

Thioredoxin-interacting protein mediates dysfunction of tubular autophagy in diabetic kidneys through inhibiting autophagic flux

Chunling Huang^{1,2}, Mike Z Lin¹, Delfine Cheng³, Filip Braet^{3,4}, Carol A Pollock¹ and Xin-Ming Chen¹

Thioredoxin-interacting protein (TXNIP) expression is ubiquitous and is induced by a variety of cellular stresses, including high intracellular glucose. TXNIP is associated with activation of oxidative stress and tubulointerstitial fibrosis in diabetic nephropathy. Autophagy is a major pathway that delivers damaged proteins and organelles to lysosomes to maintain cellular homeostasis. This study aimed to investigate the dysregulation of autophagy and the regulation of TXNIP on autophagy in renal proximal tubular cells (PTCs) under diabetic conditions. The formation of autophagosomes was measured using transmission electron microscopy, and LC3-II, and the effectiveness of autophagic clearance was determined by p62 expression in diabetic kidney and in human PTCs exposed to high glucose (HG). The results collectively demonstrated increased expression of TXNIP, LC3/LC3-II and p62 in renal tubular cells of mice with diabetic nephropathy and in cultured human PTCs exposed to HG (30 mM/l) for 48 h compared with control. The formation of autophagic vacuoles was increased in HG-induced cells. Furthermore, silencing of TXNIP by siRNA transfection reduced autophagic vacuoles and the expression of LC3-II and p62 in human PTCs exposed to HG compared with control and partially reversed the accumulation of LC3-II and p62 induced by bafilomycin A₁ (50 nM/l), a pharmacological inhibitor of autophagy which blocks the fusion of autophagosomes with lysosomes and impairs the degradation of LC3-II and p62. Collectively, these results suggest that hyperglycemia leads to dysfunction of autophagy in renal tubular cells and decreases autophagic clearance. HG-induced overexpression of TXNIP may contribute to the dysfunction of tubular autophagy in diabetes.

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Autophagy is a lysosomal protein degradation pathway. It is initiated by the formation of double-membrane-bound vesicles called autophagosomes, which are formed from cup-shaped structures known as phagophores.¹ There are four key stages involving the autophagy pathway: initiation, elongation, maturation, and fusion with the lysosomes.¹ At the last stage, the autophagosomes merge with the lysosomal compartment to form autolysosomes.¹ Autophagic flux is an indicator that the autophagy process has been completed. This catabolic process normally functions in all the cells and has a crucial role in removing protein aggregates as well as damaged or excess organelles to maintain intracellular homeostasis and cell integrity. Hence, autophagy primarily exerts a protective

role under physiological conditions and ensures cell survival under stress conditions. Renal tubular cell autophagy is known to be renoprotective in acute kidney injury induced by ischemia–reperfusion injury.^{2–4} Constitutive and induced autophagy is a major protective mechanism against podocyte aging and glomerular injury.⁵ Moreover, autophagy dysregulation is associated with intracellular stresses that are inherent in diabetes mellitus. It has been reported that high glucose (HG)-induced defective autophagy in podocytes facilitates podocyte injury.⁶ Hence, it has been hypothesized that defective autophagy may accelerate the progression of diabetic nephropathy.⁶ Despite the critical recognition of tubulointerstitial pathology in progressive renal disease, and the critical

¹Kolling Institute of Medical Research, Sydney Medical School, University of Sydney, Royal North Shore Hospital, St Leonards, NSW, Australia; ²Xiamen Center of Clinical Laboratory, Xiamen Zhongshan Hospital, Medical College of Xiamen University, Xiamen, China; ³School of Medical Sciences (Discipline of Anatomy and Histology)—The Bosch Institute, University of Sydney, Sydney, NSW, Australia and ⁴Australian Centre for Microscopy and Microanalysis, University of Sydney, Sydney, NSW, Australia
Correspondence: Professor CA Pollock, MBBS, PhD, Department of Medicine, Professorial Suite, Level 7, Kolling Building, Royal North Shore Hospital, St Leonards, NSW 2065, Australia. E-mail carol.pollock@sydney.edu.au

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involvement of the renal proximal tubular cell (PTC) in the pathogenesis of diabetic nephropathy,^{7,8} the role of tubular autophagy in diabetic nephropathy has not been defined.

Hyperglycemia leads to the activation of oxidative stress and increased production of reactive oxygen species (ROS), which in turn results in tubular cell injury and deposition of extracellular matrix proteins in the tubulointerstitium.^{9–12} Thioredoxin-interacting protein (TXNIP) is a negative regulator of thioredoxin function, inhibiting its reducing capacity¹³ and promoting cellular oxidative stress. TXNIP expression is ubiquitous and is induced by a variety of cellular stresses, including high intracellular glucose.¹⁴ TXNIP overexpression renders cells more susceptible to oxidative stress and promotes apoptosis¹⁵ and excessive matrix production, key factors in the development of tubulointerstitial fibrosis.^{16,17} We have previously demonstrated that TXNIP is the gene most significantly upregulated in human proximal tubule cells exposed to HG,¹⁸ a finding confirmed in kidneys of diabetic mice and subsequently in humans with diabetic nephropathy.^{19,20}

The present study was performed to investigate the dysregulation of tubular autophagy in diabetic nephropathy. We initially examined the formation of autophagosomes in an established mouse model of diabetic nephropathy by measuring the expression of microtubule-associated protein 1 light chain 3 phospholipid-conjugated form (LC3-II), which is specifically localized to autophagic structures throughout the autophagy process from phagophore to lysosomal degradation.^{21,22} The formation of autophagosomes was also confirmed in cultured kidney tubular cell exposed to HG using transmission electron microscope. Autophagic clearance was examined by measuring the expression of sequestosome-

MATERIALS AND METHODS

Materials

Anti-LC3-B and p62 antibodies were purchased from Abcam (Cambridge, MA), anti- α -tubulin antibody and bafilomycin A1 were from Sigma (St Louis, MO), and anti-nitrotyrosine antibody was from Millipore (Darmstadt, Germany). Anti-TXNIP and Alexa488-conjugated secondary antibodies were obtained from Invitrogen (Carlsbad, CA).

Animal Studies

Eight-week-old male C57B/6 mice (Jackson laboratory, ME) weighing approximately 20–25 g were assigned to receive either 55 mg/kg of streptozotocin (STZ) (Sigma, MO) diluted in 0.1 M citrate buffer, pH 4.5, or citrate buffer alone by intraperitoneal injection as described previously.²³ A group of C57B/6 mice ($n = 8$) received citrate buffer alone served as non-diabetic controls. All animals were housed in the Kearns Animal Facility of Kolling Institute of Medical Research with a stable environment maintained at 22 ± 1

Fluor-488 (1:1000, Invitrogen) for 40 min at room temperature. Cells were then washed with PBS and counterstained with 4', 6-diamidino-2 phenylindole (DAPI)-mounting medium (Invitrogen). The immunofluorescence images were collected by confocal fluorescence microscopy (Leica Microsystems, Mannheim, Germany).

Paraffin-embedded kidney sections were used for immunohistochemical staining. Briefly, after heat retrieval, endogenous peroxidase activity was blocked by incubation in 0.3% hydrogen peroxide. After pre-incubation with 10% protein block (Dako, CA) for 10 min at room temperature to block

osmium tetroxide in PBS for 1 h. After rinsing three times with distilled water, the samples were further stained with 1% tannic acid for 1 h. Finally, the cells were infiltrated and double-embedded in Epon. Sections of 70 nm were generated with an ultramicrotome (Ultracut 7, Leica). The specimens were examined with the JEOL 2100TEM at 200 kV.

RNA Isolation and RT-PCR Analysis

Total RNA was extracted from cells and mouse kidneys using GenElute Mammalian Total RNA Miniprep Kit (Sigma) or Trizol (Invitrogen, CA) respectively. The cDNA was synthesized using SuperScript VILO cDNA Synthesis Kit (Invitrogen, CA). Quantitative real-time PCR was performed using the SYBR Green PCR master mix kit (Invitrogen, CA) with the intron-spanning primers on ABI-Prism-7900 Sequence Detection System (Applied Biosystems). The PCR primer sets for mouse TXNIP are: (forward) 5'-ATCCCAGATACCCCA GAAGC-3'; (reverse) 5'-TGAGAGTCGTCCACATCGTC-3' and for mouse β -actin are: (forward) 5'-CAGCTGAGAGGG AAATCGTG-3'; (reverse) 5'-CGTTGCCAATAGTGATGACC-3'. The relative mRNA expression levels were calculated according to the $2^{-\Delta\Delta C_t}$ method.²⁷ The mRNA expression of β -actin was used as endogenous reference control.

Indirect Immunofluorescence

For indirect immunofluorescence, cells cultured on glass coverslips were washed with PBS, fixed with 4% formal-

dehyde at room temperature for 15 min, washed with PBS once for 5 min, then permeabilized in 0.3% (0.2–0.5%) Triton X-100 in PBS for 10 min, and blocked with 2% bovine serum albumin (BSA) in PBS for 1 h at room temperature. Cells were incubated with primary antibodies anti-rabbit-LC3 (1:200), anti-rabbit-p62 (1:100), and anti-rabbit-nitrotyrosine (1:100) in 2% BSA in PBS overnight at 4 °C. After washing with PBS, cells were incubated with secondary anti-rabbit Alexa Fluor-488 (1:1000, Invitrogen) for 40 min at room temperature. Cells were then washed with PBS and counterstained with DAPI-mounting medium (Invitrogen). The immunofluorescence images were collected by confocal fluorescence microscopy (Leica Microsystems, Mannheim, Germany).

Western Blot

Equal amount of cell lysates samples were separated by SDS-PAGE, and then transferred to Hybond ECL nitrocellulose membrane (Amersham, USA). The membranes were incubated with primary antibodies anti-rabbit-LC3 (1:500), anti-rabbit-p62 (1:500), and anti-rabbit-TXNIP (1:500) at 4 °C overnight followed with HRP-conjugated secondary antibody (Amersham, USA) (1:5000). The blots were then detected with standard ECL technique, and the bands were quantified by densitometry using LAS-4000 Imaging System (FUJIFILM, Japan).

Statistical Analysis

Results from at least four independent experiments were expressed as mean $\pm$ s.e.m. Statistical analysis of data from two groups was compared by two-tailed *t*-test. Analysis of data from multiple groups was performed by one-way ANOVA, followed by Tukey's post test. Statistical significance was determined as $P < 0.05$.

RESULTS

The Formation of Autophagosomes in Diabetic C57B/6 Mice

We examined the autophagic marker (LC3) in kidney of diabetic mice after 24 weeks of STZ induction. We have previously described that this animal model develops renal dysfunction and diabetes-induced tubulointerstitial injury.^{28,29} The formation of autophagosomes was determined by LC3 expression, which participates in the initiation and elongation processes of autophagosomes, using indirect immunofluorescence staining. As shown in Figure 1, there is basal expression of LC3 in the kidneys of normal animals, which was increased in the diabetic mice ($P < 0.05$). This accumulation of autophagosomes in diabetic mice may be caused by either induction of autophagy or inhibition of autophagosome clearance and/or degradation of autophagic substrates in the lysosome.

Autophagic Flux in Diabetic Mice

To determine tubular autophagic flux in diabetic mouse, the autophagosome clearance was examined with p62, a selective substrate of autophagy. Immunofluorescence, segmental

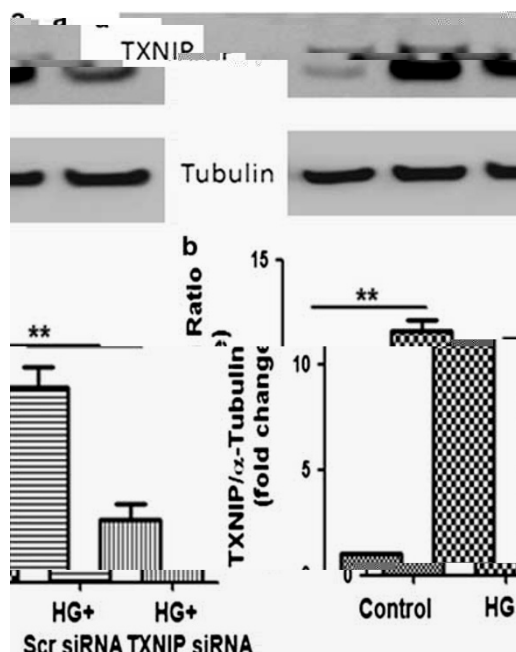


Figure 4 The expression of TXNIP in HG-induced HK2 cells. HK2 cells transfected with scramble siRNA or TXNIP-specific siRNA were untreated or treated with high glucose for 48 h. (a) Immunoblot analysis of TXNIP with or without TXNIP siRNA transfection in HG-induced HK2 cells. (b) Quantification of TXNIP expression in HG-induced HK2 cells. Results are presented as mean $\pm$ s.e.m. ** $P < 0.01$. $N = 4$.

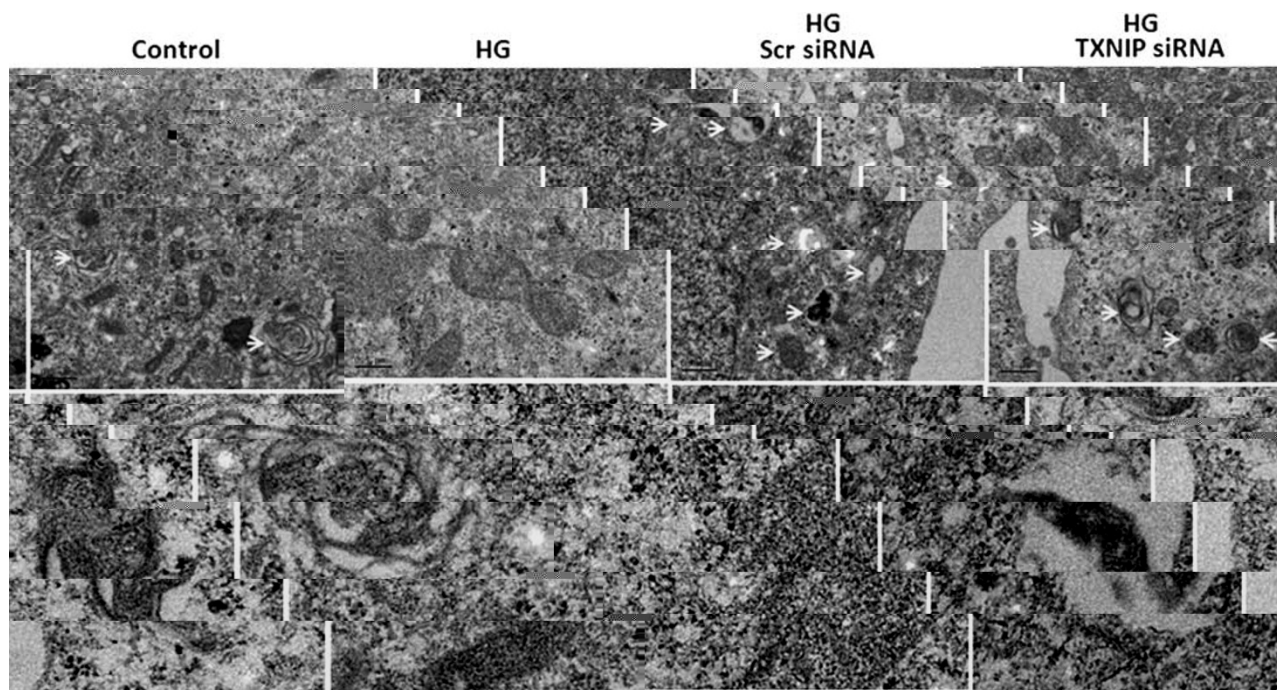


Figure 5 Electron microscopic evaluation of autophagy in HG-induced HK2 cells. HK2 cells transfected with scramble siRNA or TXNIP-specific siRNA were treated with high glucose hagic 1(gic)-537.8007993l(gic)-537.8007993(8n35)-338.5(5iabetic)-343.2999878(mice.)TJ/F41Tf9.7634000785997500l957993(8n35)-3380.1

labeling of p62 was observed, mainly in proximal tubules (Figure 2). Consistent with the LC3 findings, p62 staining was significantly increased in diabetic mice as compared with non-diabetic controls ($P < 0.01$, Figure 2). Collectively, the increase of LC3 and p62 indicate the accumulation of autophagosomes due to the suppression of autophagic flux in the proximal tubules of diabetic mice.

Overexpression of TXNIP in Diabetic Mice

Our group and others have reported that elevated TXNIP expression contributes to the oxidative stress reported in diabetic nephropathy.^{19,20} To determine whether TXNIP has a role in regulation of autophagy, we first examined the expression of TXNIP in the diabetic and control mice. RT-PCR analyses of kidney tissues demonstrated increased mRNA expression of TXNIP in the diabetic group compared with control mice ($P < 0.05$, Figure 3a), and histopathological analysis of renal cross-sections demonstrated increase of TXNIP protein significantly in diabetic mice compared with controls ($P < 0.01$, Figures 3b and c). These data confirmed that TXNIP expression was upregulated in kidney of diabetic mice.

TXNIP Gene Silence Reversed HG-Induced Suppression of Autophagic Flux

To further investigate whether TXNIP is involved in the regulation of tubular autophagy in diabetes, *in vitro* studies

were undertaken where tubular cells were exposed to HG and TXNIP was silenced using TXNIP siRNA. The inhibitory effect was determined by western blotting. As shown in Figure 4, the inhibitory efficiency is approximately 70%.

To confirm HG-induced dysfunction of autophagy and the effect of TXNIP on autophagy in kidney tubular cells, we examined autophagy using transmission electron microscopy, and the appearance of autophagosomes and related autophagic vacuoles was monitored. As shown in Figure 5 (upper panel), no obvious autophagic vacuoles were shown in HK2 cells exposed to normal glucose. However, several autophagic vacuoles appeared in HK2 cells with/without scramble siRNA transfection exposed to HG for 48 h but much fewer autophagic vacuoles were observed in HK2 cells with TXNIP siRNA transfection exposed to HG for 48 h. The structures of autophagic vacuoles were shown by higher magnification ($\times 10\,000$) electron microscopy (lower panel in Figure 5).

Consistently, western blotting results showed that the level of LC3-II is significantly increased in HK2 cells exposed to HG, while TXNIP silencing suppressed the HG-induced LC3-II expression ($P < 0.01$, Figures 6a and b). Immunofluorescence staining further confirmed the HG-induced LC3, which was reversed by TXNIP silencing ($P < 0.01$, Figures 6c and d). Similarly, HG-induced p62 expression was also abrogated by TXNIP silencing, confirmed by western blotting and immunofluorescence staining ($P < 0.01$, Figures 7a–d).

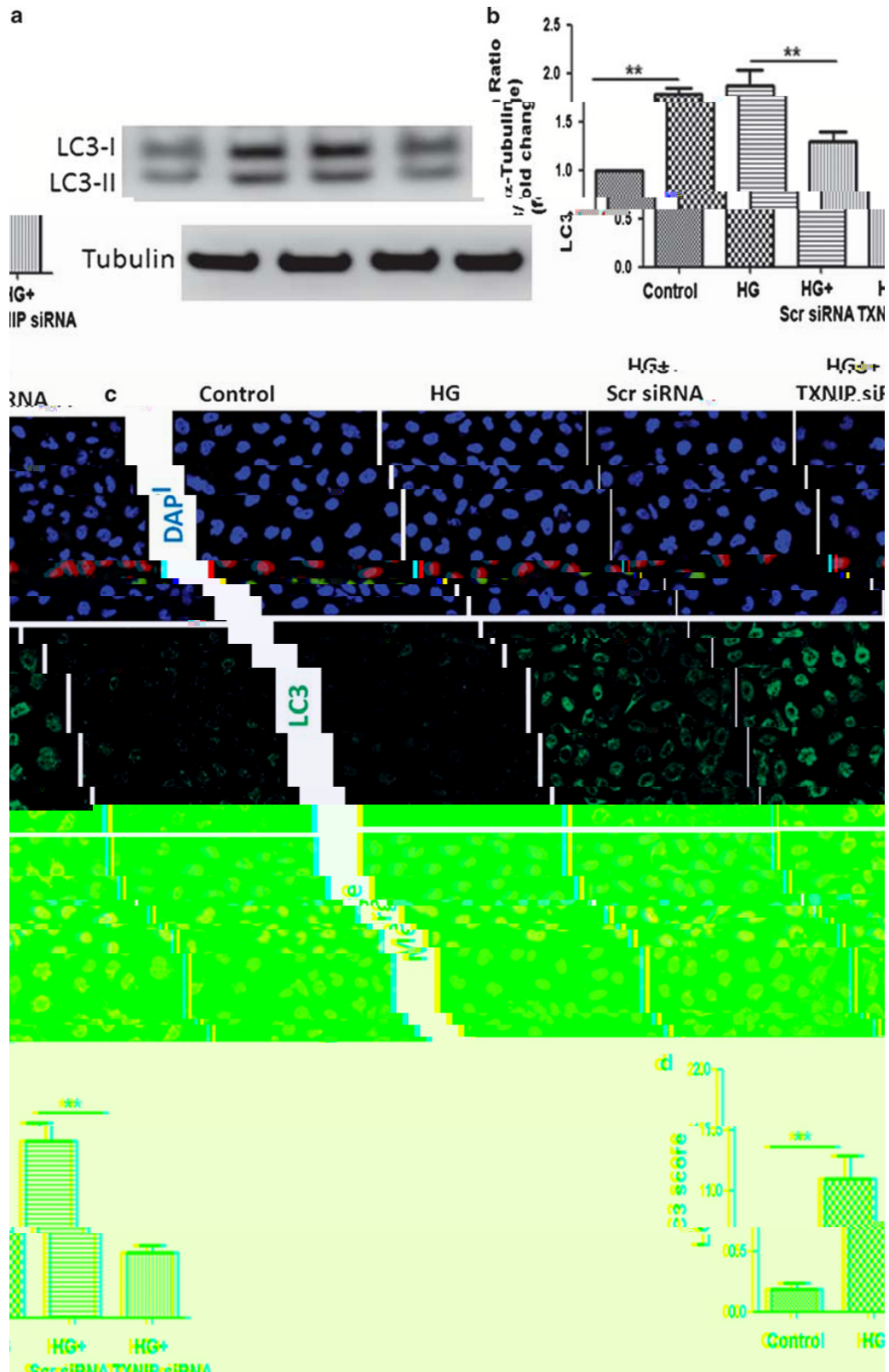


Figure 6 Effect of knockdown TXNIP on the expression of LC3 in HG-induced HK2 cells. HK2 cells transfected with scramble siRNA or TXNIP-specific siRNA were untreated or treated with high glucose for 48 h. (a) Immunoblot analysis of LC3 with or without TXNIP siRNA transfection in HG-induced HK2 cells. (b) Quantification of LC3 immunoblot expression in HG-induced HK2 cells. (c) Immunofluorescence staining of LC3 in HG-induced HK2 cells transfected with or without TXNIP siRNA. (d) Quantification of LC3 immunofluorescence expression in HG-induced HK2 cells. Results are presented as mean $\pm$ s.e.m. $**P < 0.01$. $N = 4$. Original magnification: $\times 600$.

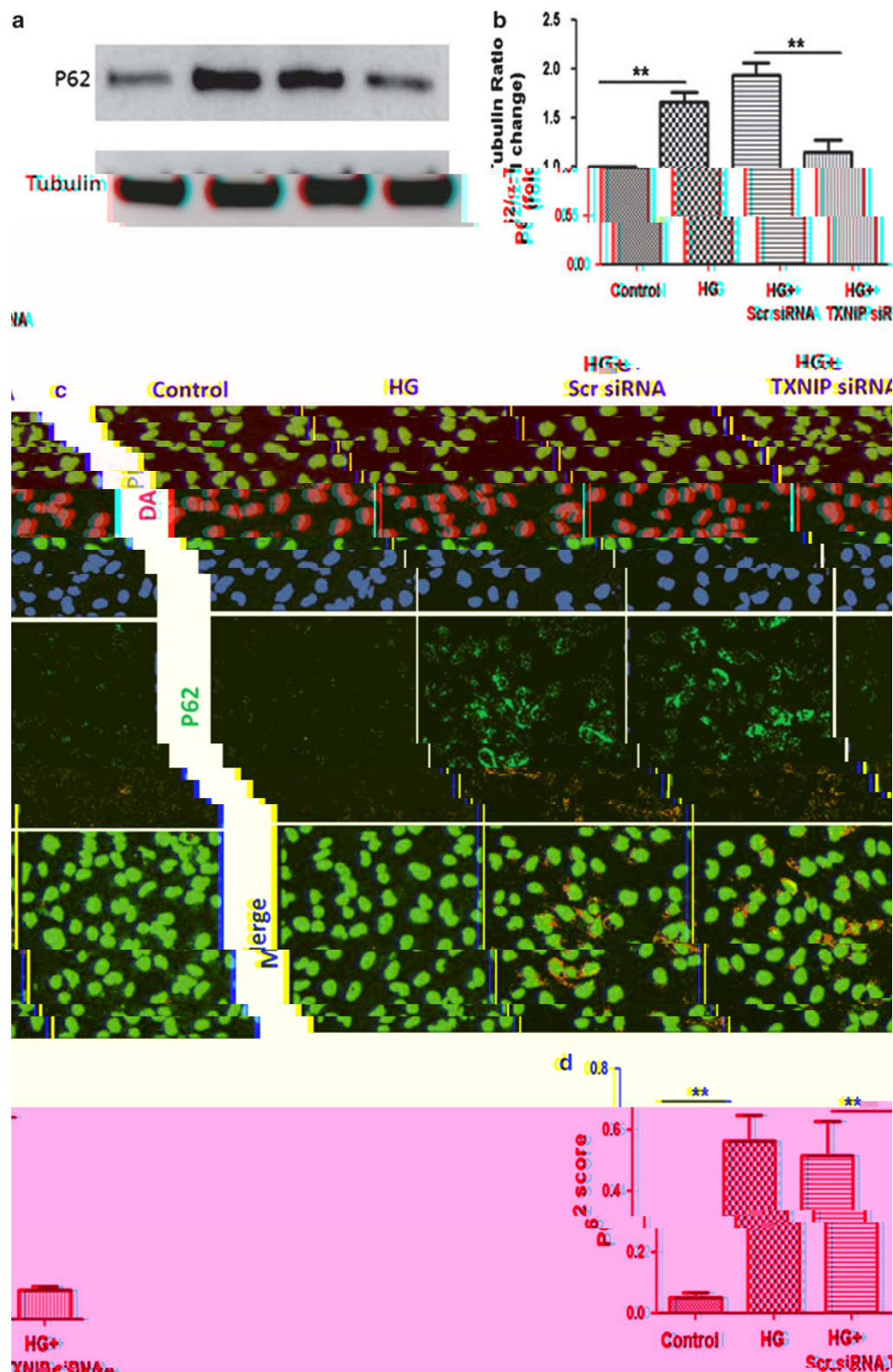


Figure 7 Effect of knockdown TXNIP on the expression of p62 in HG-induced HK2 cells. HK2 cells transfected with scramble siRNA or TXNIP-specific siRNA were untreated or treated with high glucose for 48 h. (a) Immunoblot analysis of p62 with or without TXNIP siRNA transfection in HG-induced HK2 cells. (b) Quantification of p62 immunoblot expression in HG-induced HK2 cells. (c) Immunofluorescence staining of p62 in HG-induced HK2 cells transfected with or without TXNIP siRNA. (d) Quantification of p62 immunofluorescence expression in HG-induced HK2 cells. Results are presented as mean $\pm$ s.e.m. $^{**}P < 0.01$. $N = 4$. Original magnification: $\times 600$.

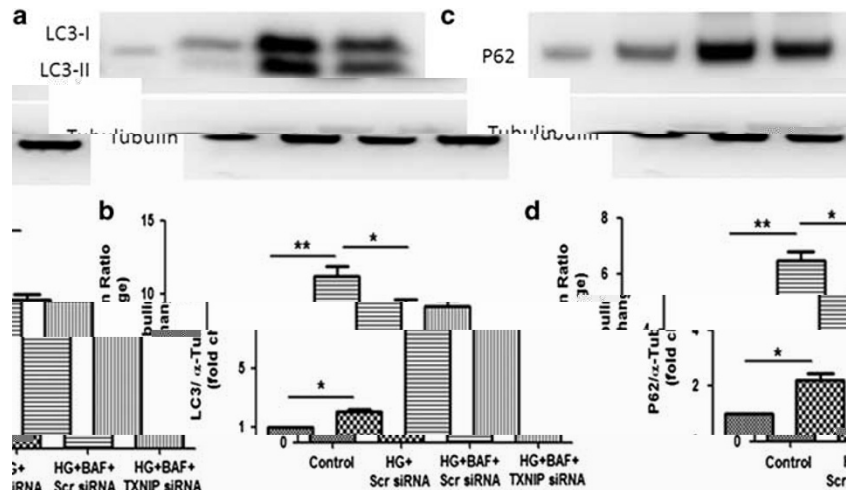


Figure 8 Effect of knockdown TXNIP on the expression of LC3 and p62 in HG-induced HK2 cells together with or without bafilomycin A₁. HK2 cells transfected with scramble siRNA or TXNIP-specific siRNA overnight were pretreated with 50 nmol/l bafilomycin A₁ for 3 h and then incubated with HG (30 mM) together for 48 h. Immunoblot analysis of LC3 (a) and p62 (c) with or without TXNIP siRNA transfection in HG-induced HK2 cells together with bafilomycin A₁. Quantification of LC3 (b) and p62 (d) expression in HG-induced HK2 cells together with bafilomycin A₁. Results are presented as mean ± s.e.m. * $P < 0.05$ and ** $P < 0.01$. $N = 4$.

Bafilomycin A₁-Induced Blockade of Autophagic Flux was Partially Reversed by TXNIP Gene Silencing in PTCs *In Vitro*

To address whether TXNIP mediates HG-induced dysregulation of tubular autophagy, we tested the effect of bafilomycin A₁, a pharmacological inhibitor of autophagy, in renal tubular cells with or without TXNIP silencing. As shown in Figure 8a, HG significantly increased the level of LC3-II as compared with control group. Treatment with bafilomycin A₁ further increased the level of LC3 in HK2 cells exposed to HG, which is partially reversed by TXNIP gene silencing ($P < 0.05$, Figure 8b). In line with these findings, the level of p62 protein was significantly increased in HG-induced HK2 cells. Treatment with bafilomycin A₁ further increased the level of p62 in HG-induced HK2 cells as well, which is partially reversed by TXNIP gene silencing ($P < 0.05$, Figures 8c and d). These data indicate that TXNIP is involved in the regulation of tubular autophagy under HG conditions.

TXNIP Mediates HG-Induced Dysfunction of Tubular Autophagy through Oxidative Stress

TXNIP is an endogenous inhibitor of the anti-oxidant thioredoxin and is highly upregulated in diabetic nephropathy, leading to oxidative stress and fibrosis.¹⁷ Recently, it has been reported that autophagy is modulated by multiple intracellular stresses, including oxidative stress.^{30,31} To investigate whether TXNIP-mediated oxidative stress is involved in regulation of autophagy, nitrotyrosine,³² a marker of NO-dependent oxidative stress, was examined in diabetic mice kidneys. Immunohistochemical staining results confirmed that the expression of nitrotyrosine is significantly increased in diabetic mice compared with non-diabetic mice ($P < 0.05$, Figures 9a and b). Furthermore, we examined the

expression of nitrotyrosine in HG-induced HK2 cells *in vitro*. As shown in Figures 9c and d, HG significantly increased the expression of nitrotyrosine in HK2 cells that was reversed by TXNIP silencing ($P < 0.01$). Together, these results indicate that HG-induced overexpression of TXNIP is likely to mediate dysregulation of tubular autophagy through oxidative stress.

DISCUSSION

The present study was conducted to investigate the dysregulation of autophagy in tubular cells in a validated mouse model of diabetic nephropathy²⁹ and in cultured human PTCs exposed to HG. The results demonstrate increased formation of autophagosomes and reduced autophagic clearance in renal tubular cells under diabetic conditions. Furthermore, silencing of the TXNIP gene, using siRNA technology, reduced formation of autophagosomes and promoted autophagic clearance in tubular cells exposed to HG. TXNIP gene silencing also partially reversed bafilomycin A₁-induced blockade of autophagosome clearance.

Established evidence indicates that the renal proximal tubular epithelial cell has a central role in the pathogenesis of diabetic nephropathy, and renal function closely correlates with the degree of tubulointerstitial injury rather than the glomerular pathology.^{7,8,33,34} Proximal tubular epithelial cells have an important role in the reabsorption and degradation of albumin and low-molecular-weight plasma proteins from the glomerular filtrate.³⁵ Numerous studies have reported on autophagy of PTCs in models of acute renal injury. Autophagy is readily induced by renal ischemia-reperfusion^{2,3} and cisplatin damage to the kidneys.^{2,36} Reduced autophagic activity worsens acute kidney injury,^{2,4,37} suggesting that stress-inducible autophagy is renoprotective. To date, dysre-

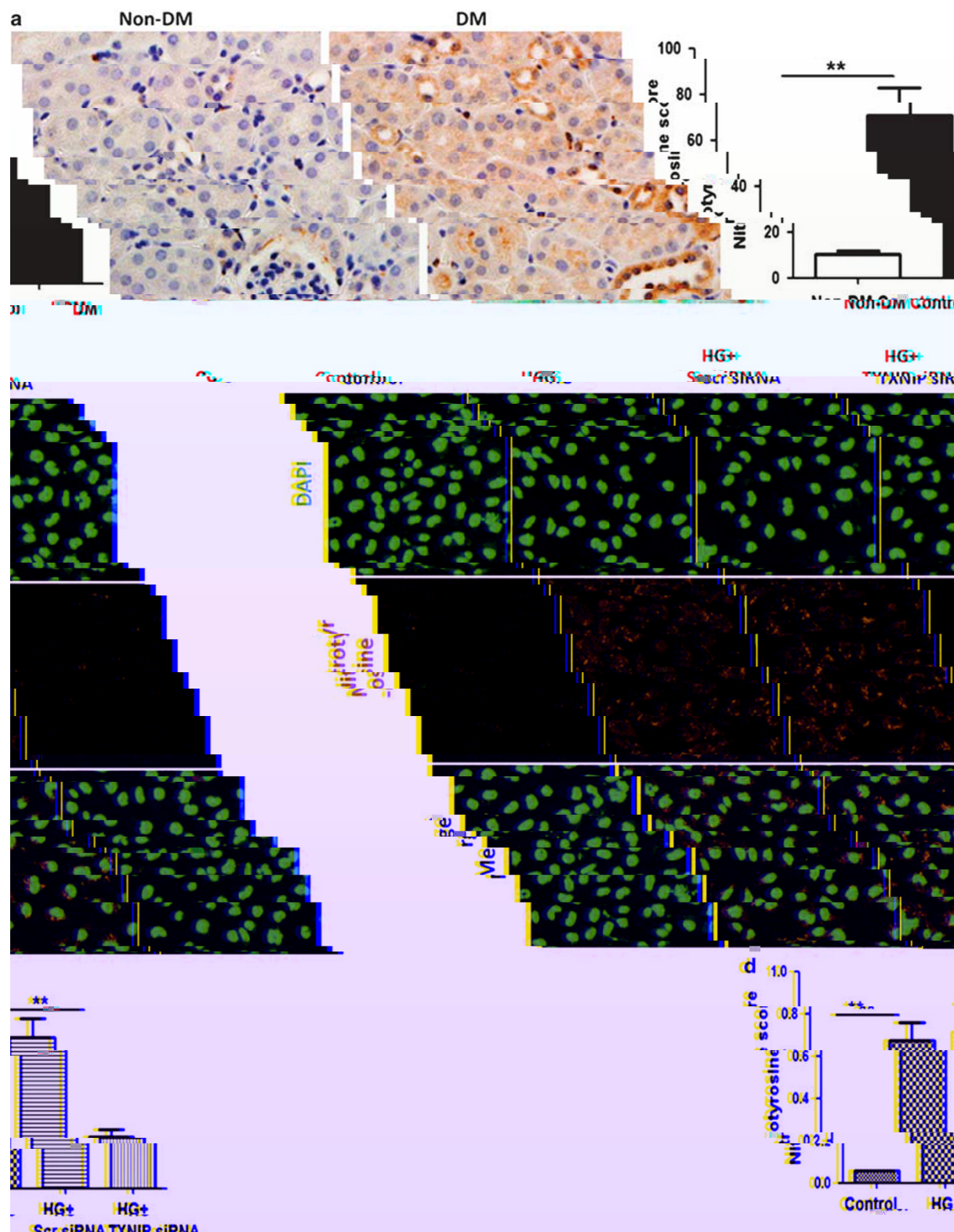


Figure 9 The expression of nitrotyrosine in diabetic mice tissues and effect of knockdown TXNIP on the expression of nitrotyrosine in HG-induced HK2 cells. C57B/6 mice were injected with STZ to induce diabetes or citrate buffer alone as non-diabetic control. After 24 weeks diabetes, kidney tissues were collected for immunostaining. (a) Immunohistochemical staining of nitrotyrosine in mice kidney tissues. $N = 8$. (b) Quantification of nitrotyrosine expression in mice kidney tissues. HK2 cells transfected with scramble siRNA or TXNIP-specific siRNA were untreated or treated with high glucose for 48 h. (c) Immunofluorescence staining of nitrotyrosine in HG-induced HK2 cells transfected with or without TXNIP siRNA. (d) Quantification of nitrotyrosine expression in HG-induced HK2 cells. Results are presented as mean $\pm$ s.e.m. ** $P < 0.01$. $N = 4$. Original magnification: $\times 600$.

gulation of tubular autophagy in kidney under diabetic conditions has not been reported.

To investigate whether chronic hyperglycemia leads to dysfunction of tubular autophagy, we initially examined the expression of LC3 and p62 in tubular cells of fibrotic kidneys. It is well documented that LC3 and p62 undergo post-translational modifications in the autophagy machinery.^{38–42} The lipidation of LC3 by the phospholipid phosphatidylethanolamine to forms LC3-II associates with forming autophagosome membranes and the conversion of LC3 to LC3-II is well accepted as a marker of autophagy induction, and p62 protein degraded by autophagy inversely reflects the autophagic flux, therefore the protein levels of LC3-II and p62 were examined to monitor autophagy and autophagic flux in this study.^{21,22,26,43–46} We found significantly increased

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