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ORIGINAL ARTICLE

NF-κB Inhibitor Parthenolide Promotes Renal Tubules Albumin Uptake in Type 2 Diabetic Nephropathy

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Key words: inflammation; insulin resistance; albuminuria; diabetic nephropathy; parthenolide

Objective Injured tubular reabsorption is highlighted as one of the causes of increased albuminuria in the early stage of diabetic nephropathy; however, the underlying mechanism has not been fully elucidated. In this study, we aimed to explore whether reducing inflammation and remodeling the insulin signaling pathway could improve albumin uptake of renal tubules.

Methods 8-week-old male db/db mice ($n=8$), a type 2 diabetic nephropathy model, administered with nuclear factor kappa-B (NF-κB) inhibitor parthenolide (PTN, 1 mg/kg) intraperitoneally every other day for 8 weeks, were as the treatment group. Meanwhile, the age-matched male db/m mice ($n=5$) and db/db mice ($n=8$) were treated with saline as the control group and type 2 diabetic nephropathy group. When the mice were sacrificed, blood and urine were collected to examine homeostasis model assessment of insulin resistance (HOMA-IR) and urine albumin creatinine ratio, and kidney samples were used to analyze histopathologic changes with periodic acid-Schiff (PAS) staining, NF-κB p65, phosphorylation of AKT (p-AKT), amnionless and cubilin expressions with immunohistochemistry as well as western blot, and the albumin uptake of renal tubules by using immunofluorescence. In addition, HKC cells were divided into the insulin group treated with insulin alone, the TNF-α group treated with insulin and tumor necrosis factor (TNF-α), and the TNF-α+PTN group exposed to PTN, insulin and TNF-α. The levels of albumin uptake and expression levels of NF-κB p65, p-IRS-1/IRS-1, p-AKT/AKT, amnionless and cubilin in HKC cells were measured.

Results Compared with the db/db group, the db/db+PTN group demonstrated decreased levels of HOMA-IR (36.83 ± 14.09 *vs.* 31.07 ± 28.05) and urine albumin creatinine ratio (190.3 ± 7.3 *vs.* 143.0 ± 97.6 mg/mmol); however, the differences were not statistically significant ($P>0.05$). Periodic acid-Schiff staining showed PTN

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could alleviate the glomerular hypertrophy and reduce the matrix in mesangial areas of db/db mice. The renal expression of NF-κB p65 was increased and p-AKT (s473) decreased in the db/db group compared with the db/m group ($P<0.05$). PTN significantly reduced the renal expression of NF-κB p65 and ameliorated the decline of p-AKT (s473) compared with the db/db group ($P<0.05$). Compared with the db/m group, the expression of amnionless and cubilin decreased and albumin uptake in tubules were reduced in the db/db group ($P<0.05$), and PTN could significantly increase the expression of cubilin ($P<0.05$), and improve albumin uptake in tubules. Insulin promoted albumin uptake and the expression of amnionless and cubilin in HKC cells ($P<0.05$). TNF-α stimulated the expression of NF-κB p65, increased p-IRS-1 (s307) and reduced p-AKT (s473) in HKC cells ($P<0.05$). In the TNF-α+PTN group, the expression of NF-κB p65 declined and p-IRS-1 (s307) and p-AKT (s473) were restored, compared with the TNF-α group ($P<0.05$). The expression of amnionless and cubilin decreased in the TNF-α group ($P<0.05$), and PTN could significantly increase the expression of cubilin ($P<0.05$).

Conclusions Inflammation caused damage to insulin signaling, which reduced amnionless-cubilin expression and albumin uptake. PTN could reduce inflammation and remodel the impaired insulin signaling pathway, which promoted the expression of cubilin and albumin uptake. Our study can shed light on the role of inflammation in the reduction of albumin uptake of renal tubules in type 2 diabetic nephropathy.

DIABETIC nephropathy (DN) is a primary cause of end-stage renal disease worldwide and is featured by increasing albuminuria and a progressive reduction of glomerular filtration rate.[1] Recent studies suggest that albuminuria in the early stages of DN is mainly caused by impaired albumin endocytosis in renal tubules.[2-4] Therefore, the identification of pathogenesis of impaired albumin tubular endocytosis is meaningful for reducing albuminuria.

Albumin reabsorption in proximal tubule cells is regulated by insulin signaling.[5] Insulin could increase the expression of albumin receptors to promote the albumin uptake.[6, 7] Moreover, clinical studies have proven that reduced insulin sensitivity is associated with increasing albuminuria.[8, 9] However, it is unclear whether insulin resistance in renal tubules contributes to the development of albuminuria. Insulin resistance induced by chronic inflammation is a well-known phenomenon in DN.[10] Albuminuria is a common phenomenon in patients with sepsis and it has been proven that renal tubular albumin endocytosis is decreased during endotoxemia.[11] Thus, it was speculated in current study that inflammation inducing insulin signaling impairment decreased tubular albumin endocytosis and promoted the development of albuminuria in DN.

Albumin uptake of proximal tubule cells is accomplished through a receptor-mediated endocytic mechanism which involves the amnionless (AMN)-cubilin complex.[12] Cubilin has been confirmed as an important receptor for albumin.[13, 14] Mice with cubilin deficiency demonstrate a reduced renal proximal tubular uptake of albumin and an increased urinary loss of albumin.[15] Cubilin interacts with AMN, which is responsible for membrane anchoring, endoplasmic reticulum export and internalization of cubilin.[16] AMN and cubilin cooperate with each other for promoting albumin reabsorption.[14] Studies have demonstrated a decreased expression of cubilin in kidneys of animals model with diabetes.[5, 17] However, the role of AMN-cubilin in the albuminuria formation of DN has not been elucidated.

Parthenolide (PTN), isolated from the European herb feverfew, exhibits anti-inflammatory activities.[18] PTN has been regarded to play a role in both modifying the activity of the nuclear factor kappa-B (NF-κB) p65 subunit and inhibiting the phosphorylation of IκB by blocking the activity of the IKK complex.[19] In our previous research, PTN was found to reduce both renal inflammation and insulin resistance in type 2 DN mice.[20] Previously, we have found that inflammatory factors, phosphorylation of IRS (p-IRS)-1/IRS-1 and gluconeogenic related enzyme were upregulated and reduced phosphorylation of AKT (p-AKT)/AKT was detected in db/db mice—a type 2 DN model, and PTN could improve these changes.[20] We speculated that PTN treatment may ameliorate albumin reabsorption in renal tubules in db/db mice.

In this study, we investigated a mechanism for the effect of insulin signaling on albumin uptake *via* regulation of AMN-cubilin expression in renal tubule cells. We further examined how perturbations of insulin

signaling caused by inflammation induce alterations in AMN-cubilin expression and albumin uptake in type 2 DN. Finally, we verified whether PTN treatment could reduce inflammation, remodel impaired insulin signaling, and subsequently increase the albumin endocytosis of renal tubules in type 2 DN.

MATERIALS AND METHODS

Mice

6-week-old male db/db (C57BLKS/J-LepR^{db}/LepR^{db}, a type 2 diabetic nephropathy model) mice and db/m (C57BLKS/J-LepR$^{db/+}$) mice were purchased from Model Animal Research Center of Nanjing University and housed in Animal Research Laboratory of Peking Union Medical College Hospital. After acclimation, 8-week-old db/m mice (n=5) and db/db mice (n=8) were intraperitoneally administered with saline every other day. Other db/db mice (n=8) were administered with PTN (1 mg/kg, Sigma-Aldrich, St Louis, MO, USA) in the same way. After 8-week administration, 24-h urine samples were collected. The mice were subsequently sacrificed and blood and kidney samples were collected for further analysis.

The present study was ethically with the consent of the Experimental Animal Center of Peking Union Medical College Hospital and Chinese Academy of Medical Sciences.

Cells

HKC cells, human kidney proximal tubular cell line, were obtained from Cell Resource Center, Peking Union Medical College and were maintained in 5 mmol/L Dulbecco's Modified Eagle Medium (DMEM, Gibco, USA) supplemented with 10% fetal bovine serum (Gibco) and 100 U/ml penicillin/streptomycin (Gibco) at 37°C and 5% CO_2.

Biochemical analysis

Measurements of blood and urine samples were conducted in Peking Union Medical College Hospital to detect blood glucose, serum insulin, serum creatinine, urine albumin, and urine creatinine.

Histopathologic analysis

Paraffin-fixed kidneys were cut into 3-μm thick sections and deparaffinized. Histopathologic observation in kidneys were examined by periodic acid-Schiff (PAS) staining.

Immunohistochemistry

The sections were blocked with 3% hydrogen peroxide at room temperature for 20 minutes after antigen retrieval. After the sections were blocked with goat serum for 1 hour, they were incubated with primary antibodies, including rabbit anti-NF-κB p65 antibody (1:2000, Abcam, UK), mouse anti-amnionless antibody (1:250, Santa Cruz Biotechnology, USA) and sheep anti-cubilin antibody (1:500, R&D Systems, USA) overnight at 4°C, and followed by incubation with the horseradish peroxidase-conjugated secondary antibodies (ZSGB Biotech. Co. Ltd., Beijing, China) for 15 minutes at 37°C. After stained with DAB for 1-3 minutes, the sections were counterstained with hematoxylin prior to examination under a light microscope. The optical density values were determined by Image Pro Plus 6.0 software (MediaCybernetics Corporation, USA) under blind conditions.

Immunofluorescence

The sections were incubated with rabbit anti-albumin antibody (1:500, Abcam, UK) overnight at 4°C. The secondary antibodies (goat anti-rabbit IgG, Alexa Fluor® 594, ZSGB Biotech. Co. Ltd., Beijing, China) were incubated for 1 hour at 37°C. Images were detected under a Leica confocal microscope (Wetzlar, Germany).

Western blotting

Kidneys or HKC cells were lysed and homogenized with RIPA lysis buffer. Protein concentrations were quantified *via* the BCA protein assay kit (Applygen, Beijing, China). The proteins were separated on 8%-10% SDS-PAGE and were subsequently transferred onto polyvinylidene difluoride membranes. The blot membranes were blocked with 5% skim milk and incubated with primary antibodies overnight at 4°C, and primary antibodies included rabbit anti-albumin antibody (1:1000, Abcam, UK), rabbit anti-NF-κB p65 antibody (1:2500, Abcam, UK), rabbit anti-Ser307-p-IRS-1 antibody (1:1000, Abcam, UK), rabbit anti-IRS-1 antibody (1:1000, Abcam, UK), rabbit anti-β-actin antibody (1:2500, Abcam, UK); rabbit anti-Ser473-p-AKT (1:1000, Cell Signaling Technology, USA), rabbit anti-AKT (1:1000, Cell Signaling Technology, USA); mouse anti-amnionless antibody (1:1000, Santa Cruz Biotechnology, USA); and sheep anti-cubilin antibody (1:500, R&D Systems, USA). The membranes were then incubated with the horseradish peroxidase-con-

jugated secondary antibodies (ZSGB Biotech. Co. Ltd., Beijing, China) for 1 hour. The proteins were shown by enhanced chemiluminescence substrate (Pierce, Thermo Fisher Scientific, USA). The analysis of the images was performed with ImageJ software (NIH, Littleton, CO, USA). The expression of proteins was normalized with β-actin.

Cell viability assay

The effects of tumor necrosis factor (TNF-α) or PTN on cell viability of HKC was assessed *via* cell counting kit-8 (CCK-8) assay. Briefly, HKC cells were plated in 96-well plates at 5×10^4 cells per well. After 12 hours, HKC cells were treated with TNF-α (0, 25, 50 and 100 ng/ml) or PTN (0, 2.5, 5 and 10 μmol/L) at 37°C for 18 hours. Then CCK-8 solution (Solarbio, Beijing, China) was added to each well. After 1 hour of incubation at 37°C, the well was assessed by measuring the absorbance at 490 nm wavelength (A). The results of cell viability (%) were calculated *via* the following formula: $(A_{\text{treated group}}-A_{\text{blank group}})/(A_{\text{control group}}-A_{\text{blank group}})\times100$.

Albumin endocytosis assay

The uptake of albumin in HKC cells was assessed by a fluorometric experiment and western blotting as previously described.[7, 21] Cells grown on 6-well cell culture plates at 5×10^5 cells per well were incubated in serum free DMEM overnight. For fluorometric experiment, cells were treated with 100 nmol/L insulin for 30 minutes and followed by 100 μg/ml FITC-labelled albumin (Solarbio, Beijing, China) for 1 hour. The control cells were without insulin exposure. For western blotting analysis, FITC-labelled albumin was replaced with 1 mg/ml human serum albumin (Solarbio, Beijing, China).

It has been demonstrated that TNF-α could induce insulin signaling damage of HKC cells.[22] In this study, to determine whether inflammation mediated insulin signaling impairment contributed to the development of albuminuria by decreasing albumin reabsorption in the proximal tubules in DN, the cells were divided into three groups: insulin group which were treated with insulin alone for 30 minutes (Sigma-Aldrich, I9278, St Louis, MO, USA), TNF-α group which were treated with TNF-α (Pepro Tech, 300-01A, RockyHill, USA) for 18 hours followed by insulin incubation for 30 minutes, and PTN+TNF-α group which were exposed to PTN, TNF-α and insulin (HKC cells were treated with TNF-α and PTN simultaneously for 18 hours, and then treated with insulin for 30 minutes) under serum free culture condition. After incubation with albumin, the cells were washed with cold PBS 6 times for stripping away membrane bound albumin and were then disintegrated by the MOPS solution with 0.1% vol/vol Triton X-100. The fluorescence intensity of FITC-albumin was measured at 493 nm wavelength excitation and 550 nm wavelength emission. The amount of protein was evaluated using the BCA method. The fluorescence values were normalized by the amount of protein. Images of the FITC-albumin endocytosis of HKC cells were captured *via* a Leica confocal microscope (Wetzlar, Germany).

Statistical analysis

All experimental data were presented as mean±SD. Unpaired Student's *t*-test was utilized to determine significant differences between two independent sets of samples and was performed on Graphpad prism 5.0 (Graphpad Software, San Diego, CA, USA). One-way analysis of variance was utilized for comparisons among multiple groups and was performed on SPSS 19.0 (SPSS Inc., Chicago, IL, USA). The values of differences were regarded as significant when $P<0.05$.

RESULTS

Metabolic and renal parameters and histopathologic changes in 16-week-old experimental mice

As shown in **Table 1**, the db/db mice developed hyperglycemia, insulin resistance (HOMA-IR increasing), and a high level of the urine albumin creatinine ratio (UACR) in comparison with the db/m mice. PTN could slightly reduce the levels of HOMA-IR and UACR in the db/db mice; however, the differences were not statistically significant (db/db *vs*. db/db+PTN: $t_{\text{HOMA-IR}}=0.4857$, $P_{\text{HOMA-IR}}=0.6359$, $t_{\text{UACR}}=0.6661$, $P_{\text{UACR}}=0.518$).

Under PAS staining (**Figure 1**), the db/db mice kidney demonstrated the glomerular hypertrophy and the increase of matrix in mesangial areas. PTN could alleviate the above pathological changes in the kidneys of the db/db mice. No obvious difference was seen in the structure of renal tubules among the three groups.

PTN ameliorated expression of NF-κB p65, restored phosphorylation of AKT, attenuated the reduction of cubilin and improved albumin uptake in mice model

Immunohistochemistry (IHC) and western blot (WB) analysis showed the db/db group presented a great-

Table 1. Comparisons of metabolic and renal parameters among the three 16-week-old mice groups§

Groups	*n*	Blood glucose (mmol/L)	Serum insulin (mIU/L)	HOMA-IR	Serum creatinine (μmol/L)	UACR (mg/mmol)
db/m	5	10.01±1.73	14.93±4.00	6.60±1.70	9.56±0.98	19.5±14.5
db/db	8	41.19±7.31*	21.02±9.95	36.83±14.09*	7.20±2.82	190.3±7.3*
db/db+PTN	8	41.00±14.78	21.30±7.30	31.07±28.05	7.56±2.42	143.0±97.6
F value		17.323	0.277	3.881	1.655	2.419
P value		<0.001	0.761	0.042	0.222	0.121

§: Plus-minus values are means ± SD.

PTN: parthenolide; HOMA-IR: homeostasis model assessment of insulin resistance; UACR: urine albumin creatinine ratio.

*$P<0.05$ compared with the db/m group.

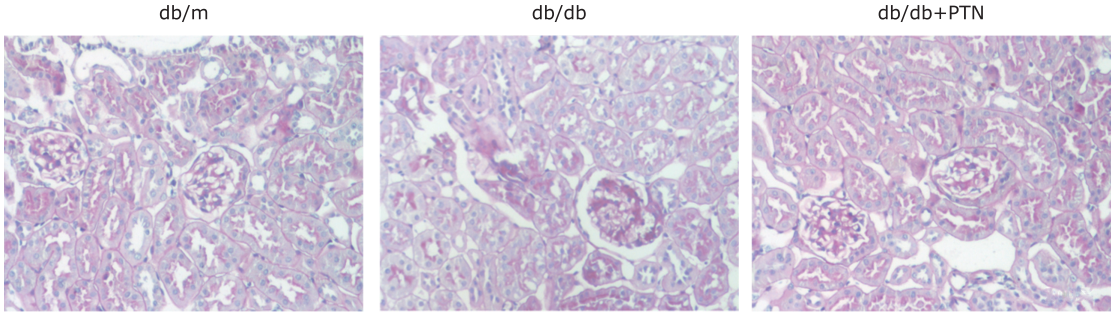

Figure 1. Histopathological changes of the kidney in mice.

Under periodic acid-Schiff staining, the db/db mice kidney demonstrated the glomerular hypertrophy and the increase of mesangial matrix. PTN could alleviate the above pathological changes in kidneys of the db/db mice. (PAS staining, 200×)

er expression of NF-κB p65 than the db/m group ($t_{IHC}=3.851$, $P_{IHC}=0.0183$, $t_{WB}=3.671$, $P_{WB}=0.0214$) and PTN could reduce the expression of NF-κB p65 in the db/db mice ($t_{IHC}=3.18$, $P_{IHC}=0.0335$, $t_{WB}=2.954$, $P_{WB}=0.0418$) (**Figure 2A, 2B**).

Moreover, western blot revealed p-AKT/AKT decreased in the db/db group compared with the db/m group ($t=4.653$, $P=0.0096$) (**Figure 2B**). And following the PTN intervention, p-AKT/AKT was restored ($t=3.386$, $P=0.0276$) (**Figure 2B**).

The AMN and cubilin expressions decreased in the db/db group, compared with the db/m group ($t_{AMN\text{-}IHC}=3.674$, $P_{AMN\text{-}IHC}=0.0213$, $t_{AMN\text{-}WB}=4.458$, $P_{AMN\text{-}WB}=0.011$, $t_{cubilin\text{-}IHC}=8.605$, $P_{cubilin\text{-}IHC}=0.001$, $t_{cubilin\text{-}WB}=3.298$, $P_{cubilin\text{-}WB}=0.030$) (**Figure 2A, 2B**). PTN could partially reverse this change. The cubilin expression in the db/db+PTN group increased compared with the db/db group ($t_{IHC}=3.819$, $P_{IHC}=0.019$, $t_{WB}=3.789$, $P_{WB}=0.019$) (**Figure 2A, 2B**). However, the difference in the AMN expression between the db/db and db/db+PTN groups was not statistically significant ($t_{IHC}=2.39$, $P_{IHC}=0.0752$, $t_{WB}=1.449$, $P_{WB}=0.221$) (**Figure 2A, 2B**).

Immunofluorescence revealed the albumin uptake of renal tubules decreased in the db/db group, compared with the db/m group and PTN could reverse this change (**Figure 2C**).

Insulin treatment induced albumin endocytosis and resulted in upregulation of AMN-cubilin expression in HKC cells

In **Figure 3A**, the fluorometric measurement showed insulin treatment resulted in an increase in albumin endocytosis in HKC cells ($t=5.997$, $P=0.0039$). Western blotting of albumin demonstrated parallel results ($t=5.134$, $P=0.0068$) (**Figure 3B**). Insulin caused the upregulation of p-AKT (s473) ($t=4.461$, $P=0.0111$) (**Figure 3C**). Moreover, insulin improved AMN-cubilin expression in HKC cells (AMN: $t=4.566$, $P=0.0103$; cubilin: $t=3.155$, $P=0.0344$) (**Figure 3C**).

Influence of TNF-α or PTN on cell viability in HKC cells

CCK-8 assay demonstrated no significant difference in the cell viability when the cells were treated with TNF-α at the concentration of no more than 25 ng/ml (**Figure 4A**) or PTN at the concentration of no more than 5 μmol/L (**Figure 4B**), compared with the group without intervention. Thus, we chose 25 ng/ml TNF-α or 5 μmol/L PTN to treat HKC cells.

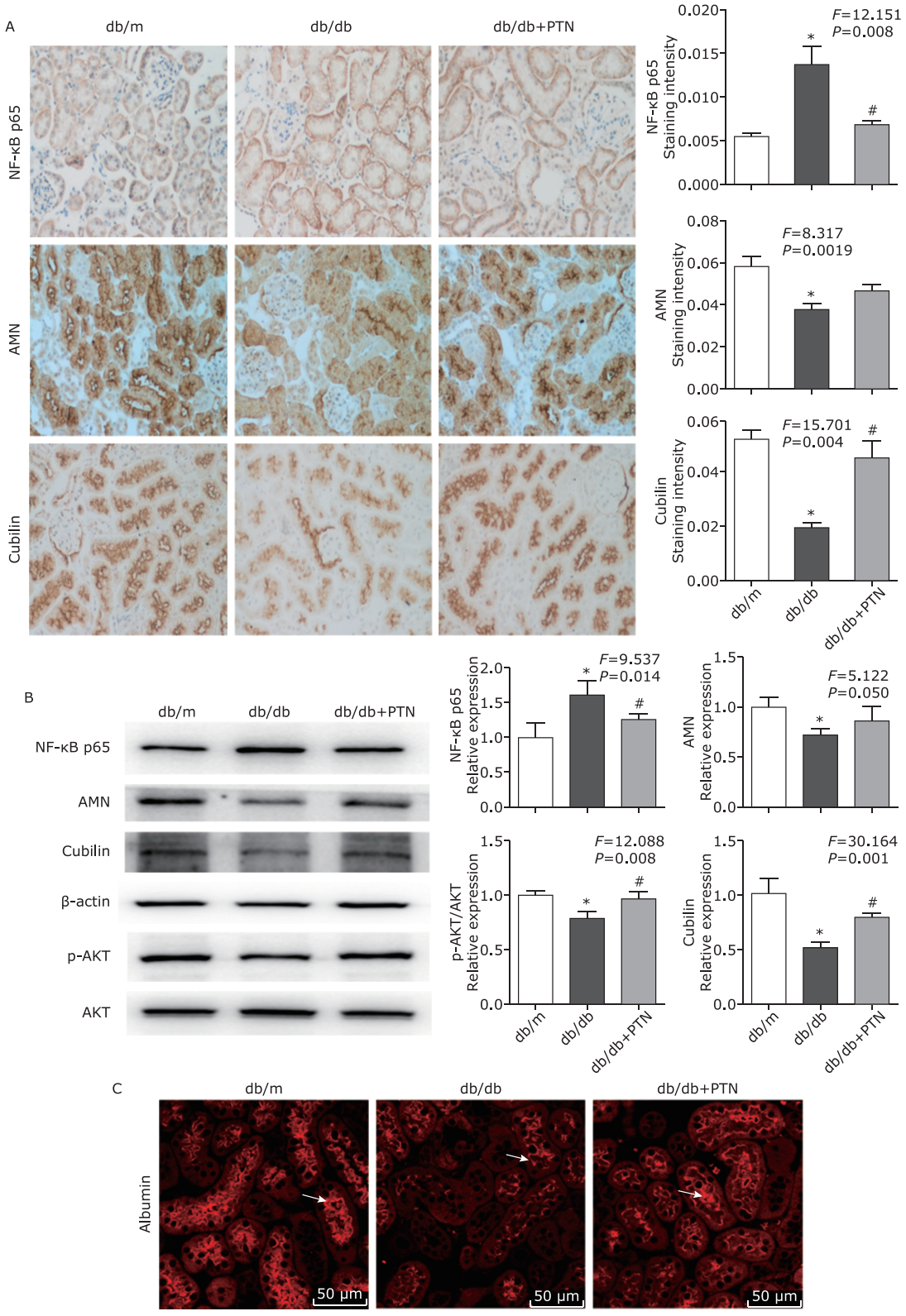

Figure 2. Expressions of NF-κB p65, p-AKT/AKT, AMN and cubilin in mice kidney were detected by immunohistochemstry (A, 200×) and western blots (B). n=3 for each group. Results of immunofluorescence for albumin uptake in renal tubules (C). White arrows indicate uptake of albumin in renal tubules.

NF-κB: nuclear factor kappa-B; AMN: amnionless.

$^{*}P<0.05$ compared with the db/m group; $^{\#}P<0.05$ compared with the db/db group.

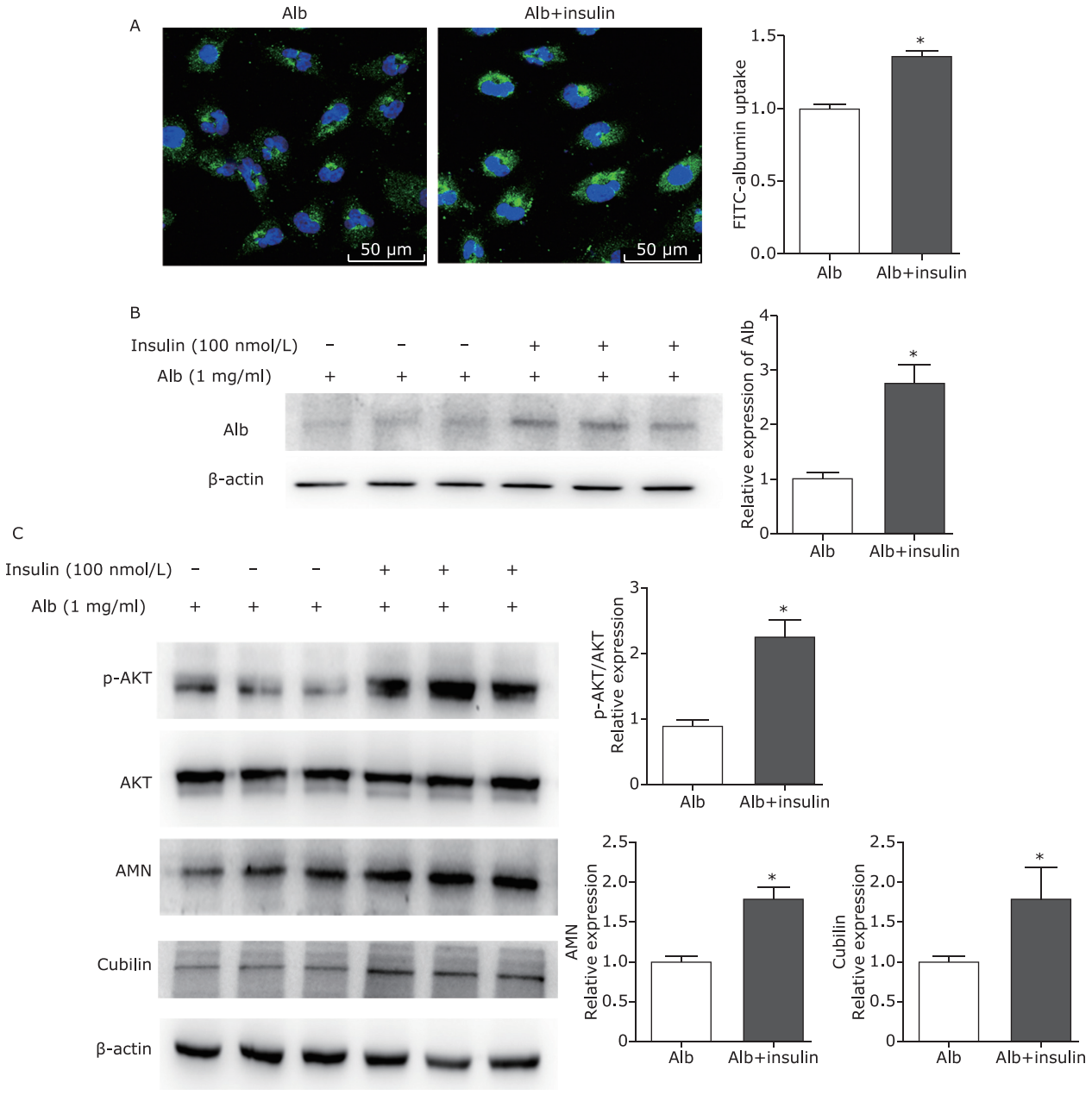

Figure 3. Albumin (Alb) endocytosis and expression of AMN-cubilin in HKC cells with insulin treatment.
Results of fluorometric measurement (A) and western blots (B) for detecting albumin uptake in HKC cells. Western bloting results of p-AKT/AKT, AMN and cubilin expressions in HKC cells (C). $n = 3$ for each group.
$^{*}P < 0.05$ compared with the group without insulin treatment.

PTN could ameliorate the reduction of albumin endocytosis induced by TNF-α

Through fluorometric measurement (FITC) and WB, we found albumin endocytosis decreased in the TNF-α group compared with the insulin group ($P_{\text{FITC-albumin}} = 0.022$, $P_{\text{WB}} = 0.001$), and PTN+TNF-α group could promote albumin endocytosis ($P_{\text{FITC-albumin}} = 0.008$, $P_{\text{WB}} = 0.009$) (**Figure 4C-4E**).

PTN could attenuate the reduction of cubilin expression, reduce expression of NF-κB p65 and restore phosphorylation of AKT and IRS-1 in HKC cells

The AMN-cubilin expression decreased in the TNF-α group, compared with the insulin group ($t_{\text{AMN}} = 3.098$, $P_{\text{AMN}} = 0.0363$, $t_{\text{cubilin}} = 7.099$, $P_{\text{cubilin}} = 0.0021$) and PTN increased the expression of cubilin ($t = 5.69$, $P = 0.0047$)

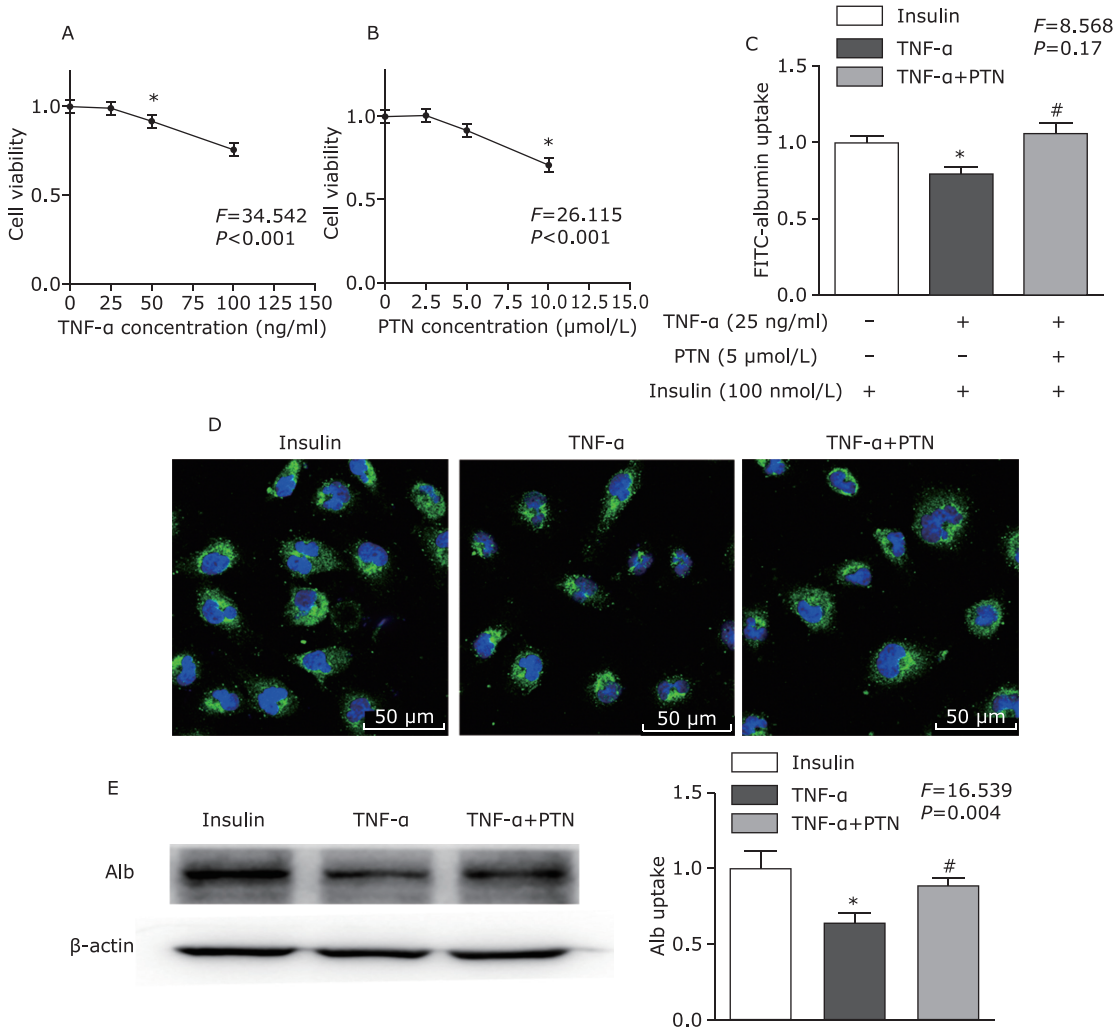

Figure 4. Albumin endocytosis in HKC cells with treatment of TNF-α and PTN.

Viability of HKC cells treated with tumor necrosis factor (TNF-α, A) or parthenolide (PTN, B). Fluorometric measurement (C, D) and western blots (E) of albumin uptake in HKC cells of the insulin, TNF-α and PTN+TNF-α groups. HKC cells in the PTN+TNF-α group were treated with TNF-α and PTN simultaneously for 18 hours, and then treated with insulin for 30 minutes. $n=3$ for each group.

$^{*}P<0.05$ compared with the insulin group; $^{\#}P<0.05$ compared with the TNF-α group.

(**Figure 5**). The difference in AMN expression between the TNF-α and TNF-α+PTN groups was not statistically significant ($t=1.599$, $P=0.185$). TNF-α induced a greater expression of NF-κB p65 than the insulin group ($t=4.59$, $P=0.0101$), and PTN could significantly reduce expression of NF-κB p65 in the TNF-α+PTN group ($t=7.88$, $P=0.0014$) (**Figure 5**). Moreover, the phosphorylation of IRS-1 (s307) increased and phosphorylation of AKT (s473) decreased in the TNF-α group, compared with the insulin group ($t_{\text{IRS-1}}=4.14$, $P_{\text{IRS-1}}=0.0143$, $t_{\text{AKT}}=4.07$, $P_{\text{AKT}}=0.0152$) (**Figure 5**). Following intervention with PTN, p-IRS-1 (s307) and p-AKT (s473) were restored compared with the TNF-α group ($t_{\text{IRS-1}}=3.46$, $P_{\text{IRS-1}}=0.026$, $t_{\text{AKT}}=3.46$, $P_{\text{AKT}}=0.0259$) (**Figure 5**).

DISCUSSION

Albuminuria is closely related to the diagnosis and development of DN. Impaired albumin reabsorption of renal proximal tubules is emphasized in the early stage of DN and a reduction of tubular albumin reabsorption contributes to the development of albuminuria.[2-4] To date, the pathophysiologic mechanism of the reduction in tubular albumin reabsorption in the early stage of DN remains poorly understood. Thus, we aimed to

determine whether inflammation mediated insulin signaling impairment contributed to the development of albuminuria by decreasing albumin reabsorption in the proximal tubules in DN. Our findings suggest that the upregulation of inflammatory factor NF-κB p65, the decreased phosphorylation of AKT (s473), and the reduction of albumin receptors AMN-cubilin could be detected in db/db mouse kidneys and TNF-α stimulating HKC cells. Consistent with our hypothesis, PTN could mitigate inflammation and remodel impaired insulin signaling. Moreover, PTN could promote the expression of cubilin in db/db mice and improve the albumin uptake in renal tubule cells. These results indicate that inflammation impairing insulin signaling may be one of the mechanisms that accounts for the reduction in tubular albumin reabsorption in the early stage of DN.

It is well established that albumin could be transported from primary urine into the blood by transcytosis.[2, 23-25] Albumin retrieval is achieved by a complex network of endocytosis apparatus involving megalin, AMN-cubilin, ORAI, NHE3 and so on.[7, 12] Several studies have reported reduced expression of albumin receptors megalin or cubilin in type 1 diabetes mellitus models[4, 5, 17, 25] or type 2 diabetes mellitus models.[26, 27] These studies may lead researchers to association of the albumin receptor reduction with less albumin reabsorption in DN.[28] In previous research, we well proved that inflammation induced insulin signaling impairment and PTN could reduce renal expression of NF-κB and insulin resistance in db/db mice.[20] So we further studied the possible relationship among inflammation, insulin signaling and albumin uptake in renal tubules based on the previous results in db/db mice. In this study, the data in Table 1 showed that UACR

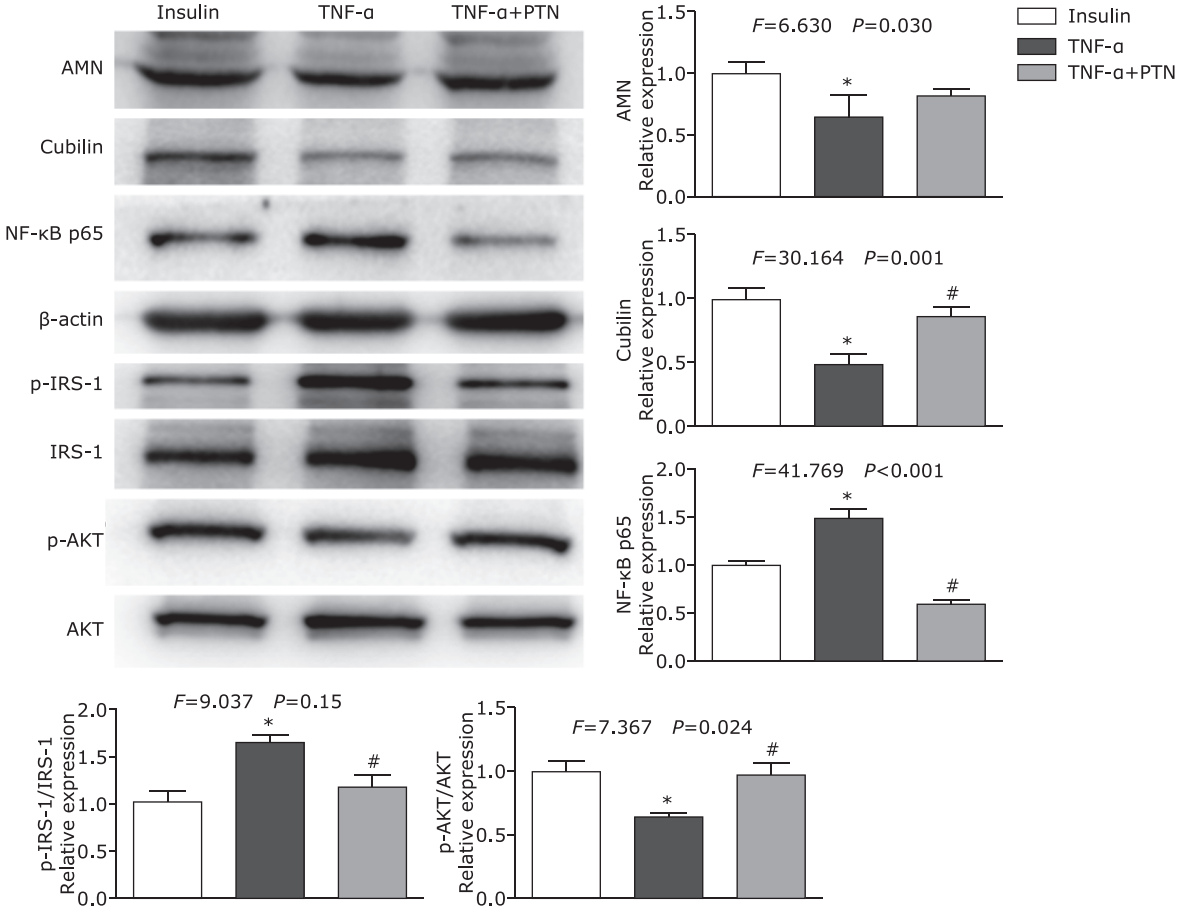

Figure 5. Expression of AMN-cubilin, NF-κB p65, and phosphorylation of AKT and IRS-1 in HKC cells with treatment of TNF-α and PTN detected by Western blot.

HKC cells were treated with TNF-α and PTN simultaneously for 18 hours, and then treated with insulin for 30 minutes. $n = 3$ for each group.

$^{*}P < 0.05$ compared with the insulin group; $^{\#}P < 0.05$ compared with the TNF-α group.

level in db/db mice significantly increased compared with the db/m mice. Moreover, we examined the HOMA-IR and renal p-AKT (s473) and ensured that db/db mice demonstrated obvious insulin resistance. The PAS staining of db/db mice kidney demonstrated hypertrophy in glomerulus and increase of matrix in mesangial areas. Thus, we confirmed the type 2 DN model is successfully established. However, the change of tubules in db/db mice is too slight to be detected by the PAS stain. The db/db mice demonstrated slight reduction of serum creatinine and higher level of UACR. In the initial phase of DN, the glomerulus might be in hyperfiltration with an increase in glomerular filtration rate (GFR).[29] In db/db mice, the creatinine clearance rate may be higher and serum creatinine showed slightly reduced. The increase of albuminuria in db/db mice may be attributed to glomerular hyperfiltration and impaired tubular reabsorption. To determine the condition of tubular albumin uptake, we further examined the expression of albumin endocytic receptors AMN and cubilin. Both AMN and cubilin play important roles in the albumin endocytosis of proximal tubules.[16] Many conditions that cause tubuar injury and albuminuria are related to a reduced expression of cubilin.[24] In this study, db/db mice exhibited lower expression of AMN-cubilin than db/m mice. These findings indicated that a reduction of albumin receptors may be associated with albuminuria in early DN.

We may subsequently consider the question regarding why the expression of albumin receptors decreased. We found many factors would affect the expression of albumin receptors, including insulin, the RAS system, and substrate albumin. Studies have indicated that insulin increases the expression of megalin[6] and ORAI[7], and further promotes the albumin uptake in proximal tubular cells.[5] Some researchers have found that deletion of insulin receptor in the proximal tubule augmented albumin excretion.[30] Insulin signaling and the albumin endocytosis are closely linked through AKT. AKT mediates the albumin uptake through its interaction with the endocytic apparatus proteins.[5, 21, 31] In HKC cells, we found that insulin could activate AKT phosphorylation at the serine 473 residue and increase the albumin endocytosis, which was in accordance with Coffey *et al*.'s results.[5] We subsequently confirmed that insulin could stimulate the expression of AMN and cubilin. These results showed that insulin could phosphorylate AKT and increase albumin receptors expression to promote albumin endocytosis in tubular cells. Thus, in diabetes mellitus, insulin resistance or a lack of insulin could disturb the expression of albumin receptors and the subsequent reabsorption of albumin in renal tubules.

It is well established that metabolic inflammatory processes could induce insulin resistance through interfering with insulin signaling in type 2 diabetes mellitus.[32] The excess of proinflammatory cytokines could disturb the physiologic action of insulin by phosphorylating IRS-1 at the serine 307 residue.[32] Researchers found inflammation could directly induce insulin resistance through activating NF-κB signaling pathway.[33] Type 2 diabetes mellitus is featured by chronic inflammation and insulin resistance. A high NF-κB p65 expression has been observed in DN.[34] Our study demonstrated that NF-κB p65 increased in db/db mice, which indicates a clear inflammatory response in DN. We further treated HKC cells with TNF-α, which has been shown to induce inflammation and insulin resistance,[22, 35, 36] and we found NF-κB p65 expression increased. We subsequently detected that inflammation impaired insulin signaling, presented as the phosphorylation of IRS-1 (s307) increasing and phosphorylation of AKT (s473) decreasing, which was in agreement with previous studies.[35, 36] We expected to attenuate inflammation and improve insulin resistance through inhibiting the NF-κB activity. Our previous results showed that PTN could attenuate renal inflammation and ameliorate insulin signaling impairment in db/db mice.[20] In this study, we also found PTN played the same role as they did in the research we had done before.

In the kidney, albumin is filtrated across the glomerulus and reabsorped in renal tubules. The amount of abumin in urine is affected by the glomerular filtration and tubular reabsorption. In db/db mice, we found albuminuria was elevated. With the treatment of PTN, albuminuria in db/db mice was slightly reduced, but the difference was not statistically significant. We speculated that the amount of albumin by glomerular filtration was an interfering factor, the amount of albumin in urine could not directly reflect reabsorption of albumin in renal tubules. We further examined the expression of AMN and cubilin to determine the condition of tubular albumin uptake. We found PTN could significantly increase cubilin expression, compared with db/db group with no PTN treatment. Then we detected the expression of albumin in renal tubules by the immunofluorescence and we found al-

bumin mainly localized at the apical surface of tubular cells. We also found that albumin uptake decreased in db/db mice and PTN could improve the reduction of albumin uptake. So we considered that PTN may affect the uptake of albumin in renal tubules. There are different results about the albumin uptake in renal tubules of DN.[2, 25, 37, 38] We guessed that the amount of albumin filtered by glomerulus may affect the albumin uptake in renal tubules. To determine the condition of albumin reabsorption, we further investigated the condition of albumin encytosis in HKC cells. We found that TNF-α could decrease the AMN-cubilin expression and reduce the albumin endocytosis. In the TNF-α+PTN group, PTN could attenuate the reduction of cubilin expression and promote albumin endocytosis. The results in HKC cells further confirmed the hypothesis drawn in animal experiments.

Taken together, the study indicates a novel mechanism to illustrate the genesis of tubular albuminuria in the early stage of type 2 DN. Inflammation caused damage to the insulin signaling pathway in type 2 DN, which reduced the expression of the albumin receptor complex and the albumin reabsorption. PTN could reduce inflammation, remodel the impaired insulin signaling pathway, and improve expression of cubilin and albumin uptake. This study may provide a novel clue for the reduction of albuminuria in type 2 DN.

Conflict of Interests Statement

The authors have no conflict of interests to disclose.

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