Experimental Physiology

Research Paper

A plasminogen activator inhibitor type 1 mutant retards diabetic nephropathy in *db/db* mice by protecting podocytes

Jiandong Zhang[1], Chunyan Gu[1], Daniel A. Lawrence[2], Alfred K. Cheung[1,3] and Yufeng Huang[1]

[1]*Division of Nephrology & Hypertension, University of Utah School of Medicine, Salt Lake City, UT, USA*
[2]*Department of Internal Medicine, University of Michigan School of Medicine, Ann Arbor, MI, USA*
[3]*Medical Care Center, Veterans Affairs Salt Lake City Health Care System, Salt Lake City, UT, USA*

New Findings

- **What is the central question of this study?**
  The mechanism by which PAI-1R reduces proteinuria has not been previously explored.
- **What is the main finding and its importance?**
  Using cultured podocytes and diabetic *db/db* mice, we show that, similar to the effects of transforming growth factor-β1, PAI-1 directly injures podocytes. Reducing the increased PAI-1 activity by PAI-1R in diabetic *db/db* mice, in fact, reduces podocyte injury, thereby reducing albuminuria. Therefore, PAI-1R may provide an additional therapeutic effect in slowing progression of diabetic nephropathy by maintaining podocyte integrity.

A mutant non-inhibiting plasminogen activator inhibitor type 1 (PAI-1), termed PAI-1R, which reduces endogenous PAI-1 activity, has been shown to inhibit albuminuria and reduce glomerulosclerosis in experimental diabetes. The mechanism of the reduction of albuminuria is unclear. This study sought to determine whether the administration of PAI-1R protected podocytes from injury directly, thereby reducing albuminuria in the *db/db* mouse, a model of type 2 diabetes. Untreated uninephrectomized *db/db* mice developed significant mesangial matrix expansion and albuminuria at week 22 of age, associated with segmental podocyte foot-process effacement, reduction of renal nephrin, podocin and zonula occludin-1 production and induction of renal desmin and B7-1 generation. In contrast, treatment with PAI-1R at 0.5 mg (kg body weight)$^{-1}$ i.p., twice daily from week 20 to 22, reduced glomerular matrix accumulation, fibronectin and collagen production and albuminuria by 36, 62, 65 and 31%, respectively ($P < 0.05$), without affecting blood glucose level or body weight. Podocyte morphology and protein markers were also significantly attenuated by PAI-1R administration. Importantly, recombinant PAI-1 downregulated nephrin and zonula occludin-1 but increased desmin and B7-1 mRNA expression and protein production by podocytes *in vitro*, similar to the effects of transforming growth factor-β1. These observations provide evidence that PAI-1, in a manner similar to transforming growth factor-β1, directly induces podocyte injury, particularly in the setting of diabetes, where elevated PAI-1 may contribute to the progression of albuminuria. Reducing the increased PAI-1 activity by administration of PAI-1R, in fact, reduces podocyte

injury, thereby reducing albuminuria. Therefore, PAI-1R provides an additional therapeutic effect in slowing the progression of diabetic nephropathy via the protection of podocytes.

Introduction

Diabetes is the leading cause of end-stage renal disease, and almost half of new end-stage renal disease patients have diabetes (Molitch *et al.* 2004). Despite the use of angiotensin-converting enzyme inhibitors or angiotensin receptor blockers (Brenner *et al.* 2001; Parving *et al.* 2001), progression of diabetic nephropathy is common. Hence, it is important to identify other interventions that might retard the progression of diabetic nephropathy.

Recently, plasminogen activator inhibitor type 1 (PAI-1) has emerged as a powerful fibrogenic mediator in kidney diseases, including diabetic nephropathy (Eddy & Fogo, 2006; Brown, 2010; Ghosh & Vaughan, 2012). Enhanced glomerular expression of PAI-1 has been reported in both human and animal models of diabetes (Pauekshakon *et al.* 2002; Huang *et al.* 2008). Besides diabetes, increased deposition of PAI-1 in the kidney has been demonstrated in most other human kidney diseases (Rondeau *et al.* 1990; Shihab *et al.* 1995; Yamamoto *et al.* 1996, 1999; Grandaliano *et al.* 2000; Lee *et al.* 2001). The elevated expression of *PAI-1* is found in podocytes, in endothelial and mesangial cells of the glomeruli, in proximal tubular epithelial cells and in small arteries and fibroblast cells in the interstitium (Keeton *et al.* 1993). Importantly, genetic PAI-1 deficiency has been shown to be protective in experimental diabetic nephropathy, five-sixth nephrectomy, crescentic anti-GBM nephritis and the spontaneous renal fibrosis that develops in mice that overexpress transforming growth factor-β (TGFβ; Oda *et al.* 2001; Krag *et al.* 2005; Nicholas *et al.* 2005; Eddy & Fogo, 2006). In contrast, the renal fibrosis is worse in *PAI-1*-overexpressing mice (Matsuo *et al.* 2005). Together, these observations strongly implicate tissue PAI-1 in the renal fibrotic process.

The profibrotic actions of PAI-1 have not been completely elucidated. Plasminogen activator inhibitor type 1 possesses two distinct functions that could mediate its profibrotic effects alone or in combination, which are an antiprotease activity that inhibits the generation of plasmin and a vitronectin-binding ability that interferes with cell adhesion to this extracellular matrix protein (Huang & Noble, 2007). As the primary physiological inhibitor of urokinase (uPA) and tissue-type plasminogen activator, PAI-1 controls the formation and activity of plasmin and, consequently, promotes fibrin deposits and accumulation of fibrotic matrix.

The binding of PAI-1 to vitronectin could also be important in the pathogenesis of fibrosis. When bound to vitronectin, the antiprotease activity of PAI-1 is significantly stabilized (Podor *et al.* 2000). The binding of PAI-1 to vitronectin also blocks the interactions of vitronectin with cell-surface integrin required for cellular motility and with the receptor for cell-bound uPA (uPAR). Furthermore, the binding of PAI-1 to vitronectin induces epithelial-to-mesenchymal transition in epithelial cells by modulating their interaction with the extracellular matrix, a process that is involved in tissue fibrosis (Scaffidi *et al.* 2001; Vial & McKeown-Longo, 2008; Senoo *et al.* 2010). Whether the properties of PAI-1 observed *in vitro* are also operative in diseased subjects or animal models remains unclear.

In order to understand the actions of PAI-1 further and to develop a potential therapeutic agent that inhibits PAI-1 *in vivo*, a non-inhibitory mutant PAI-1 (PAI-1R) was designed (Stefansson *et al.* 2001). This PAI-1R mutant does not inhibit uPA or tissue-type plasminogen activator, but it retains the ability to bind vitronectin, thereby decreasing the availability of vitronectin for PAI-1 and effectively shortening its half-life. The administration of PAI-1R to nephritic rats and diabetic *db/db* mice increased glomerular plasmin generation and reduced proteinuria and glomerulosclerosis (Huang *et al.* 2003, 2008). Using the plasminogen/plasmin blocker tranexamic acid, we further observed that the therapeutic effect of PAI-1R on glomerular extracellular matrix accumulation was largely mediated through plasmin, because this effect was blocked by cotreatment with tranexamic acid (Huang *et al.* 2006).

However, the effect of PAI-1R on the reduction of proteinuria was not affected by blocking plasmin generation, suggesting that PAI-1R has multiple therapeutic actions on the measures of disease severity. Conversely, elevated PAI-1 may have multiple actions on the induction and progression of kidney disease. It is likely that PAI-1 may act as a direct cause of proteinuria independent of plasmin generation, which could be prevented by inhibition of PAI-1. This notion was strengthened by the observations that *PAI-1* deletion significantly reduced the severity of proteinuria in diabetes (Nicholas *et al.* 2005; Collins *et al.* 2006; Lassila *et al.* 2007). It has been shown that proteinuria is often the result of podocyte injury in human diseases and animal models, especially in diabetes (Yu *et al.* 2005; Shankland,

2006; Ziyadeh & Wolf, 2008). Thus, we postulate that PAI-1 plays a role in podocyte injury and proteinuria. We further hypothesize that PAI-1R reduces proteinuria by the amelioration of podocyte injury via PAI-1 inhibition.

To test our hypothesis, we examined whether PAI-1 directly damaged podocytes in cell culture and determined the therapeutic efficacy of PAI-1R on podocyte injury in a murine model of uninephrectomized diabetic nephropathy.

Methods

Reagents

Unless otherwise indicated, materials and chemicals were obtained from Sigma-Aldrich (St Louis, MO, USA). The recombinant human PAI-1R and active stable PAI-1 have been described previously (Huang *et al.* 2009) and were purified as described (Kvassman & Shore, 1995).

Study 1: therapeutic effects of PAI-1R on diabetes-associated podocyte injury in *db/db* mice

Animals. Diabetic male *db/db* mice (BKS.Cg-*Dock7ᵐ* +/+ *Leprᵈᵇ*/J homozygotes) and their littermate male *db/m* mice were obtained from the Jackson Laboratory (Bar Harbor, ME, USA) and housed in accordance with the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH publication no. 85-23, revised 1996). The experimental protocols were approved by the Animal Care Committee at the University of Utah. The *db/db* mice were determined to be diabetic by the vendor on the basis of the appearance of obesity at ~5 weeks of age and were demonstrated to be hyperglycaemic in our laboratory at week 7. Mice were subjected to right uninephrectomy under general anaesthesia induced by inhalation of 2.5% isoflurane through a complete anaesthesia system (VetEquip IMPAC6; VetEquip Inc., Pleasanton, CA, USA) at week 8 to hasten the development of diabetic nephropathy as described previously (Huang *et al.* 2008). The *db/m* mice were subjected to uninephrectomy at week 8 to serve as the operation control. Our previous work on model development showed that uninephrectomized diabetic *db/db* mice developed overt nephropathy by 18 weeks of age, and disease progression was remarkable between 18 and 22 weeks of age, determined by expansion of the mesangium and significant albuminuria (Huang *et al.* 2008). In the present study, this model was used to determine the impact of PAI-1R on podocyte injury from 20 to 22 weeks of age.

Experimental design. Groups of eight or nine uninephrectomized mice were assigned and treated at 20 weeks of age as follows: untreated non-diabetic *db/m* mice ($n = 9$) as healthy control animals; diabetic *db/db* mice treated with PBS by I.P. injection from week 20 to 22 ($n = 8$) as disease control animals; and diabetic *db/db* mice treated with 0.5 mg (kg body weight)$^{-1}$ PAI-1R injected I.P., twice daily, from week 20 to 22 ($n = 8$; the effective dose of PAI-1R was determined previously, see Huang *et al.* 2008). The blood glucose level and glycosylated haemoglobin (HbA$_{1c}$) level were monitored in tail blood samples by using a blood glucose metre (Glucometer Elite XL; Bayer Healthcare, Elkhart, IN, USA) and the DC 2000+ HbA1C kit (Bayer Healthcare), respectively. Collection of urine for 24 h was carried out for each mouse after placement in metabolic cages. Urine albumin was measured using the DC2000+ microalbumin reagent kit (Bayer Healthcare). Regarding the reproducibility of this assay, the coefficients of variance were <3% when the same sample was measured three times consecutively.

Mice were killed under isoflurane anaesthesia in accordance with the US National Institutes of Health *Guide for the Care and Use of Laboratory Animals.* Briefly, general anaesthesia was induced by inhalation of 2.5% isoflurane through a complete anaesthesia system (VetEquip IMPAC6; VetEquip Inc., Pleasanton, CA, USA). When mice were deeply anaesthetized as determined by absence of the toe-pinch reflex, blood samples were obtained by cardiac puncture, and the kidneys were perfused through the heart with cold PBS and then excised. Mice died of massive blood loss. The renal cortex was harvested by dissection and saved for further analysis as described previously (Huang *et al.* 2008). Briefly, three pieces of cortex were either snap-frozen in 2-methylbutane at –80°C or fixed in 10% neutral-buffered formalin for immunohistological examination or fixed in 2.5% glutaraledhyde and 0.1 M sodium cacodylate buffer at 4°C for electron microscopic examination. Other pieces of cortex were treated with TRIzol™ Reagent (GibcoBRL, Gaithersburg, MD, USA) for isolation of RNA or treated with 100 mM NaCl and 20 mM Hepes to be sonicated for 30 s, three times on ice and centrifuged at 13,100 *g* for 15 min at 4°C. The supernatant from the sonication was then collected and stored at –80°C for fibronectin (FN) ELISA (Huang *et al.* 2008) and protein measurement by a BCA protein assay kit (Pierce, Rockford IL, USA). The total collagen level in renal cortex was determined by a colorimetric method according to the manufacturer's instructions (Biocolor Ltd, Carrickfergus, UK), in which the Sirius Red dye binds to the side-chains of amino acids found in collagen.

Histological analyses. Formalin-fixed renal cortex tissues were subsequently embedded in paraffin. Three-micrometre-thick sections were cut from the tissue blocks and stained with periodic acid–Schiff. The periodic

acid–Schiff-positive glomerular matrix was quantified in a blinded fashion by a computer-assisted method as previously described (Huang *et al.* 2008). At least 20 glomeruli from each mouse were assessed. The matrix surface area in the mesangium was normalized by that of the total glomerular tuft, where the percentage of mesangial matrix occupying each glomerulus was rated on a 0–4 scale (0 = 0, 1 = 25, 2 = 50, 3 = 75 or 4 = 100%), as described previously (Huang *et al.* 2008).

Immunofluorescent staining for nephrin and podocin was performed on frozen sections. The polyclonal goat anti-nephrin IgG and the polyclonal goat anti-podocin IgG (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) were used as the primary antibodies. The rhodamine (TRITC)-conjugated donkey anti-goat IgG (reacts with both heavy and light chains; Jackson ImmunoResearch Laboratories Inc., West Grove, PA, USA) was used as the secondary antibody. Intraglomerular positive staining of both molecules was also quantified separately in a blinded fashion by a computer-assisted method. Briefly, images (×400 magnification) of 20 random glomeruli per slide were captured using a Nikon D50 digital camera (Inkle's-Ritz Camera, www.ritzcamera.com, Salt Lake City, UT, USA; Nikon Capture 4 version 4.3, Nikon Inc., Melville, NY, USA), and the total staining intensity in each glomerulus was quantified using a computer-assisted colour image analysis system (ImageJ 1.38 for Windows; National Institutes of Health http://rsb.info.nih.gov; Abramoff *et al.* 2004). The results were expressed as the ratio of the staining intensity in the experimental groups to that in the normal glomeruli.

Transmission electron microscopic examination. Glutaraledhyde-fixed renal cortex tissues were further processed with OsO_4 and Epon 812. Ultrathin sections were cut and stained with uranyl acetate and lead citrate. Specimens were placed on copper grids and examined in an electron microscope (JEOL JEM-1010, Tokyo, Japan). Foot-process density was calculated by dividing the number of foot processes by the glomerular basement membrane (GBM) length (Zheng *et al.* 2008).

Western blot analysis. Equal amounts of renal cortex tissue (15 mg) from each mouse of each group were homogenized in lysis buffer (Cell Signaling Technology, Inc., Beverly, MA, USA) with 1% octylphenoxypolyethoxyethanol (Nonidet P-40), 1 mM phenylmethylsulfonyl fluoride and one tablet per 5 ml protease inhibitor mix (Complete, Mini; Roche Diagnostics Corporation, Indianapolis, IN, USA). Protein samples from eight or nine mice of each group were pooled for further examination.

For Western blot analysis, protein samples (20 μg each) were subjected to SDS-PAGE in 4–12%

gradient gels (Invitrogen, Carlsbad, CA, USA) and immunblotted onto immobilon-P transfer membranes (Millipore Corporation, Bedford, MA, USA). Desmin and B7-1 were assessed on the Western blots using mouse monoclonal anti-desmin IgG2a (Santa Cruz Biotechnology, Inc.) and rabbit monoclonal anti-B7-1/CD80 IgG (OriGene Technologies, Inc., Rockville, MD, USA). The immunostaining band was visualized and quantified as described previously (Huang *et al.* 2008). Desmin and B7-1 protein levels were corrected by the densitometric intensity of the glyceraldehyde 3-phosphate dehydrogenase (GAPDH; using goat anti-GAPDH IgG antibody; GenScript, Piscataway, NJ, USA) or β-actin (using mouse monoclonal anti-β-actin IgG) in each loading. For comparison, this ratio was set at unity for normal control samples, and other lanes on the same gel were expressed as the fold-change over this value. Three blots were performed for each primary antibody.

Preparation of RNA and real-time RT-PCR. Total RNA was extracted from renal cortex tissue using TRIzol^{TM} Reagent (GibcoBRL) according to the manufacturer's instructions. Two micrograms of total RNA were reverse transcribed using the Superscript III first-strand synthesis system for RT-PCR kit (Invitrogen). Real-time RT-PCR was performed using the SYBR green dye I (Applied Biosystems, Foster City, CA, USA) and the ABI 7900 Sequence Detection System (Applied Biosystems) as described previously (Huang *et al.* 2008). Samples were run in triplicate in separate tubes to permit quantification of the target gene normalized to β-actin. Sequences of primers used are listed in Table 1. The specificity of the PCR products was confirmed on a 1.5% agarose gel by showing a specific single band with the expected size.

Study 2: effect of wild-type PAI-I on cultured podocytes

Cell culture. Conditionally immortalized mouse podocytes were kindly provided by Dr Peter Mundel (Massachusetts General Hospital and Harvard Medical School). These cells were cultured and induced to differentiate as described previously (Reiser *et al.* 2004). Briefly, podocytes were cultured in permissive conditions (33°C) in RPMI-1640 supplemented with 10% fetal calf serum, penicillin–streptomycin and interferon-γ (all from Invitrogen). Differentiation was induced by thermoshift to 37°C without interferon-γ in type I collagen-coated six-well plates (Fisher Science Education, Hanover Park, IL, USA) before experimental treatment. All experiments were performed with cells that had been allowed to differentiate for at least 14 days. Podocytes between passages 10 and 15 were used for all experiments.

Table 1. Primers used for real-time RT-PCR

Gene	Primer	Location (complementary to nucleotides)	Sequence 5′–3′
Mouse nephrin	Forward	4344–4363	AGATTTTGGGTTGCAGGTTG
(NM_019459)	Reverse	4476–4495	TGACCCATCTTTCCAGTTCC
Mouse zonula occludin-1	Forward	5975–5994	GGGGTGCATTTGAGTTCTGT
(NM_009386.1)	Reverse	6219–6238	ACATGCTTTGGTGTCCTTCC
Mouse desmin	Forward	1374–1393	CAAAGGGGTTCTGAAGTCCA
(NM_010043.1)	Reverse	1552–1571	GAAAAGTGGCTGGGTGTGAT
Mouse B7-1	Forward	297–316	CCATGTCCAAGGCTCATTCT
(NM_009855.2)	Reverse	480–499	TTCCCAGCAATGACAGACAG
Mouse β-actin	Forward	857–876	GCTCTTTTCCAGCCTTCCTT
(NM_007393.2)	Reverse	1132–1151	TGATCCACATCTGCTGGAAG

Effect of PAI-1 on podocyte injury. To examine the effect of PAI-1 on podocyte injury, differentiated podocytes were rendered quiescence in serum-free medium for 24 h, then incubated with 150 nM of recombinant active stable human PAI-1 for various times or incubated for 24 h in incremental concentrations of PAI-1 (Huang *et al.* 2009). Podocytes treated with serum-free medium only or with 2 ng ml^{-1} TGFβ1 (R&D, Minneapolis, MN, USA) for 24 h served as normal and positive controls, respectively. After incubation, the podocytes were washed with ice-cold PBS. Total cellular RNA or protein was isolated from the podocytes as described previously for the analyses of the mRNA and protein expression of podocyte-associated molecules by real-time RT-PCR and Western blotting respectively (Huang et al., 2007). The rabbit polyclonal anti-nephrin antibody (TA306037) and rabbit polyclonal anti-zonula occludin-1 (ZO-1) antibody (61-7300) were obtained from OriGene Technologies and Invitrogen, respectively. All experiments were performed at least three times in duplicate wells.

Statistical analyses

Data are expressed as means ± SD. The disease-induced increase in a variable was defined as the mean value for the disease control group minus the mean value of the normal control group. The percentage of reduction in disease severity in a treated group was calculated as described previously (Huang *et al.* 2008). Statistical analyses of differences between the groups were performed by one-way ANOVA and subsequent Student–Newman–Keuls or Dunnett testing for multiple comparisons. A value of $P < 0.05$ was considered statistically significant.

Results

Study 1: therapeutic efficacy of PAI-1R

Systemic parameters. There was no significant difference in survival rate among the groups of non-diabetic *db/m*

mice, diabetic *db/db* mice treated with PBS and diabetic *db/db* mice treated with PAI-1R. The baseline and final parameters of the three groups of mice are presented in Table 2. The body weights of *db/db* mice at 20 weeks of age were significantly greater than the corresponding values in *db/m* control animals. There was no significantly change in body weight from week 20 to 22 in *db/db* mice. Diabetic mice remained hyperglycaemic throughout the experimental period, as indicated by blood glucose and glycosylated haemoglobin levels. The PAI-1R treatment had no effect on body weight or blood glucose levels in the *db/db* mice.

At the beginning of intervention (20 weeks of age), urinary albumin excretion of *db/db* mice in both groups treated with or without PAI-1R was similar and was significantly higher compared with *db/m* mice. Albumin excretion stayed high between weeks 20 and 22 in PBS-treated *db/db* mice. The PAI-1R treatment, in fact, lowered the urinary albumin excretion by 31.4% (359.5 ± 45.3 *versus* 247.4 ± 19.1 μg day^{-1}; $P < 0.05$).

Glomerular histology. At the end of the study (22 weeks), the glomeruli of *db/db* mice demonstrated increased accumulation of periodic acid–Schiff-positive extracellular matrix proteins in the mesangium, compared with glomeruli of *db/m* mice (Fig. 1*A* and *B*). Treatment with PAI-1R ameliorated extracellular matrix deposition from the diabetic glomeruli by 36.3% ($P < 0.05$).

Collagen and FN levels in renal cortex. The levels of both collagen and FN, two major components of glomerular extracellular matrix, were quantified in the renal cortex. Consistent with the accumulation of glomerular periodic acid–Schiff-positive extracellular matrix, levels of FN and collagen were significantly increased in *db/db* mice at week 22. The PAI-1R treatment reduced the disease-induced increases in protein levels of FN and collagen by 62.5 and 67.7%, respectively ($P < 0.05$; Fig. 1*C* and *D*).

Table 2. Parameters of the experimental groups of mice

Parameter	db/m (n = 9)	db/db (n = 8)	db/db-PAI-lR (n = 8)
Body weight, initial (g)	24.5 ± 0.8	43.1 ± 6.0*	46.0 ± 3.3*
Body weight, final (g)	25.1 ± 1.3	43.1 ± 5.6*	42.4 ± 3.5*
Plasma glucose, initial (mg dl^{-1})	106.2 ± 16.8	512.0 ± 96.8*	553.0 ± 59.1*
Plasma glucose, final (mg dl^{-1})	98.1 ± 43.7	560.0 ± 65.9*	595.4 ± 5.9*
Glycosylated haemoglobin (%)	3.8 ± 0.2	9.8 ± 2.4*	10.2 ± 2.2*
Urinary albumin excretion, initial (μg day^{-1})	7.4 ± 0.5	345.8 ± 0.6*	359.5 ± 45.2*
Urinary albumin excretion, final (μg day^{-1})	6.1 ± 1.3	356.9 ± 3.5*	247.4 ± 19.1*†‡

Non-diabetic (*db/m*) or diabetic mice (*db/db*) were treated for 2 weeks with either PBS or PAI-1R between 20 and 22 weeks of age. *$P < 0.05$ *versus db/m*; †$P < 0.05$ *versus db/db*; ‡$P < 0.05$ *versus db/db*-PAI-R at 20 weeks of age.

Electronic microscopy of podocyte ultrastructure. As shown in Fig. 2*A–C*, segmental podocyte foot-process effacement and a decrease in foot-process density were evident in the glomeruli from diabetic mice at 22 weeks, in comparison to glomeruli from non-diabetic mice (*db/db*

versus db/m, 1.56 ± 0.35 *versus* 3.42 ± 0.34, $P < 0.05$). The PAI-1R treatment effectively restored foot-process density (PAI-1R *versus db/db*, 2.83 ± 0.67 *versus* 1.56 ± 0.35, $P < 0.05$). The changes of the podocyte foot process can be seen clearly in the high-resolution images (Fig. 2*D–F*).
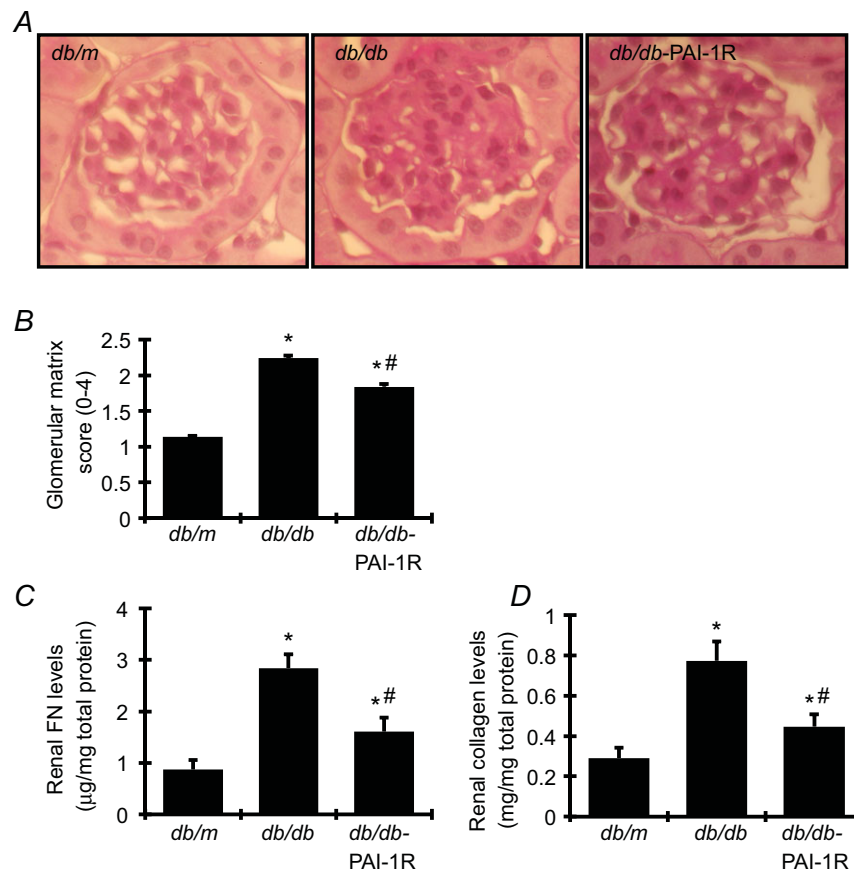

Figure 1. *In vivo* effect of mutant plasminogen activator inhibitor type 1 (PAI-1R) on glomerular matrix protein accumulation
A, representative photomicrographs (at original magnification ×400) of glomeruli at 22 weeks from normal control mice (*db/m*), diabetic *db/db* mice treated with PBS (*db/db*) and diabetic *db/db* mice treated with mutant plasminogen activator inhibitor type 1 (*db/db*-PAI-1R). Graphical representation of the mean periodic acid–Schiff staining scores (*B*). Fibronectin (FN; *C*) and total collagen (*D*) of each group are also shown. *$P < 0.05$ *versus db/m*. #$P < 0.05$ *versus db/db*.

Expression of podocyte slit-diaphragm-associated proteins. In order to confirm the protective effects of PAI-1R on podocyte injuries observed using electronic microscopy, the podocyte slit-diaphragm-associated proteins nephrin and podocin were assessed by immunofluorescent staining. As shown in Fig. 3, non-diabetic *db/m* mice showed intense and linear staining for both nephrin and podocin along the capillary in the glomeruli. In contrast, diabetes resulted in an attenuation of staining by 48.4% for nephrin and 36.9% for podocin ($P < 0.05$) in the glomeruli of *db/db* mice at 22 weeks of age. The administration of PAI-1R reduced the loss of podocyte-specific nephrin and podocin staining by 58.6 and 73.7%, respectively ($P < 0.05$).
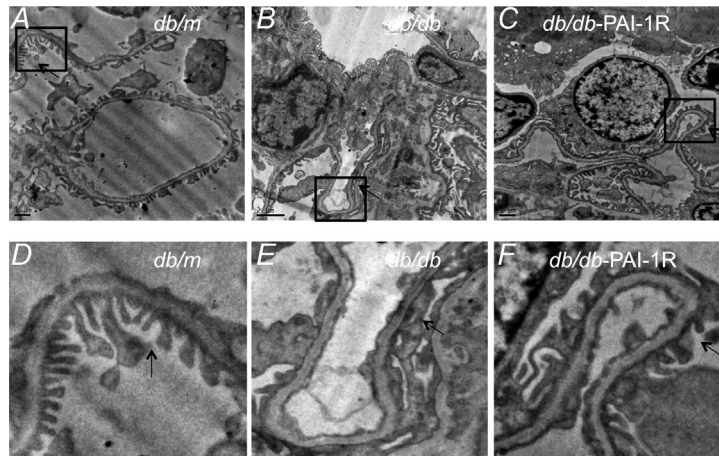

Figure 2. *In vivo* effect of PAI-1R on podocytes assessed by transmission electron microscope
Representative photomicrographs of glomerular podocytes obtained at 22 weeks from a normal control mouse (*db/m*; *A*), a diabetic *db/db* mouse treated with PBS (*db/db*; *B*) and a diabetic mouse treated with PAI-1R (*db/db*-PAI-1R; *C*). The podocyte foot process is indicated by an arrow. The zoom images (*D–F*) are shown below the original photographs. Scale bar in *A* and *C* represents 1 μm and represents 2 μm in *B*.
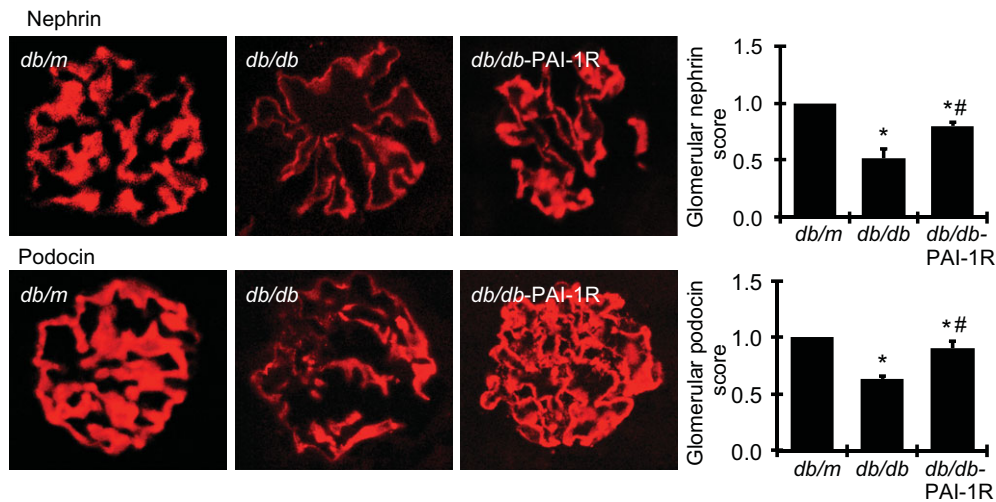

Figure 3. Effect of PAI-1R on immunofluorescent staining for glomerular nephrin and podocin at week 22
Representative photomicrographs (original magnification ×400) of the glomeruli obtained at 22 weeks from a normal control mouse (*db/m*), a diabetic *db/db* mouse treated with PBS (*db/db*) and a diabetic *db/db* mouse treated with PAI-1R (*db/db*-PAI-1R). Graphical representations of the mean nephrin and podocin staining scores of each group are shown on the right. *$P < 0.05$ *versus db/m*. #$P < 0.05$ *versus db/db*.
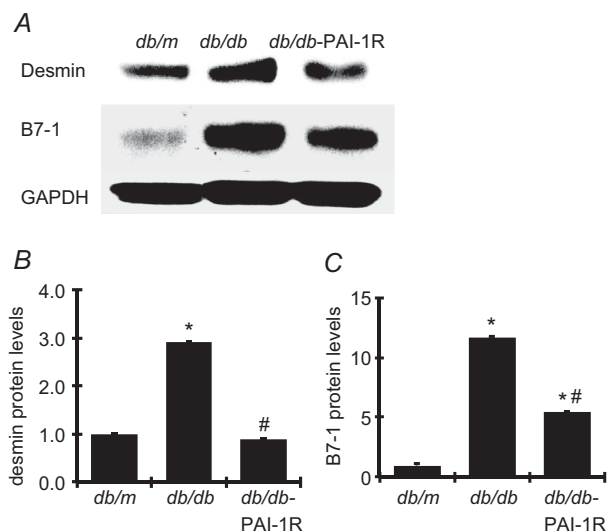
Figure 4. Effect of PAI-1R on desmin and B7-1 production in renal cortical tissues
A, representative Western blots showing desmin and B7-1 protein expression. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was included in the blot for normalization. *B* and *C*, graphical representations of the mean band intensity of desmin and B7-1, respectively, normalized against the band intensity of GAPDH. *$P < 0.05$ *versus db/m*. #$P < 0.05$ *versus db/db*.

Expression of markers of podocyte injury. By Western blotting, we observed that the protein levels of desmin in renal cortex tissues, a conventional marker for podocyte injury, were upregulated by 190% in diabetic *db/db* mice ($P < 0.05$), as shown in Fig. 4. The PAI-1R treatment reversed the disease-induced increase of desmin protein. In addition, it has been shown that B7-1 is synthesized in podocytes in response to split-diaphragm protein rearrangement and foot-process effacement, and serves as another marker of injury (Reiser *et al.* 2004). As shown in Fig. 4, B7-1 protein levels were markedly increased in diabetic renal tissue in *db/db* mice. Although we could not confirm whether the elevated production of desmin and B7-1 was from podocytes, the changes of both desmin and B7-1 were consistent with the changes of the two slit-diaphragm-associated proteins nephrin and podocin shown in Fig. 3, which may indicate that increased renal production of desminin and B7-1 could somehow be related to podocyte injury in diabetic *db/db* mice. Consistent with the results for nephrin and podocin, PAI-1R treatment also successfully reduced the elevated B7-1 protein expression in *db/db* mice by 58.3% ($P < 0.05$).

In agreement with protein levels, over 50% mRNA expression of nephrin was lost in diabetic *db/db* mice at week 22, compared with that of *db/m* control animals. The PAI-1R treatment rescued 71.5% of the reduction of nephrin mRNA levels in diabetic renal tissue (Fig. 5*A*).
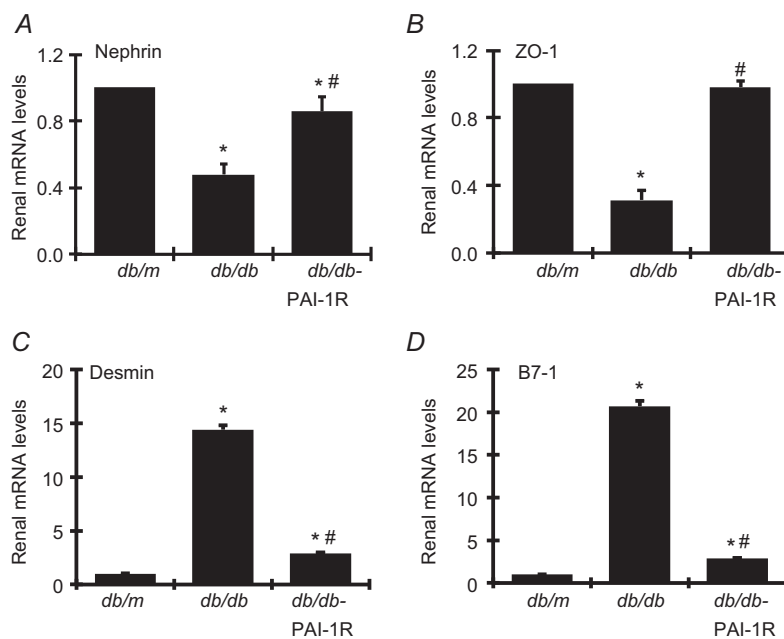

Figure 5. Effect of PAI-1R on expression of nephrin, zonula occludin-1 (ZO-1), desmin and B7-1 mRNAs in renal cortical tissues
Expression of mRNA in renal cortical tissues obtained from various mouse groups at 22 weeks was determined by real-time RT-PCR and corrected for *β*-actin for each sample. *A*, expression of nephrin mRNA. *B*, expression of ZO-1 mRNA. *C*, expression of desmin mRNA. *D*, expression of B7-1 mRNA. *$P < 0.05$ *versus db/m*. #$P < 0.05$ *versus db/db*.

In contrast, mRNA expression of desmin and B7-1 was markedly increased in diabetic renal tissue by 14- and 20-fold, respectively ($P < 0.05$), compared with that found in normal renal tissue ($P < 0.05$). The PAI-1R treatment reversed the increased mRNA expression of desmin and B7-1 (Fig. 5C and D), similar to the response of the corresponding proteins. We also measured mRNA expression of the tight junction protein ZO-1 in podocytes. Unsurprisingly, ZO-1 mRNA was 68.6% lower in diabetic renal tissues than in normal control tissues ($P < 0.05$). This reduction in ZO-1 expression was completely reversed by PAI-1R treatment ($P < 0.05$; Fig. 5B).

Collectively, these data suggest that PAI-1R treatment protects podocyte integrity in diabetic *db/db* mice by preserving the expression of slit-diaphram proteins and counter-regulating pathogenic molecules involved in podocyte injury.

Study 2: direct effect of PAI-1 on podocyte injury *in vitro*

Effect of PAI-1 on mRNA expression of nephrin, ZO-1, desmin and B7-1 by podocytes. It has been shown that the therapeutic efficacy of PAI-1R is largely due to its ability to reduce endogenous activity of PAI-1, by competion for binding to vitronectin at the site of injury but lack of protease inhibitory activity (Huang *et al.* 2006, 2009). In order to delineate the mechanism of action of PAI-1R further, we examined the direct effect of PAI-1 in a cultured podocyte model. Based on our previous studies on cultured mesangial cells (Huang *et al.* 2006), a recombinant active stable human PAI-1 at the concentration of 150 nM was chosen. As shown in Fig. 6, addition of PAI-1 to podocytes for up to 48 h caused a time-dependent inhibition of both nephrin and ZO-1 mRNA expression. The inhibition at 6 h was 13.4% for nephrin and 27.2% for ZO-1, and >80% ($P < 0.05$) at 48 h (Fig. 6A and B). Importantly, these changes seen in PAI-1-treated podocytes were similar to those observed in podocytes exposed to 2 ng ml^{-1} TGFβ1 for 24 h. The 24 h time point was thus used for further experiments as described below.

A dose-dependent decrease in mRNA levels of both nephrin and ZO-1 ($P < 0.05$; Fig. 7A and B) and a dose-dependent increase in mRNA levels of B7-1 ($P < 0.05$; Fig. 7D) in podocytes were seen after 24 h of treatment with PAI-1. While there was also an increase in mRNA levels of desmin with PAI-1 treatment, the dose dependence of this phenomenon was less apparent (Fig. 7C), In comparison, the addition of 2 ng ml^{-1} TGFβ1 to podocytes for 24 h resulted in dramatic decreases in both nephrin and ZO-1 mRNA expression and dramatic increases in both desmin and B7-1 mRNA expression ($P < 0.05$ *versus* normal control for each comparison). These data indicate that PAI-1 directly alters

gene expression of podocyte molecules in a manner similar to TGFβ1.

Effect of PAI-1 on protein production of nephrin, ZO-1 and B7-1 by podocytes. Consistent with the results on mRNA expression, 150 nM PAI-1 and 2 ng ml^{-1} TGFβ1 decreased the protein levels of nephrin and ZO-1 (by 39.4 and 45.4% for nephrin and 18.7 and 32.0% for ZO-1, respectively; all $P < 0.05$; Fig. 8). Meanwhile, both PAI-1 and TGFβ1 significantly increased B7-1 protein production by 230 and 246%, respectively. These results indicate that PAI-1, similar to TGFβ1, induces podocyte injury by altering the protein production of normal slit-diaphram molecules and B7-1.

Discussion

In the present experiments, we repeatedly observed that uninephrectomized, diabetic *db/db* mice developed overt nephropathy associated with progressive albuminuria and glomerular mesangial matrix expansion and increased renal production of fibronectin and collagen at 22 weeks of age. Treatment with human PAI-1R from week 20 to 22 had no effect on body weight, plasma glucose and HbA$_{1c}$ levels
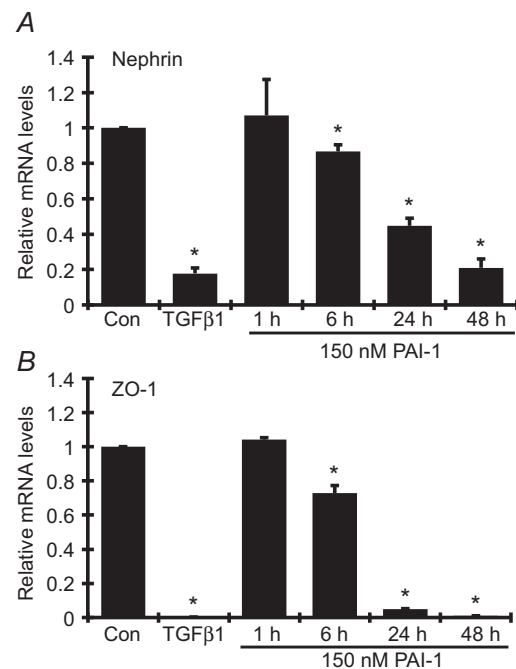

Figure 6. Time effects of wild-type PAI-1 on expression of nephrin mRNA (*A*) and ZO-1 mRNA (*B*) by mouse podocytes
Quiescent mouse podocytes were incubated in the absence (normal control; Con) or the presence of transforming growth factor-β1 (TGFβ1; 2 ng ml^{-1}) or wild-type PAI-1 (150 nmol) for the indicated durations, and real-time RT-PCR was performed. The mRNA expression in the normal controls was used as the reference. *$P < 0.05$ *versus* normal control.

but reduced albuminuria and markers of renal fibrosis seen in *db/db* mice. Although we have not dissected the plasmin-dependent and plasmin-independent actions of PAI-R in the *db/db* mouse model, we have previously shown that the effects of PAI-1R on pathological matrix accumulation in the model of anti-thy-1 nephritis were completely reversed by tranexamic acid treatment (Huang *et al.* 2003, 2006), indicating the plasmin dependence of these therapeutic actions. In contrast, tranexamic acid had no effect on other disease measures, including proteinuria, in an anti-thy-1 nephritis model with PAI-1R treatment (Huang *et al.* 2006). This latter observation suggests that PAI-1R has other actions in addition to enhancing plasmin generation.

Increasing evidence shows that the development of proteinuria is typically associated with podocyte foot-process effacement and/or slit-diaphragm disruption in proteinuric kidney diseases, including diabetic nephropathy (Fornoni, 2010). Consistent with this notion, untreated diabetic *db/db* mice in the present study developed significant segmental podocyte foot-process effacement associated with reduced renal production of nephrin, podocin and ZO-1 and increased renal

production of B7-1 and desmin. Importantly, these changes in podocyte morphology and protein molecules that are indicative of podocyte injury were all ameliorated by the treatment with PAI-1R. Nephrin and podocin are critical for maintaining the barrier function of the podocyte slit-diaphragm (Liu *et al.* 2003; Kawachi *et al.* 2006). Dysfunction or loss of nephrin and podocin may cause changes in podocyte shape, such as foot-process effacement, and attenuate the contractile function of podocytes, leading to detachment from the underlying GBM (Liu *et al.* 2003; Kawachi *et al.* 2006). The PAI-1R treatment may therefore reduce podocyte injury by preserving the normal structure of podocyte foot processes and slit-diaphragms.

B7-1 is a transmembrane protein normally expressed on the surface of B lymphocytes and antigen-presenting cells (Abbas & Sharpe, 1999; Chambers & Allison, 1999). It has been demonstrated to be synthesized *de novo* in podocytes and involved in the disruption of the slit-diaphragm protein complex and the induction of foot-process effacement in response to a variety of stress conditions (Reiser *et al.* 2004). Thus, B7-1 also acts as an autocrine factor to modulate podocyte integrity beyond its
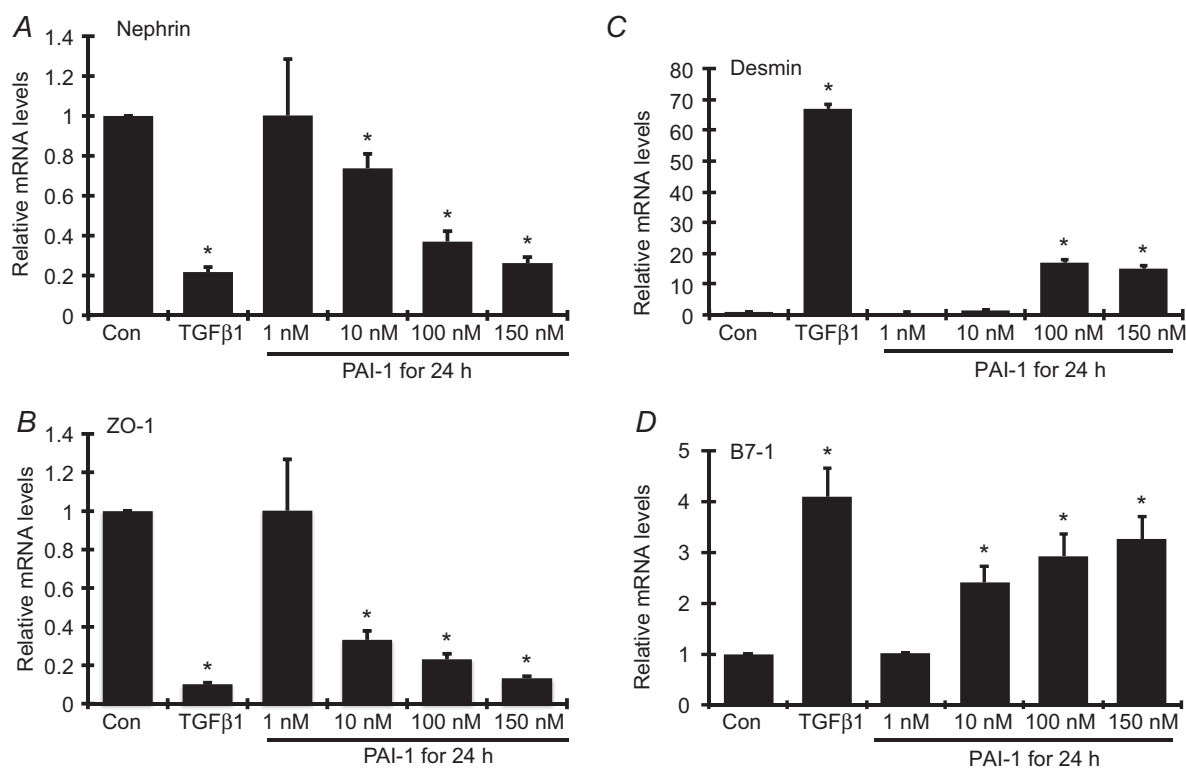

Figure 7. Dose effect of wild-type PAI-1 on mRNA expression of nephrin (*A*), ZO-1 (*B*), desmin (*C*) and B7-1 (*D*) by mouse podocytes
Quiescent mouse podocytes were incubated in the absence (normal control; Con) or the presence of various doses of wild-type PAI-1 or TGFβ1 (2 ng ml^{-1}) for 24 h, and real-time RT-PCR was performed. The mRNA expression in the normal controls was used as the reference. *$P < 0.05$ *versus* normal control.

role in immune cells. Indeed, it has been shown that the mRNA expression of B7-1 in urinary sediments correlates with the severity of proteinuria in patients with kidney diseases (Navarro-Muñoz *et al.* 2011). We observed that the mRNA expression and protein production of B7-1 were significantly increased in diabetes in the present study (Figs 4 and 5). However, PAI-1R treatment substantially reversed the diabetes-induced elevation of B7-1, which was accompanied by the recovery of podocyte morphology and slit-diaphragm protein production and a reduction in albuminuria.

In addition, podocyte epithelial-to-mesenchymal transition, characterized by the loss of epithelial markers, such as ZO-1, and gain of mesenchymal markers, such as desmin, has been involved in podocyte foot-process effacement (Li *et al.* 2008; Yamaguchi *et al.* 2009). In the present study, we also observed a reduction in ZO-1 and an increase in desmin in diabetic renal tissue in *db/db* mice at 22 weeks of age. Treatment with PAI-1R largely reversed these changes in the signalling molecules of podocyte epithelial-to-mesenchymal transition. Together, these results provide evidence that PAI-1R protects the podocyte from injury by counter-regulating the expression profile of slit-diaphragm proteins and pathogenic molecules in *db/db* mice. Consequently, podocyte protection may be quantified as a marked reduction in albuminuria in the *db/db* mice (Table 2).

In concordance with the protective effect of PAI-1R on podocyte injury is the notion that PAI-1 directly regulates podocyte protein expression resulting in cellular injury and proteinuria. Notably, *PAI-1* deletion prevented diabetic renal complications, including albuminuria, in both *db/db* diabetic mice and streptozotocin-induced diabetic mice (Nicholas *et al.* 2005; Collins *et al.* 2006; Lassila *et al.* 2007), while transgenic overexpression of *PAI-1* exaggerated proteinuria in a model of immune nephritis (Kitching *et al.* 2003), although podocyte structures were not examined in those animals. In a mouse model of angiotensin II-mediated hypertensive kidney disease (Knier *et al.* 2011), the knockout of *PAI-1* resulted in less glomerulosclerosis. Coincidentally, more intense nephrin immunostaining was found in the podocytes. Our present observation in cultured podocytes provides the first evidence that PAI-1 directly induces podocyte injury by altering the expression of nephrin and B7-1, similar to the actions of TGFβ1 (Figs 6–8), a well-known mediator of podocyte injury (Reiser *et al.* 2004). In addition, PAI-1 appeared to be sufficient to induce podocyte epithelial-to-mesenchymal transition in cell culture by changing the cellular expression of ZO-1 and desmin, similar to the effects of TGFβ1. Collectively, these observations suggest a new mechanism for the role of PAI-1 in the pathogenesis of kidney disease via induction of podocyte injury, in addition to the known injurious
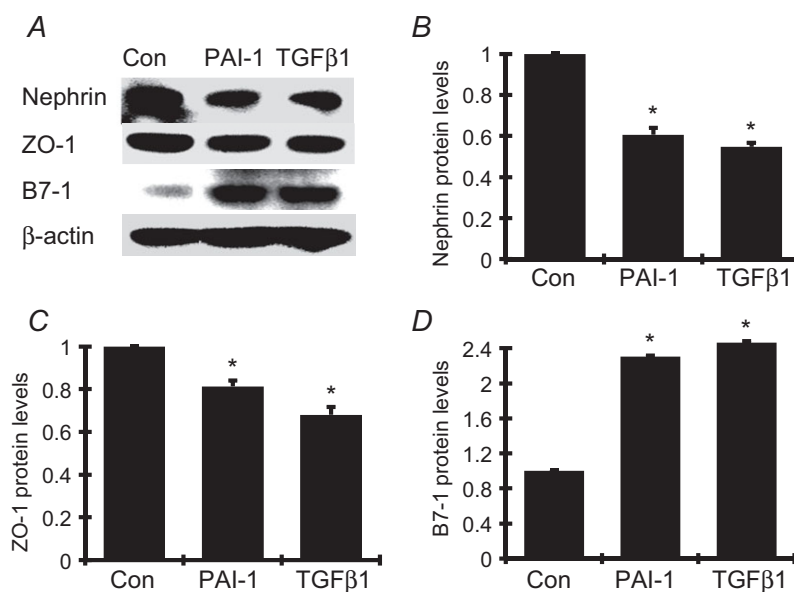

Figure 8. Effect of PAI-1 on protein production of nephrin, ZO-1 and B7-1 by mouse podocytes
Quiescent mouse podocytes were incubated in the absence (normal control; Con) or the presence of TGFβ1 (2 ng ml^{-1}) or PAI-1R (150 nmol) for 24 h, and Western blot analysis was performed in cell lysates. *A*, representative Western blots showing nephrin, ZO-1 and B7-1 protein expression; β-actin was included in the blot as a protein loading control. The graphs in *B–D* summarize the results of the respective band density measurements, normalized against the band intensity of β-actin. *$P < 0.05$ *versus* normal control.

effects of PAI-1 on the matrix degradation (Huang *et al.* 2006).

The signalling pathway by which PAI-1 directly modulates podocyte-associated molecules is unclear. A new signalling pathway in podoctyes that involves vitronectin, uPAR and $\alpha_v\beta_3$ integrin has recently been identified, which leads to podocyte foot-process effacement and urinary protein loss (Wei *et al.* 2008). These findings, together with the ability of PAI-1 to bind to vitronectin and complex with uPAR through PAI-1 complex formation with uPA, have led to the exciting possibility that PAI-1 induces podocyte injury through activation of this uPAR signalling pathway. This further suggests that the plasmin-independent benefit of PAI-1R may stem from its inability to interact stably with uPAR, and thus not activate this signalling pathway.

In conclusion, the present study provides evidence that, similar to TGFβ1, PAI-1 directly injures podocytes in diabetes. Reducing the increased PAI-1 activity by treatment with PAI-1R may, in fact, reduce podocyte injury, thereby reducing albuminuria. Therefore, PAI-1R may provide additional therapeutic effect in slowing the progression of diabetic nephropathy by maintaining podocyte integrity.

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Additional Information

Competing interests

None declared.

Author contributions

and drafted the article. Daniel A. Lawrence produced the recombinant human PAI-1R and active stable PAI-1 and edited the article. Alfred K. Cheung edited the paper. Yufeng Huang designed and supervised the experiments, performed the uninephrectomy in mice, interpreted the data and revised the article critically for important intellectual content. All authors approved the final version of the manuscript for publication.