailed analysis of the regulatory system by which DPP4 influences hemoglobin gene expression.

Hemoglobin is expressed by mesangial cells and it plays a cytoprotection role against oxidative insults (Nishi et al. 2008). In addition, the decrease in hemoglobin levels causes hypoxia. Oxidative stress and hypoxic stress induce endoplasmic reticulum (ER) stress (Ihara & Ikezaki 2010), which is involved in a wide range of renal pathophysiology (Kitamura 2008). Our findings indicate that soluble DPP4 enhances hemoglobin gene expression in the kidneys, and that hemoglobin protects renal function from diabetes by acting in a cytoprotective manner against oxidative, hypoxic, or ER stress.

In conclusion, we have shown for the first time, to our knowledge, that DPP4 contributes to the regulation of hemoglobin gene expression in the kidney. Although further studies are required, these findings indicate that enhanced hemoglobin expression with DPP4 expression play a key role in the preservation of renal function in diabetes.

Supplementary data
This is linked to the online version of the paper at http://dx.doi.org/10.1530/JOE-14-0016.

Declaration of interest
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement
The authors have made the following declaration about their contribution: Y S designed the study, collected and analyzed data, and wrote the manuscript. T K collected and analyzed data. A Y contributed to the discussion.

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References


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