Loss of TIMP3 selectively exacerbates diabetic nephropathy

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Loss of TIMP3 selectively exacerbates diabetic nephropathy. Am J Physiol Renal Physiol 303: F1341–F1352, 2012. First published August 15, 2012; doi:10.1152/ajprenal.00349.2012.—Diabetic nephropathy is the most common cause of end-stage renal disease. Polymorphism in the tissue inhibitor of metalloproteinase-3 (TIMP3) gene, and the ECM-bound inhibitor of matrix metalloproteinases (MMPs), has been linked to diabetic nephropathy in humans. To elucidate the mechanism, we generated double mutant mice in which the TIMP3 gene was deleted in the genetic diabetic Akita mouse background. The aggravation of diabetic injury occurred in the absence of worsening of hypertension or hyperglycemia. In fact, myocardial TIMP3 levels were not affected in Akita hearts, and cardiac diastolic and systolic function remained unchanged in the double mutant mice. However, TIMP3 levels increased in Akita kidneys and deletion of TIMP3 exacerbated the diabetic renal injury in the Akita mouse, characterized by increased albuminuria, mesangial matrix expansion, and kidney hypertrophy. The progression of diabetic renal injury was accompanied by the upregulation of fibrotic and inflammatory markers, increased production of reactive oxygen species and NADPH oxidase activity, and elevated activity of TNF-α-converting enzyme (TACE) in the TIMP3+/–/Akita kidneys. Moreover, while the elevated phospho-Akt (S473 and T308) and phospho-ERK1/2 in the Akita mice was not detected in the TIMP3+/–/Akita kidneys, PKCβ1 (but not PKCα) was markedly elevated in the double mutant kidneys. Our data provide definitive evidence for a critical and selective role of TIMP3 in diabetic renal injury consistent with gene expression findings from human diabetic kidneys.

diabetes; Akita mouse model; renal injury; human diabetic nephropathy

DIABETIC NEPHROPATHY AND CARDIOMYOPATHY are serious long-term complications of diabetes, accounting for ~40% of new cases of end-stage renal disease (ESRD) in the United States (10, 16, 31). Polymorphism in tissue inhibitor of metalloproteinase-3 (TIMP3) is associated with type I diabetic nephropathy (13), and TIMP3 is differentially expressed in human diabetic glomeruli (51). TIMP3 is one of the four identified endogenous inhibitors of matrix metalloproteinases (MMPs). The dynamic physiological equilibrium between TIMPs and MMPs determines the extracellular matrix (ECM) integrity and the tissue microenvironment. TIMP3 is ECM bound and the most highly expressed TIMP in the kidney (26), and it is also known to inhibit the ADAM (a disintegrin and metalloproteinate domain) and ADAM-TS (a disintegrin and metalloproteinate with thrombospondin motif, also known as aggrecanase) families, which are proteases controlling the bioactivity of many growth factors and cytokines (1, 24). Loss of TIMP3 in mice enhances susceptibility to cardiomyopathy (27) and interstitial nephritis and fibrosis (26). Given the critical role of inflammation and MMP activation in diabetic nephropathy and renal injury (7, 37, 49), we hypothesize that loss of TIMP3 will exacerbate diabetic nephropathy and cardiomyopathy.

The Akita mouse (Ins2WT/C46EY) develops spontaneous hyperglycemia and reduced β-cell mass as a result of a mutation of the insulin 2 gene (Ins2; Cys96Tyr) that disrupts a disulfide bond between A and B chains of the insulin molecule (21, 28, 52). The Akita model is a well-established type I diabetic mouse model and the most useful platform by which to study diabetic nephropathy due to less variability in renal structural responses (18, 19, 39, 50).

We previously reported that Akita mice develop cardiac diastolic dysfunction (4). In the present study, we cross-bred TIMP3 knockout (KO) and diabetic Akita mice and generated the TIMP3-deficient Akita mouse model to examine the role of TIMP3 in the progression of diabetic nephropathy. Our data revealed that lack of TIMP3 exacerbates diabetic nephropathy without altering diabetic cardiomyopathy.

MATERIALS AND METHODS

Experimental animals and protocol. Wild-type (Ins2WT/WT) C57BL/6d and diabetic heterozygous Akita (Ins2WT/C46EY) male mice were purchased from The Jackson Laboratory and bred at the University of Alberta Health Sciences Laboratory Animal Services facility. TIMP3-deficient (TIMP3–/–/Ins2WT/WT) mice were generated on a C57BL/6d background as described (22, 26). TIMP3 deficiency in diabetic heterozygous Akita mice (TIMP3–/–/Ins2WT/C46EY) was generated by breeding TIMP3-deficient (TIMP3–/–) females with Akita (Ins2WT/C46EY) male mice. Only male Akita (Ins2WT/C46EY), Akita/TIMP3–/– (TIMP3–/–/Ins2WT/C46EY), wild-type (TIMP3+/+/Ins2WT/WT), and TIMP3–/– (TIMP3–/–/Ins2WT/WT) mice at 3 and 6 mo of age were used in all experiments. Throughout the period of study, animals were provided with free access to water and standard rodent chow (Harlan Teklad, Madison, WI). All experiments were conducted in accordance with the guidelines of the University of Alberta Animal Care Committee and the Canadian Council of Animal Care.

Blood glucose and tail-cuff blood pressure measurements. Fasting (8 h) blood glucose levels were measured every month starting from 4 wk until 24 wk of age between 8 and 9 a.m. using an Ascensia Contour glucometer (Bayer). Akita/TIMP3–/– (TIMP3–/–/Ins2WT/C46EY) showed a stable and sustained hyperglycemia from an early age and comparable to age-matched Akita (Ins2WT/C46EY) mice. Tail-cuff systolic blood pressure (TC-SBP) at 3 and 6 mo of age was measured in conscious mice using the IITC Life Science Blood Pressure System (Woodland Hills, CA), and the recordings were analyzed using IITC software as previously described (53).

Echocardiography and tissue Doppler imaging. Transthoracic echocardiography was performed and analyzed as described previ-
ously (4) using the Vevo 770 high-resolution imaging system with a 30-MHz transducer (RMV-707B; VisualSonics, Toronto, ON) to assess systolic and diastolic function. For diastolic function assessment, we employed the E′/A′ and E/E′ ratios, where the early wave (E-wave) due to ventricular relaxation and the late filling wave (A-wave) due to atrial contraction were acquired using transmitted flow Doppler imaging, and the early diastolic (E′) and late diastolic (A′) peak annular velocities (4, 44) were measured using Tissue Doppler Imaging (TDI) of the mitral valve annulus.

Western blot analysis and gelatin zymography. Total protein was extracted from flash-frozen heart or kidney tissues, cleansed in PBS, homogenized using an electric homogenizer (Omni TH_2, Kennesaw, GA) in EDTA-free RIPA buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% NP-40, 0.1% SDS with protease inhibitor, Calbiochem, EMD Chemicals, Gibbstown, NJ), and phosphatase inhibitor cocktails (Sigma-Aldrich Canada, Oakville, ON), and was quantified using a Bio-Rad DC protein assay. Western blot analysis was performed as previously described (26). Briefly, SDS-PAGE was performed using a rabbit polyclonal antibody raised against amino acids 46–100 mapping within an integral region of TIMP3 (1:500 dilution, sc-30075, Santa Cruz Biotechnology, Santa Cruz, CA). Phosphorylated and total protein levels of ERK1/2 and Akt/PKB (New England Biolabs, Santa Cruz, CA) were detected using 10% SDS-PAGE gels as previously described (39). α-Actin was used as the endogenous control in kidney samples. Gelatin zymography was performed as before (26).

TNF-α-converting enzyme activity assay. TNF-α-converting enzyme (TACE) activity was assessed using an in vitro fluorogenic assay as described (42). A total of 5 μg of protein extracts from the cortex was incubated with 10 μM of fluorogenic substrate for TACE [7-methoxycoumarin-PLAQAV-(2,4-dinitrophenyl)-RSSSR-NH2, R&D Systems] at room temperature. The fluorescence emitted from the cleavage products of the substrate was quantified by spectrofluorometry using an excitation wavelength of 320 nm and emission wavelength of 405 nm. TACE-specific activity is expressed as RFU per minute.

mRNA expression analysis by real-time TaqMan PCR. Flash-frozen whole kidney samples from mice were used to dissect cortex in an RNA-stabilizing solution (RNaider, Ambion, Austin, TX) followed by extraction of total RNA using the TRIzol reagent (Invitrogen Canada, Burlington, ON). A total of 1 μg of total RNA was used to synthesize cDNA by performing RT, and RNA expression levels of the genes of interest were analyzed by TaqMan real-time PCR with a sequence detection system (ABI 7900 Sequence Detection System) using primers and probes as previously described (26, 53). Premixed primer/probe mixes were purchased from ABI for WT1 (Mm0128147_m1), NOX2 (Mm01287743_m1), p47phox (Mm00447920_g1), connective tissue growth factor (CTGF; Mm01192933_g1), transforming growth factor (TGF)-β (Mm00177819_m1), and nephrin (Mm00497828_m1). All samples were run in triplicate, and 18S rRNA was used as an internal control as previously described (4, 26).

Glomerular mesangial matrix score and histology. Mice were euthanized at 3 or 6 mo of age, kidneys were excised, weighed, and fixed in 10% neutral-buffered formalin (Sigma-Aldrich) for 24 h followed by 90% ethanol. Formalin-fixed, paraffin-embedded kidney tissue was used for assessment of mesangial sclerosis by light microscopy. All glomerular profiles (~90) in a single 3-μm-thick periodic acid-Schiff (PAS)-stained section from each animal were scored for mesangial matrix (MM) increase. Each glomerulus was scored as 0 (normal), 1 (mild MM increase, ~2 times the width of a mesangial cell nucleus), 2 (moderate MM increase, 3–4 times the width of a mesangial cell nucleus), or 3 (severe MM increase, >4 times the width of a mesangial cell nucleus). The glomerular MM score for each animal was expressed as percent MM increase [(total score/total no of glomeruli) × 100]. Picrosirius red and Gomori trichrome staining was performed as previously described (4, 26).

Urine albumin analysis. Each mouse was housed in individual metabolic cages (Nalgene, model 650–0311; Nalge Nunc International, Rochester, NY) with freely accessible water and rodent mash. The 24-h urine was collected, centrifuged at 8,000 g for 5 min (to remove debris), and flash-frozen for further analysis. An indirect competitive ELISA kit (Albuwell M; Exocell, Philadelphia, PA) was used according to the manufacturer’s instruction to measure urinary albumin concentration.

NADPH oxidase activity, dihydroethidium fluorescence, and nitrotyrosine staining. NADPH oxidase activity in kidney cortex (homogenized in PBS containing a phosphatase inhibitor cocktail from Roche Applied Sciences) of mice was quantified using a lucigenin-enhanced chemiluminescence assay using a single-tube luminometer (Berthold FB12, Berthold Technologies) with NADPH (1 mM) and lucigenin (50 μM) at 37°C as previously described (39). The specific peptide inhibitor of NOX2, gp91phox ds tat (50 μM), was used to confirm superoxide generation from NADPH oxidase (5, 40). Dihydroethidium (DHE), an oxidative fluorescent dye, was used to measure superoxide levels in kidney samples as previously described (39). Briefly, optimal cutting temperature (OCT)-embedded frozen kidney tissues were cut into 15-μm-thick sections, washed with Hank’s balanced salt solution (HBSS), and then incubated with DHE (20 μM DHE in HBSS; Sigma-Aldrich) at 37°C for 30 min in the dark. Fluorescence images were subsequently taken with an Olympus IX81 fluorescence microscope (10). Nitrotyrosine immunofluorescence staining was performed on OCT-embedded kidney cryosections (5 μm) using mouse anti-nitrotyrosine primary antibody (Santa Cruz Biotechnology) and Alexa Fluor 594-conjugated goat anti-mouse secondary antibody (Invitrogen) as previously described (40). Quantitative measurements of DHE and nitrotyrosine fluorescence intensity were carried out using Metamorph Basic (version 7.7.0.0; Molecular Devices, Sunnyvale, CA).

WT-1 staining and podocyte counting. Wilms tumor protein (WT-1) is a podocyte marker (43). WT-1 staining was performed on deparaffinized mouse kidney slides that were blocked with serum for 20 min at room temperature. Slides were then incubated with WT-1 antibody (1:200 in 1% BSA, Santa Cruz Biotechnology) for 2 h at room temperature. After 3 times washing in PBS 10 min each, slides were incubated with rabbit IgG ( Vectastain ABC Kit, Vector Laboratories, Burlington, ON) for 1 h. Slides were washed in PBS three times, 10 min each, and then quenched with 1% H2O2 in methanol for 45 min. Slides were incubated with ABC Reagent for 1 h, then stained with DAB substrate for 1 min. Dehydrated slides were then mounted with permount. All slides were scanned digitally by the Advanced Optical Microscopy Facility (Princess Margaret Hospital, Toronto, ON), and Aperio ImageScope software (Aperio Technologies, Vista, CA) were used to count WT-1-positive cells.

Statistical analysis. Data were analyzed using either an unpaired Student’s t-test (Fig. 1) or a two-way ANOVA followed by Bonferroni post hoc testing (Fig. 2; see also Figs. 3–6) using SPSS Statistics 19 software.

RESULTS

TIMP3 is upregulated in Akita kidneys but not in the heart. TIMP3 is a highly expressed TIMP in the kidneys and the heart, and TIMP3 deficiency has been linked to both cardiomyopathy (27) and kidney disease (26). We assessed the expression of TIMP3 by Western blot analysis in hearts and kidneys of 3- and 6-mo-old Akita mice. While TIMP3 levels remained unaltered in Akita hearts (Fig. 1A), kidney levels of TIMP3 were significantly increased in the Akita model at 3 and 6 mo of age (Fig. 1B). These results show that in the diabetic Akita model, TIMP3 is selectively upregulated in the kidneys.

Loss of TIMP3 does not worsen heart function in Akita mice. TIMP3 is also a critical determinant of cardiac function (27, 36) and as such we used echocardiography to assess heart function in our murine models. Our results confirmed diastolic dysfunction with preserved systolic function in Akita mice which was not
altered by the loss of TIMP3 in 6-mo-old mice (Fig. 2). While M-mode images showed preserved systolic function (Fig. 2A), a transmitral flow profile showed prolongation of isovolumetric relaxation time (IVRT) with increased deceleration time (Fig. 2B). Tissue Doppler imaging revealed a reduction in E′ and an increase in A′ velocities (Fig. 2C). Quantitative assessment of heart function confirmed no change in ejection fraction and fractional shortening with equivalent reduction in the E′/A′ and E/E′ ratios in Akita and Akita/TIMP3 double mutant mice (Table 1).

**TIMP3 deficiency exacerbates diabetic renal injury.** Gross morphological assessment of the kidneys revealed increased renal size in Akita mice as previously reported (18, 21), while the absence of TIMP3 in the Akita mice resulted in significantly greater enlargement of the kidneys (Fig. 3, A and B). As diabetic injury is characterized by mesangial expansion and increased microalbuminuria, we assessed these parameters in our murine models. The mesangial matrix in the Akita/TIMP3 double mutant mice as shown using PAS staining and mesangial matrix score was significantly greater than in the Akita mice (Fig. 3C). Consistent with the expansion of the mesangium, urinary albumin excretion showed a twofold increase in the Akita/TIMP3−/− mice compared with the increase in the Akita mice (Fig. 3D), which occurred in the absence of differential effects on systolic blood pressure (Fig. 3E) and hyperglycemia (Fig. 3F). Loss of glomerular podocytes has been linked to worsening diabetic nephropathy (43, 45). However, the mRNA (Fig. 4A) and protein levels (Fig. 4B, i and ii) of nephrin, a marker of podocytes, and the average number of podocytes positively labeled with WT-1 protein or WT-1 mRNA levels (Fig. 4C) were not different among the various experimental groups.

**Lack of TIMP3 activates the PKC pathway and increases inflammation in Akita kidneys.** Given the exacerbation of diabetic kidney injury by the loss of TIMP3, we next examined alterations in signaling pathways such as Akt, ERK1/2, and PKC in the kidneys. The phosphorylation of Akt on both serine-473 and threonine-308 were activated in Akita kidneys but not in the
Akita/TIMP3 double mutants (Fig. 5, A and B) with a similar trend seen for the ERK1/2 pathway (Fig. 5C). Activation of the PKC system plays a key pathophysiological role in diabetic nephropathy (29, 38). While the protein expression of PKCα was unaltered (Fig. 5D), PKCβ1 levels were increased about twofold in Akita/TIMP3−/− kidneys (Fig. 5E). This indicates that loss of TIMP3 compromises the activation of important renoprotective signaling pathways while increasing PKCβ1 levels in diabetic kidneys. TIMP3 has also been shown to be involved in control of the tissue microenvironment by regulating tissue fibrosis and inflammation (25, 26). Deletion of TIMP3 in Akita kidneys was associated with increased mRNA expression of various profibrotic markers such as pro-collagen type I-α1, CTGF, and TGF-β (Fig. 6, A–C) while picrosirius red (Fig. 6D) and Masson trichrome staining (Fig. 6E) confirmed increased tubulointerstitial fibrosis. Expression analysis of inflammatory markers showed increased chemokine monocyte chemotactic protein-1 (MCP-1) levels while IL-1β and TNF remained unchanged (Fig. 6, F–H) in Akita/TIMP3−/− kidneys.

**Increased reactive oxygen species generation in Akita/TIMP3−/− kidneys without a differential effect on MMP2 activation.** Activation of the NADPH oxidase and generation of reactive oxygen species

Table 1. Echocardiographic assessment of diastolic and systolic function in WT, Akita, TIMP3KO, and Akita/TIMP3KO mice at 6 mo of age

<table>
<thead>
<tr>
<th></th>
<th>WT (n = 10)</th>
<th>Akita (n = 12)</th>
<th>TIMP3KO (n = 10)</th>
<th>Akita/TIMP3KO (n = 12)</th>
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</thead>
<tbody>
<tr>
<td>HR, beats/min</td>
<td>489 ± 16</td>
<td>474 ± 13</td>
<td>480 ± 20</td>
<td>470 ± 20</td>
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<tr>
<td>E-wave, mm/s</td>
<td>685 ± 24.8</td>
<td>702 ± 31.6</td>
<td>690 ± 22.5</td>
<td>735 ± 19.9</td>
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<tr>
<td>A-wave, mm/s</td>
<td>445 ± 47.3</td>
<td>441 ± 40</td>
<td>492 ± 37.6</td>
<td>494.73 ± 14.25</td>
</tr>
<tr>
<td>E'/A' ratio</td>
<td>1.54 ± 0.1</td>
<td>1.59 ± 0.09</td>
<td>1.41 ± 0.06</td>
<td>1.48 ± 0.08</td>
</tr>
<tr>
<td>IVRT ms</td>
<td>15.8 ± 0.66</td>
<td>18.7 ± 0.94*</td>
<td>14.5 ± 0.75</td>
<td>15.4 ± 0.82</td>
</tr>
<tr>
<td>E', mm/s</td>
<td>26.3 ± 1.7</td>
<td>21.8 ± 1.86</td>
<td>32.44 ± 1.6</td>
<td>24.4 ± 2.1*</td>
</tr>
<tr>
<td>E/E' ratio</td>
<td>27.9 ± 1.8</td>
<td>33.1 ± 1.7*</td>
<td>24.54 ± 1.07</td>
<td>30.1 ± 1.7*</td>
</tr>
<tr>
<td>A'</td>
<td>24.4 ± 1.4</td>
<td>29.2 ± 1.5*</td>
<td>26.8 ± 1.3</td>
<td>29.07 ± 2.52</td>
</tr>
<tr>
<td>E'/A'</td>
<td>1.14 ± 0.06</td>
<td>0.74 ± 0.07*</td>
<td>1.23 ± 0.07</td>
<td>0.84 ± 0.08*</td>
</tr>
<tr>
<td>LVESD, mm</td>
<td>3.79 ± 0.05</td>
<td>3.71 ± 0.04</td>
<td>3.53 ± 0.08</td>
<td>3.67 ± 0.08</td>
</tr>
<tr>
<td>LVESD, mm</td>
<td>2.59 ± 0.06</td>
<td>2.52 ± 0.05</td>
<td>2.27 ± 0.12</td>
<td>2.43 ± 0.11</td>
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<tr>
<td>LVFS, %</td>
<td>31.6 ± 2.1</td>
<td>32.1 ± 1.9</td>
<td>36.02 ± 2.17</td>
<td>34.09 ± 1.83</td>
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<td>LVEF, %</td>
<td>62.7 ± 2.4</td>
<td>60.4 ± 2</td>
<td>62.2 ± 2.13</td>
<td>60.21 ± 1.01</td>
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<td>VCFe, circ/s</td>
<td>6.3 ± 0.50</td>
<td>6.25 ± 0.38</td>
<td>7.00 ± 0.60</td>
<td>6.40 ± 0.30</td>
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<tr>
<td>LVPWT, mm</td>
<td>0.69 ± 0.03</td>
<td>0.71 ± 0.02</td>
<td>0.72 ± 0.01</td>
<td>0.7 ± 0.03</td>
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</tbody>
</table>

Values are means ± SE; n = sample size. WT, wild-type; TIMP3, tissue inhibitor of metalloproteinase-3; KO, knockout; HR, heart rate; E-wave, peak early transmitral inflow mitral E velocity; A-wave, transmitral inflow velocity due to atrial contraction; IVRT, isovolumetric relaxation time; DT, deceleration time; EWDR, E-wave deceleration rate (E-wave/DT); E', early diastolic tissue Doppler velocity; LVESD, left ventricular end-diastolic diameter; LVESD, left ventricular end-systolic diameter; LVFS, LV fractional shortening; LVEF, LV ejection fraction; VCFe, velocity of circumferential shortening corrected for heart rate; LVPWT, left ventricular posterior wall thickness. *P < 0.05 compared with WT and TIMP3KO groups.
(ROS) play a key role in diabetic nephropathy (23, 39, 47). Staining for DHE (Fig. 7A) and nitrotyrosine (Fig. 7B), and the corresponding quantifications (Fig. 7, C and D), confirmed higher levels of superoxide and nitrotyrosine in diabetic Akita kidneys which was increased further in the Akita/TIMP3 double mutant kidneys, indicating that loss of TIMP3 amplified ROS generation in the diabetic kidneys. The renal cortical NADPH activity based on the lucigenin chemiluminescence assay was significantly increased in Akita compared with wild-type kidneys in association with increased mRNA expression of the NADPH oxidase subunit.

Fig. 3. Loss of TIMP3 exacerbates diabetic renal injury in 6-mo-old Akita mice. Representative images are shown of whole kidneys (A) and averaged kidney weight-to-tibial length (TL) ratio (B) of the various genotypes showing greater enlargement of the kidneys in Akita/TIMP3−/− mice. Also shown are representative periodic acid-Schiff staining (C), glomerular mesangial matrix score (D), and urinary albumin excretion (E) showing exacerbation of glomerular mesangial injury and worsening of albuminuria in the absence of a differential effect on systolic blood pressure (F) and fasting blood glucose (G) in the Akita/TIMP3−/− mice. Values are means ± SE; n = 10. *P < 0.05 compared with WT or TIMP3−/−. #P < 0.05 compared with all other groups.
The NADPH oxidase activity was elevated further in the Akita/TIMP3−/− kidneys, likely driven by the greater elevation in p47phox mRNA levels and increased NOX2 (gp91phox) levels (Fig. 7, E–G). The specific peptide inhibitor of NOX2 gp91phox ds tat (50 μM) and its inactive scrambled form (scr) were used to confirm superoxide generation from NADPH oxidase (6) (Fig. 7E).

Since TIMP3 is the only identified physiological inhibitor of ADAM-17 or TACE (1), a key regulator of MMP2 activation (12) and MMP9 (26, 35), we next investigated whether loss of TIMP3 impacted activity of TACE, MMP2, and MMP9, the key proteases in kidney tissue remodeling in the background of type I diabetes. While mRNA expression of TACE was not different among groups (Fig. 8A), its activity was significantly elevated with TIMP3 deficiency. The increase in TACE activity in the Akita group did not reach statistical significance, whereas when combined with TIMP3 deficiency, it was significantly elevated compared with all other groups (Fig. 8Aii).

Gelatin zymography showed elevated pro-MMP9 levels in the TIMP3−/−/Akita kidneys while active MMP2 band intensity was only detectable in the TIMP3−/− and

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**Fig. 4.** Wilms tumor (WT)-1 and nephrin levels are not altered by the loss of TIMP3 in 6-mo-old Akita kidneys. Shown are mRNA expression levels (A; n = 8/group) and representative Western blot and averaged quantification (B; n = 4/group) of nephrin protein levels in the indicated genotypes. B: representative images of WT-1 staining (i), analysis of WT-1-positive cells per glomeruli (ii), and WT-1 mRNA expression levels (iii) in the indicated genotypes. RE, relative expression. Values are means ± SE; n = 8/group. *P < 0.05 compared with WT. #P < 0.05 compared with all other groups.
TIMP3<sup>−/−</sup>/Akita kidneys (Fig. 8B, i-iii). As such, loss of TIMP3 led to increased TACE activity and active MMP2 levels, which could contribute to oxidative stress and worsening of diabetic nephropathy.

**DISCUSSION**

Diabetic nephropathy and cardiomyopathy are well-recognized complications of diabetes and is now the most common cause of ESRD in the United States (10, 16, 31). Studies in patients with diabetic nephropathy have implicated a possible role of TIMP3 in diabetic nephropathy (13, 51). In this study, we demonstrate that loss of TIMP3 leads to the aggravation of diabetic renal injury as exemplified by significantly increased kidney mass, glomerular mesangial matrix score, and urinary albumin excretion, which are all early features of human diabetic nephropathy (9). These changes occurred in the absence of worsening of hypertension or glycemic control. Importantly, TIMP3 levels were increased in the Akita/TIMP3 double mutant kidneys.
Fig. 6. Loss of TIMP3 increases the expression of profibrotic, tubulointerstitial fibrosis and inflammatory markers in 6-mo-old Akita kidneys. Expression analysis shows greater mRNA levels of profibrotic markers, pro-collagen type I-α1 (A), connective tissue growth factor (CTGF; B), and transforming growth factor (TGF)-β1 (C), while histological analysis using picrosirius red (D) and Gomori trichrome (E) staining illustrate greater tubulointerstitial fibrosis in the Akita/TIMP3−/− kidneys. Expression analysis shows greater mRNA levels of monocyte chemotactic protein-1 (MCP-1; F) without altering IL-1β (G) and TNF-α (H) expression in Akita/TIMP3 double mutant kidneys. Scale bar = 100 μM. Values are means ± SE; n = 8. *P < 0.05 vs. all other groups.
increased in diabetic kidneys, suggesting an early compensatory role of TIMP3 in the pathogenesis of diabetic nephropathy. We previously reported that Akita mice develop cardiac diastolic dysfunction (4). However, TIMP3 deficiency did not exacerbate this diabetic cardiomyopathy, consistent with unaltered cardiac TIMP3 levels in diabetic Akita mice. These results demonstrate that TIMP3 plays a key and organ-specific role in diabetic nephropathy and is consistent with observations made in human diabetic nephropathy (13, 51).

Several canonical pathways are key mediators of diabetic nephropathy, including increased renal NADPH oxidase activity, altered intracellular signaling such as activation of the PKC system, and altered remodeling of the glomerular mesangium (7, 15, 29, 38, 48, 49). Activated NADPH oxidase (39) coupled with other sources of oxidative stress such as the mitochondria-dependent (8) and hyperglycemia-induced (11, 23) generation of ROS are clearly linked to the exacerbation of diabetic renal injury. We found increased renal cortical NADPH oxidase activity and expression of the p47phox subunit in agreement with previous findings (39). Exacerbation of diabetic renal injury by deletion of TIMP3 was associated with increased generation of NADPH oxidase-dependent ROS and nitrotyrosine, and increased expression of the renal cortical NADPH subunits p47phox and NOX2 (gp91phox) in Akita/TIMP3 double mutant kidneys. The peaked NADPH oxidase activity in Akita/TIMP3 double mutant kidneys was reduced dramatically by gp91phox ds-tat, the specific peptide inhibitor of NADPH oxidase, confirming superoxide generation from NADPH oxidase. The elevated oxidative stress could be brought about by the elevated TACE activity in the double mutant mice as TACE mediates cell surface processing of membrane-bound TACE.

Fig. 7. TIMP3 deficiency increases oxidative stress in 6-mo-old Akita diabetic kidneys. Shown are representative images of glomerular Dihydroethidium (DHE) staining (A) and nitrotyrosin staining (red) with nuclear DAPI staining (blue; B), with quantification of superoxide (C) and nitrotyrosin levels (D) showing increased superoxide levels in the Akita/TIMP3–/– kidneys. Also shown are increased NADPH oxidase activity (E), p47phox (F) and NOX2 (G) mRNA subunit expression in Akita/TIMP3–/– kidneys; n = 8/group. Gp91-tat is a specific peptide inhibitor of NOX2, gp91phox ds-tat; Scr tat is an inactive scrambled form of gp91phox ds-tat. Values are means ± SE. *P < 0.05 compared with WT. #P < 0.05 compared with all other groups. ##P < 0.05 compared with scrambled (Scr) tat peptide group (using unpaired Student’s t-test).
TNF-α into its soluble form (14, 27) whereby it can activate its receptors, mainly TNFR1/p55, triggering various tissue responses including superoxide production and oxidative stress (2, 3, 17, 30, 34). Loss of TIMP3 increased the expression of PKC1, which has been linked to exacerbation of diabetic nephropathy (29, 38), and suppressed the activation of the Akt/PI3K and ERK1/2 MAPK pathways, which have been associated with protective outcomes in diabetic nephropathy (32, 46), although these pathways have also been reported to be involved in different aspects of kidney disease (20, 33).

TIMP3 is also a critical player in regulating the tissue microenvironment including control of inflammation and fibrosis (25, 26). Inflammation has been well documented as a cardinal pathogenic mechanism in diabetic nephropathy (37). We investigated the role of inflammatory molecules in the deterioration of diabetic renal injury due to the lack of TIMP3

Fig. 8. Differential alterations in TNF-α-converting enzyme (TACE) and matrix metalloproteinase 2 (MMP2) and MMP9 levels in kidneys of different genotypes. A: TACE mRNA expression (i) and activity (ii) in all groups; n = 5/group. B: representative gelatin zymography (i) and quantification of the band intensity for pro-MMP9 (ii), MMP9 (iii), pro-MMP2 (iv), and MMP 2 (v) in each genotype; n = 6/group. +ve, Positive control for MMP2 and MMP9. *P < 0.05 compared with WT. #P < 0.05 compared with all other groups.
by examining the mRNA expression of various fibrinolytic and inflammatory markers. Indeed, pro-collagen type I-α1, CTGF, TGF-β and MCP-1 substantially increased which was associated with increased tubulointerstitial fibrosis in Akita/TIMP3 double mutant kidneys which is consistent with our previous findings showing that loss of TIMP3 enhances interstitial nephritis and fibrosis in a model of unilateral ureteral obstruction (9). TIMP3 is a known key suppressor of MMP2 activation (26, 36) and loss of TIMP3 led to increased active MMP2 in the diabetic Akita kidneys. Altered glomerular mesangium constituency due to relative increase in proteolysis secondary to active MMP2 may worsen glomerular injury and increase the susceptibility to diabetic nephropathy (7, 48, 49). In summary, TIMP3 is a key player in diabetic renal injury and strategies aimed at enhancing TIMP3 levels in diabetic kidneys may minimize diabetic renal injury.

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

AUTHOR CONTRIBUTIONS


