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# Plasminogen activator inhibitor-1 deficiency retards diabetic nephropathy

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**Plasminogen activator inhibitor-1 deficiency retards diabetic nephropathy.**

*Background.* Plasminogen activator inhibitor-1 (PAI-1) is increased in kidneys of humans and animals with diabetic nephropathy and is associated with extracellular matrix (ECM) accumulation. PAI-1 may promote ECM buildup by preventing plasmin and matrix metalloproteinase (MMP) activation. However, the importance and mechanism of PAI-1 action in the pathogenesis of diabetic nephropathy is unknown.

*Methods.* We investigated the effect of streptozotocin (STZ)-induced diabetes in wild-type (PAI-$1^{+/+}$) mice and mice null for PAI-1 (PAI-$1^{-/-}$). After 1 month of diabetes, animals were placed in metabolic cages for 24-hour urine collection. Total RNA was isolated from kidney cortex for reverse transcription-polymerase chain reaction (RT-PCR) and Northern blot analysis, and Western blots were quantitated from cortical protein. Primary mesangial cells were grown from Sprague-Dawley rats and used in signal transduction studies.

*Results.* Urinary albumin excretion (UAE) in diabetic PAI-$1^{+/+}$ mice increased >threefold, but remained unchanged in PAI-$1^{-/-}$ mice. Transforming growth factor-β (TGF-β) and fibronectin message and protein levels were lower in diabetic PAI-$1^{-/-}$ vs. PAI-$1^{+/+}$ mice, suggesting that PAI-1 deficiency impaired TGF-β expression despite diabetes. Indeed, recombinant PAI-1 directly stimulated TGF-β message and protein via mitogen-activated protein kinase (MAPK) signal transduction in cultured mesangial cells. Urokinase plasminogen activator (uPA) inhibited this PAI-1 action in a dose-dependent manner. The inhibitory effect of antibody to uPA receptor (uPAR) on PAI-1–induced TGF-β function suggested that uPAR mediated the cellular effect of PAI-1.

*Conclusion.* PAI-1 can regulate TGF-β expression by binding to uPAR and activating the extracellular-regulated signal kinase (ERK)/MAPK pathway. Therefore, PAI-1 contributes to diabetic nephropathy by regulating TGF-β and renal ECM production and may be a therapeutic target in diabetic nephropathy.

**Key words:** transforming growth factor-β, MAPK, albuminuria, fibronectin, knockout, urokinase receptor.

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Plasminogen activator inhibitor-1 (PAI-1) prevents conversion of tissue plasminogen activator and urokinase plasminogen activator (uPA) to plasminogen [1]. Plasmin, the active form of plasminogen, is a broad-spectrum protease that degrades fibrin clots and extracellular matrix (ECM) proteins. As a result, PAI-1 is an important regulator of both fibrinolysis and tissue remodeling. PAI-1 levels are normally tightly regulated to maintain homeostasis of both processes. A deficiency in PAI-1 results in hemorrhage [2–4], while overexpression is prothrombotic contributing to coronary events and deep venous thrombosis [1]. PAI-1 is thought to promote tissue fibrosis by inhibiting plasmin and metalloproteinase ECM degradation [5]. It has been implicated in experimental glomerulonephritis [6], chronic renal transplant rejection [7], and pulmonary fibrosis [8]. Transgenic mice overexpressing PAI-1 develop significantly greater pulmonary fibrosis when administered bleomycin, while similarly treated PAI-1–deficient mice have substantially less fibrosis compared to wild-type mice [9]. PAI-1 deficiency also protects against renal interstitial fibrosis induced by unilateral ureteral obstruction (UUO) [10]. More recently, a mutant human PAI-1 that could bind to vitronectin but not inhibit plasminogen activator–decreased ECM accumulation in experimental glomerulonephritis [11].

In diabetic nephropathy, accumulation of ECM proteins in the mesangium leads to glomerulosclerosis, the hallmark of diabetic nephropathy [12]. In normal human kidney, PAI-1 levels are undetectable, but in diabetes, PAI-1 expression is up-regulated in renal glomeruli and arteries [13]. The increased glomerular PAI-1 accompanies microangiopathic injury and accumulation of ECM in patients with diabetes and may

indicate a worse prognosis [14]. The increase in tissue PAI-1 likely occurs in response to factors inherent to the diabetic environment such as increased transforming growth factor-β (TGF-β), hyperglycemia, very low-density lipoprotein (VLDL) triglycerides, and angiotensin II (Ang II), all of which activate the PAI-1 promoter [15].

To more precisely determine the role of PAI-1 in diabetic nephropathy, we induced diabetes in genetically deficient PAI-1 (PAI-$1^{-/-}$) mice with streptozotocin (STZ) (Sigma Chemical Co., St. Louis, MO, USA). Lack of PAI-1 prevented albuminuria and accumulation of fibronectin mRNA and protein, a major ECM component. However, renal matrix metalloproteinase (MMP)-9 and MMP-2 activities were not increased in diabetic PAI-$1^{-/-}$ mice. On the other hand, both TGF-β mRNA and protein levels were significantly reduced in diabetic PAI-$1^{-/-}$ mice compared to diabetic wild-type (PAI-$1^{+/+}$) mice. Consistent with this observation, we found that PAI-1 directly regulates TGF-β expression in mesangial cells by binding to uPA receptor (uPAR) and activating the extracellular-regulated signal kinase (ERK)/mitogen-activated protein kinase (MAPK) pathway. Thus, the lack of PAI-1 likely is associated with a decrease in ECM production contributing to decreased ECM accumulation in diabetes. Targeting PAI-1 may be a useful therapeutic strategy to prevent glomerular fibrosis and proteinuria in diabetic nephropathy.

## METHODS

### Mouse breeding and measurements

PAI-1–deficient (PAI-$1^{-/-}$) mice on a C57B/L6 background (breeding pairs) and wild-type C57B/L6 (PAI-$1^{+/+}$) mice (Jackson Laboratory, Bar Harbor, ME, USA) were housed in accordance with the NIH Guide for the Care and Use of Laboratory Animals and placed on a protocol approved by the Animal Research Committee of the University of California, Los Angeles. Mice were ad libitum in isolation-topped cages under a 12-hour light-dark regime. Ten-week-old mice were divided into nondiabetic PAI-$1^{+/+}$ ($N = 14$), diabetic PAI-$1^{+/+}$ ($N = 15$), nondiabetic PAI-$1^{-/-}$ ($N = 16$), and diabetic PAI-$1^{-/-}$ ($N = 13$) mice. Diabetes was induced with a single intraperitoneal injection of STZ (Sigma Chemical Co.), 120 to 180 mg/kg body weight to achieve plasma glucose ≥300 mg/dL after 5 to 7 days. Plasma glucose, from retro-orbital blood, was determined by glucose oxidase reaction (Beckman Glucose Analyzer 2; Beckman Instruments, Fullerton, CA, USA). Weekly blood pressures were obtained from trained, prewarmed mice by computerized, noninvasive tail-cuff system (BP 2000 Blood Pressure Analysis System) (Visitech Systems, Apex, NC, USA) [16].

Animals were placed in metabolic cages for 24-hour urine collection. Albumin concentrations were measured using the Albuwell M Assay Kit (Exocel, Philadelphia, PA, USA). Albumin standards and samples were placed in 96-well plates, and washed with rabbit antimurine albumin antibody and antirabbit horseradish peroxidase (HRP) conjugate for color detection at 450 nm absorbance used to calculate albumin concentration.

### Quantitative reverse transcription (RT) real-time polymerase chain reaction (PCR)

Total RNA (45 μg) isolated from mouse kidney cortex or primary mesangial cells by Trizol method (Invitrogen Life Technologies, Carlsbad, CA, USA) was DNase-treated and (200 ng) reverse transcribed using Applied Biosystems Reverse Transcription Reagents (Applied Biosystems, Foster City, CA, USA) with a parallel negative control. Triplicate PCR amplifications of cDNA (5 ng) were performed with the following primers (200 nmol/L) and probes (100 nmol/L) (Perkin-Elmer/Applied Biosystems PE/ABD Primer Express Software): PAI-1 forward primer 5′-TGC ATC GCC TGC CAT TG-3′; PAI-1 reverse primer 5′-GGA CCT TGA GAT AGG ACA GTG CTT-3′; PAI-1 probe 5′-6FAM TGG AGG GTG CCA TGG GCC A TAMRA-3′; fibronectin forward primer 5′-TGT AGG AGA ACA GTG GCA GAA AGA-3′; fibronectin reverse primer 5′-CCG CTG GCC TCC GAA-3′; fibronectin probe 5′-6FAM TCG GAG CCA TTT GTT CCT GCA CGT TAMRA-3′; TGF-β forward primer 5′-CCA TCC ATG ACA TGA ACC GA-3′; TGF-β reverse primer 5′-CAG GTG TTG AGC CCT TTC CA-3′; and TGF-β probe 5′-/56-FAM/CCA-TCC-TTT-CCC-ATA-TCC-TGT-CC/36-TAMTPH/−3′. PCR amplification [including glyceraldehyde-3-phosphate dehydrogenase (GAPDH)] was performed (Perkin-Elmer/Applied Biosystems 7700 Sequence Detector) with negative RT controls to detect contaminating genomic DNA. The data were analyzed using Perkin-Elmer Applied Biosystems Sequence Detection System software.

### Northern blot analysis

Northern blot analysis was performed on total RNA (20 μg) from mouse kidney cortex which was carefully dissected from the renal capsule and medulla [17]. RNA separated by electrophoresis was transferred to nylon membranes (Hybond N+) (Amersham Biosciences, Buckinghamshire, UK) which were probed with $^{32}$P-labeled TGF-β or fibronectin cDNA (generously provided by Matthew Breyer, Vanderbilt University). Signals were detected by autoradiography (Amersham Biosciences) scanned and quantitated using NIH Image 1.60 and Image J version 1.28U scan software.