

## Original Research Article

# Annexin A1 mimetic peptide Ac2-26 alleviates renal inflammatory injury in a diabetic mice model through the suppression of p38MAPK/NF- $\kappa$ B

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### Abstract

**Purpose:** To examine the protective effect of mimetic peptide Ac2-26 of Annexin A1 (ANXA1) against renal inflammatory injury in a diabetic mice model.

**Methods:** Twenty-four mice were randomized into three groups with eight mice per group. These included control group (CG), model group (MG) given intraperitoneal injection of streptozotocin (60 mg/kg), and AC2-26 group (AG) given AC2-26, 72 h after the induction of diabetes. Fasting blood glucose (FBG), blood lipids, and renal function in the mice were determined by Sysmex-180 Biochemistry Analyzer, while serum inflammatory factors in renal tissue were also determined.

**Results:** Compared with the model group, there was a significant decrease in the levels of FBG, triglyceride (TG), total cholesterol (TC), low-density lipoprotein (LDL), high-density lipoprotein (HDL), oxidized LDL (ox-LDL), as well as in 24 h urinary protein, creatinine (Cr) and blood urea nitrogen (BUN), but a significant increase in body weight in AC2-26 group ( $p < 0.001$ ). There was a significant decrease in TNF- $\alpha$ , IL-6, IL-1 $\beta$ , and IL-18 levels, as well as in mRNA levels and protein expressions of p38MAPK, NF- $\kappa$ B, ANXA1 in AC2-26 group when compared with the model group ( $p < 0.001$ ).

**Conclusion:** Annexin A1 is the target gene of p38MAPK. Annexin A1 mimetic peptide Ac2-26 alleviates renal inflammation by suppressing p38MAPK/NF- $\kappa$ B pathway, thus improving renal function in the diabetic mice model. Hence, the findings of this study provide a potential avenue for the development of an effective treatment for renal disease in diabetics.

**Keywords:** Diabetic mice model, Renal inflammatory injury, Annexin A1, p38 Mitogen-activated protein kinases, Nuclear factor-kappa B

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## INTRODUCTION

Diabetic nephropathy (DN) is a chronic inflammatory disease resulting from the activation of natural immune responses induced by glucose and lipid metabolism disorders [1]. The inflammatory factors are highly expressed in

the serum of DN patients, positively correlating with the degree of proteinuria, and directly contributing to renal damage DN [2]. Therefore, controlling the inflammatory response might be crucial in the treatment of childhood DN.

Annexin A1 (ANXA1) is a natural endogenous

inflammation-regulatory protein with core and N-terminal domains [3]. Specifically binding to the relevant receptor, the N-terminal domain inhibits body tissue inflammation and exerts a protective effect on body tissues. Studies have shown that ANXA1 can control inflammation in rat pneumonia, as well as inhibit the expression of phospholipase A2 (PLA2) from the formation of inflammatory complexes. This results in the inhibition of inflammatory cytokine secretion such as TNF- $\alpha$ , and IL-1 $\beta$ , and alleviates inflammatory responses [4]. However, the effect of ANXA1 on renal inflammation in a diabetic mice model has not been reported yet.

After activation by various inflammatory stimuli, p38 mitogen-activated protein kinase (p38MAPK) transmits the extracellular stimulus signals to the cell, promotes the secretion of inflammatory factors, and induces inflammatory injury on tissues and cells [5]. The nuclear factor-kappa B (NF- $\kappa$ B) p65 is a nuclear transcription factor which phosphorylation can also promote the transcription of inflammatory factors, with p38MAPK as the upstream kinase. Activating p38MAPK stimulates and induces NF- $\kappa$ B p65 phosphorylation, and promotes an inflammatory response process [6]. The aim of this study was to explore the protective role of mimetic peptide Ac2-26 of ANXA1 in renal inflammation and its impact on the p38MAPK/NF- $\kappa$ B signaling pathway, as a possibly new therapeutic approach to DN.

## EXPERIMENTAL

### Model preparation and grouping

Twenty-four male SPF BALB/c mice (6-week-old, 18 – 22 g) were supplied by Animal Experiment Center, West China Hospital, Sichuan University. They were raised in a clean environment at room temperature ( $25 \pm 2$  °C), with a light-dark cycle for 12 h, commercial feed and water ad libitum. The Ethics Committee of Chongqing Public Health Medical Center approved all experimental procedures (approval no. 20220519013). Each experimental procedure was implemented in line with the Guide for the Care and Use of Laboratory Animals [7].

Twenty-four mice were randomized into three groups, with eight mice per group. The mice in the three groups were free to eat and drink. The control group (CG) was not given any other intervention. The model group (MG) received intraperitoneal injection of streptozotocin (60 mg/kg) (Sigma-Aldrich), in order to establish the Type I diabetic model. Fasting blood glucose (FBG) and urine glucose were determined 72 h

after streptozotocin administration. If blood glucose was above 16.7 mmol/L with urine glucose (+++), the model was considered successful [8]. During modeling, 2 mL of normal saline injection was given through the tail vein seven times, once every three days. In the meantime, the AC2-26 group (AG) was modeled by injecting AC2-26 1 mg/kg of 2 mL through the tail vein seven times, once every three days, same with the other groups. The FBG and body weight were measured weekly.

### Assessment of body weight, fasting blood glucose, and lipid metabolism

The collection of urine and determination of body weight were done before the end of the experiment. Blood samples were taken from the heart, and FBG, triglyceride (TG), total cholesterol (TC), low-density lipoprotein (LDL), high-density lipoprotein (HDL), and oxidized LDL (ox-LDL) were determined by Sysmex-180 Biochemistry Analyzer (Sysmex, Japan).

### Evaluation of renal function

The kidney was weighed with the kidney weight index (kidney weight/body weight, KI) calculator. The urine was collected after 24 h, and centrifuged for 15 min (3000 rpm, 4 °C) to obtain the supernatant. Blood (5 mL) was collected from the abdominal aorta, left to stand for 30 min at room temperature, and subsequently centrifuged at 3000 rpm for 15 min. Up test kit (Beijing Leadman Biochemistry Co. Ltd., Beijing, China) was used to determine the 24 h urinary protein, then immunoturbidimetric assay was implemented to identify creatinine (Cr), while Sysmex-180 biochemistry analyzer (SYSMEX, Kobe, Japan) was used to measure blood urea nitrogen (BUN).

### Hematoxylin and Eosin (H & E) and PAS staining

Kidney tissue was fixed with 4 % paraformaldehyde for two days, then dehydrated with gradient ethanol, embedded into paraffin, and sectioned into 5  $\mu$ m slices. The tissue was dewaxed, put into water, and then dried to remove extra water.

### Hematoxylin-Eosin (H&E) stain

The sample was put in hematoxylin and 1 % ammonia to stain for 3 min, and then rinsed with water. Afterwards, it was stained with eosin for 2 min, dehydrated with gradient ethanol, cleared with xylene, and sealed with mount.

### Periodic Acid-Schiff (PAS) stain

The sample was stained with Schiff reagent for 10 min and rinsed in running water for 5 min. The sample was stained with Myaer hematoxylin, then rinsed, blued, washed, dehydrated with gradient ethanol, cleared with xylene, and then sealed with mount. The samples were observed under an optical microscope.

### ELISA assay

Blood samples were collected for centrifugation (3000 rpm, 10 min) after removing eyeballs, with serum stored at -80 °C. Fresh kidney tissue (50 mg) was homogenized on ice using a tissue homogenizer, and the ratio of homogenization was chosen to be 10 %, equivalent to 1 g of tissue and 9 mL of homogenization solution, and PBS was selected as the homogenization solution (pH = 7.2 - 7.4, concentration of 0.01 mol/L) to centrifuge at 5000 rpm for 15 min and extract the supernatant. With the serum or renal tissue supernatant sample collected, ELISA kits (R & D Systems, Minneapolis, USA) were applied to measure ox-LDL, TNF- $\alpha$ , IL-6, IL-1 $\beta$ , and IL-18. In details, 0.1 mL diluted test sample was added to the coated reaction wells separately, and the sample was kept standing at 37 °C for one hour. Then the sample was added with the HRP-conjugated antibody after washing, and allowed to stand for another hour, washed again, added with the substrate, and set to stand for 30 min at room temperature. Afterwards, the reaction was terminated by adding stopping solution. The absorbance of samples was recorded at 450 nm with a micro-plate reader (Bio-Tek ELX800, Bio-Tek, Biotek Winooski, VT, USA). The absorbance values of the control tube and test tube were measured separately, and the ratio was calculated to obtain the sample content of the test antigen.

### Determination of mRNA expression

TRIzol reagent was used for total RNA extraction from frozen renal tissues, which was quantified using ultraviolet spectrophotometry, and reverse transcribed through a reverse transcription kit (Invitrogen, Carlsbad, USA). Reaction condition: 37 °C for 15 min, and 85 °C for 5 sec. After reverse transcription, GenBank showed the

required sequence, and the primers were designed by Premier 5.0 software. The synthesis and validation of the primers were conducted by Shanghai Boya Biotechnology Co. Ltd. The same quantity of RNA was taken from each sample in a qRT-PCR device, and  $\beta$ -actin served as internal reference for amplification. The primer sequence is shown in Table 1.

The PCR reaction mixture consisted of SYBR 10  $\mu$ L, 2  $\mu$ L of each primer (5  $\mu$ mol/L), cDNA 2  $\mu$ L, and PCR was undertaken at 95 °C for 5 min, then at 95 °C for 5 sec and at 60 °C for 30 sec for 45 cycles, and the temperature increased at the rate of 1 °C/sec from 65 to 90 °C. The calculation of target gene expression was performed using the formula  $2^{-\Delta\Delta Ct}$ .

### Determination of protein expression

Renal tissue (100 mg) was added with 0.25 mL radioimmunoprecipitation assay (RIPA) lysate (Beyotime Biotechnology Co. Ltd, Shanghai, China) for homogenization, followed by centrifugation (12000  $\times$  g, 2 min, 4 °C) (Centrifuge 5702, Eppendorf, Hamburg, Germany), with the supernatant retained. The BCA test kit (Beyotime Biotechnology Co. Ltd) was applied for protein concentration analysis. The protein (30 mg) was boiled for 5 min and deformed. Separation gel (10 %) and concentrated gel (5 %) were prepared, and the sample was added with a measurement of 50  $\mu$ g/well. SDS-PAGE was performed to separate proteins, which were then electrotransferred onto a nitrocellulose membrane for 2 h, and then blocked with buffer containing 5 % skimmed milk powder for a further two hours.

The NC membrane received rabbit anti-rat p38MAPK, p-p38MAPK, NF- $\kappa$ B p65, p-NF- $\kappa$ B p65 antibodies (1:1000, Santa Cruz Biotechnology, Santa Cruz, USA). The membrane was stored overnight at 4 °C, rinsed and incubated at 37 °C with horse radish peroxidase (HRP) conjugated secondary antibody (1:20000, Beyotime Biotechnology Co. Ltd.) for an hour. After washing the membrane, protein bands were measured using electrochemiluminescence (ECL) (Beyotime Biotechnology Co., Ltd.).

**Table 1:** Primer sequence

Gene	Forward (5'-3')	Reverse (5'-3')
p38MAPK	GCGTGaATGATGGACTGaAA	CCCGaACGATACCAGaACC
NF- $\kappa$ B p65	cacTGTCACcTGGaagcaga	GacctGGagCAAGCCattag
ANXA1	TCGCAATGaagGACGATag	ATATCCTCTTACAGTC
$\beta$ -actin	CGTTGACATCCGTAAAGACC	AACagtCCGCCTAGaAgcac

Image1.43 software was used for semi-quantitative analysis, and  $\beta$ -actin was regarded as internal reference to calculate the relative protein expression.

### Dual-luciferase reporter gene assay

Wild type p38MAPK WT 3' non-coding region (UTR) and mutant type p38MAPK MUT 3' UTR were constructed and inserted into the detection plasmids. ANXA1 mimic, NC mimic, and plasmids containing p38MAPK WT 3'UTR and p38MAPK MUT 3'UTR were transfected into human kidney epithelial cell line 293T cells (Shanghai Yiyuan Biotechnology Co., Ltd., Shanghai, China) for 48-h incubation. Dual-luciferase assay kit was used to analyze the fluorescence intensity of the cells, as well as to calculate the relative luciferase activity.

### Statistical analysis

Statistical Package for the Social Sciences (SPSS, version 19.0) was utilized for statistical analysis. Measurement data were presented as mean  $\pm$  standard deviation (SD), while ANOVA was used for comparing multiple groups, and t-test for comparing two groups with normal distribution. When  $p < 0.05$ , the difference was deemed statistically significant.

## RESULTS

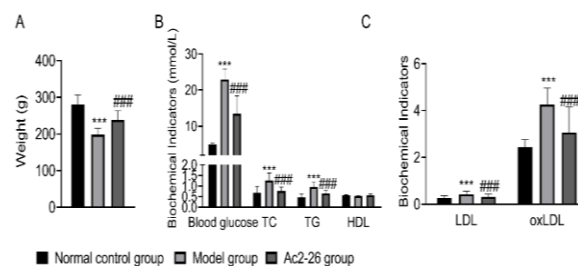
### AC2-26 relieved hyperglycemia and lipid metabolism disorder

No mouse in each group died during the experiment. The CG, FBG, TG, TC, LDL, and ox-LDL increased markedly, while the bodyweight reduced significantly ( $p < 0.001$ ) in the MG. There was a significant decrease in the FBG, TG, TC, LDL, and ox-LDL levels, and a significant increase in body weight ( $p < 0.001$ ) in AG compared with MG. None of the groups exhibited a significant difference in HDL level ( $p > 0.05$ ). Based on these findings, AC2-26 effectively improved hyperglycemia and lipid metabolism disorders in mice with diabetes, as shown in Figure 1.

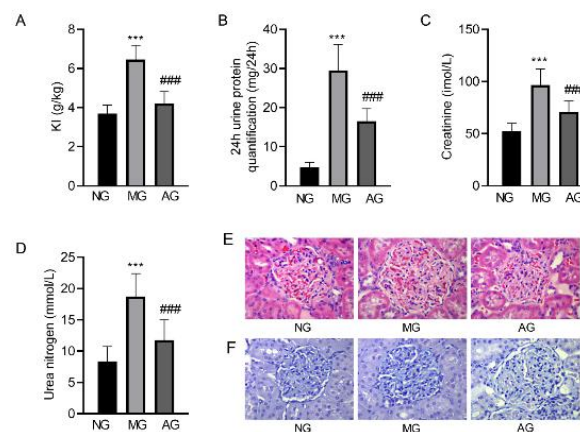
### AC2-26 alleviated kidney tissue damage and improved renal function

As observed from H&E and PAS stain, MG showed balloon adhesion, mesangial matrix expansion, hyperplasia of the basement in glomeruli, vacuolar and granular degeneration of epithelial cell, and capillary luminal stenosis in the renal tubule when compared with CG. The Ac2-26 significantly lessened renal tissue

damage compared with MG. The KI, 24 h urinary protein, Cr, and BUN levels in MG were elevated ( $p < 0.001$ ) in comparison with CG, and the levels were reduced in AG when compared with MG ( $p < 0.001$ ). These results suggested that AC2-26 can substantially reduce kidney tissue damage and improve renal function in diabetic mice, as shown in Figure 2.



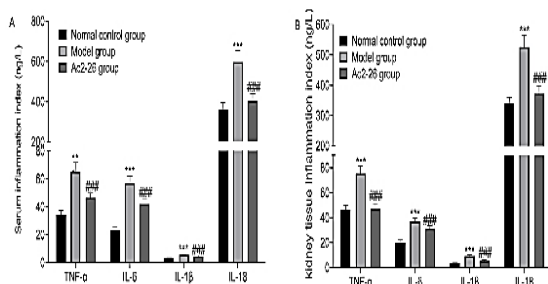
**Figure 1:** Comparison of general status and serological indices of mice in each group. AC2-26 substantially increased (A) body weight and reduce (B) FBG, TC, TG (C) LDL, and ox-LDL levels in diabetic mice, without obvious effect on (B) HDL. \*\*\* $p < 0.001$  versus CG, ### $p < 0.001$  versus MG



**Figure 2:** Comparison of indices of the renal function in each group. The AC2-26 effectively reduced (A) KI, (B) 24 h urinary protein quantification, (C) Cr, and (D) BUN levels in diabetic mice. (E and F) showed that MG exhibited balloon adhesion, mesangial matrix expansion, hyperplasia of the basement in glomeruli, vacuolar and granular degeneration of epithelial cells, and capillary luminal stenosis in renal tubule after H & E and PAS stain when compared with CG. \*\*\* $p < 0.001$  versus CG, ### $p < 0.001$  versus MG

### AC2-26 reduced inflammatory cytokines

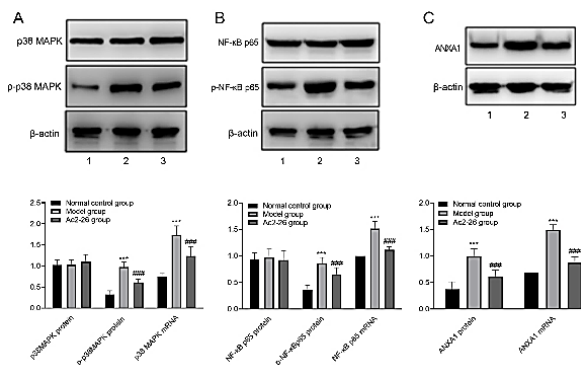
There was a significant elevation in TNF- $\alpha$ , IL-6, IL-1 $\beta$ , and IL-18 contents in serum and renal tissues of MG ( $p < 0.001$ ) in contrast to CG. The AG showed a significant decrease in TNF- $\alpha$ , IL-6, IL-1 $\beta$ , and IL-18 contents ( $p < 0.001$ ) in contrast to MG, indicating that AC2-26 reduced the inflammatory response in serum and renal tissue of diabetic mice (Figure 3).



**Figure 3:** Comparison of inflammatory cytokines in, (A) serum and, (B) kidney tissues. The AC2-26 significantly reduced TNF-α, IL-6, IL-1β, and IL-18 in the serum and renal tissues of diabetic mice. \*\*\**p* < 0.001 versus CG, ###*p* < 0.001 versus MG

**AC2-26 downregulated the expression of p38MAPK/NF-κB p65 pathway-related factors**

There was a significant increase in p38MAPK, NF-κB p65, and ANXA1 mRNA expression levels in the renal tissues of mice in MG (*p* < 0.001) in contrast to CG, and they decreased significantly in AG, in comparison with MG (*p* < 0.001). There was a significant increase in the relative protein expression levels of p-p38MAPK, p-NF-κB p65, and ANXA1 in the renal tissues of mice in the MG than in the CG (*p* < 0.001), and the levels in the AG decreased significantly in contrast to the MG (*p* < 0.001). The result suggested that AC2-26 can inhibit the activation of p38MAPK/ NF-κB p65 pathway in the renal tissues of mice with diabetes, as displayed in Figure 4.

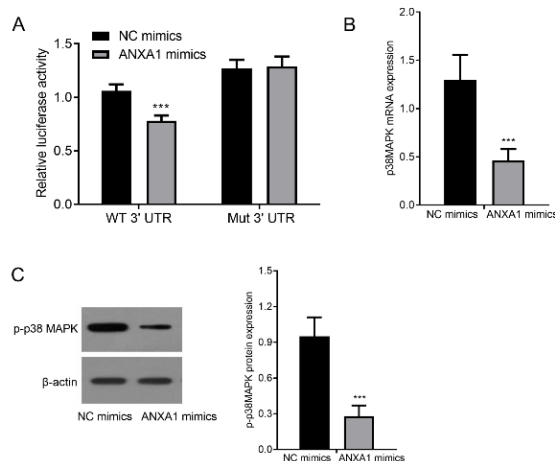


**Figure 4:** Expression of p38MAPK/NF-κB p65 proteins pathway-related factors in each group, (A) AC2-26 suppressed p-p38MAPK expression, (B) AC2-26 suppressed p-NF-κB p65 expression, (C) AC2-26 suppressed ANXA1 expression. Note: \*\*\**p* < 0.001 versus CG, ###*p* < 0.001 versus MG

**p38 MAPK is the target gene of ANXA1**

Compared with NC mimics, ANXA1 mimics had a significant inhibition on the luciferase intensity of p38 MAPK 3' UTR WT (*p* < 0.001) but had no noticeable impact on that of p38MAPK 3' UTR

MUT (*p* > 0.05, Figure 5 A). The mRNA expression of p38MAPK in the ANXA1 mimics decreased in contrast to NC mimics (*p* < 0.001, Figure 5 B). The p-p38MAPK protein expression in the ANXA1 mimics was significantly inhibited in contrast to NC mimics (*p* < 0.001, Figure 5 C). It suggested that ANXA influence p38MAPK expression, as shown in Figure 5.



**Figure 5:** Results of dual luciferase report assay, (A) ANXA1 mimics significantly suppressed the luciferase intensity of p38MAPK 3' UTR WT, (B) ANXA1 mimics significantly suppressed p38MAPK mRNA expression, (C) ANXA1 mimics significantly suppressed p-p38MAPK protein expression. \*\*\**P* < 0.001 when compared with NC mimics

**DISCUSSION**

Diabetic nephropathy is the most common diabetic complication, which, if not actively and properly controlled could rapidly lead to end stage renal failure [9], thereby harming the patient's health. Therefore, actively investigating approaches to prevent or delay DN has become the focus of medical research. It has been pointed out that ANXA1 mimetic peptide AC2-26 can effectively alleviate myocardial injury in diabetic mice [10]. This study reported that ANXA1 might substantially slow down the development of renal injury through regulation of p38MAPK/NF-κB p65 pathway in mice with type I diabetes.

The type-1 DN model induced by streptozotocin administration is a classic model [11], which has been extensively used to screen drugs and explore the mechanisms of human DN. This study demonstrated that streptozotocin-treated diabetic mice had lower body weight, higher FBG, and higher lipometabolic disturbance of TG, TC, LDL, and ox-LDL. In addition, there was an increase in the levels of KI, 24 h urinary protein, Cr, and BUN in the MG in contrast to the

CG, suggesting that the ANXA1 mimetic peptide AC2-26 effectively reduced renal tissue injury and improved renal function in diabetic mice. Wu *et al* [12] showed that AC2-26 has potential to reduce renal injury in db/db mice and diabetic ANXA1 knockout mice. However, this study aims to examine the pathogenesis of diabetic kidney injury. This DN is a significant microvascular complication of diabetes, which occurrence and development of inflammatory response play a vital role. The pro-inflammatory cytokines of TNF- $\alpha$ , IL-6, IL-1 $\beta$ , and IL-18 are released by an inflammatory response, and they participate in the immune responses [13]. The P38MAPK/NF- $\kappa$ B mediated immune-inflammatory signaling can promote an increase in TNF- $\alpha$ , IL-6, IL-1 $\beta$ , and IL-18 production after activation [14], which further escalates diabetic kidney injury.

This study showed that TNF- $\alpha$ , IL-6, IL-1 $\beta$  and IL-18 contents in serum and renal tissue of diabetic mice increased significantly, possibly accounting for the renal inflammation and impaired renal function in diabetic mice. A previous report exhibited a remarkable elevation in the serum levels of IL-6, IL-18, and TNF- $\alpha$  in DN patients at the early-stage, similar to the findings of this study [15]. In contrast, the administration of ANXA1 mimetic peptide AC2-26 decreased the contents of inflammation-related indicators in the renal tissue of the diabetic mice, and the renal function impairment reduced significantly, which indicated that mimetic peptide AC2-26 of ANXA1 reduce renal tissue inflammation in diabetic mice.

Activated p38MAPK stimulates the production and release of various pro-inflammatory factors by activating the relevant signaling pathways, thus inducing inflammatory responses and causing inflammatory tissue damage [16]. The NF- $\kappa$ B is a reverse transcriptional regulator that binds to promoters of pro-inflammatory genes after activation, up-regulates pro-inflammatory gene expression, enhances inflammatory response, and causes inflammatory tissue damage. Inhibiting the transcriptional activity of NF- $\kappa$ B is a critical way to inhibit inflammatory response [17].

Studies have demonstrated that p38MAPK is an upstream kinase of NF- $\kappa$ B, which regulates the NF- $\kappa$ B's phosphorylation of NF- $\kappa$ B, facilitates its nuclear translocation, and stimulates the transcription of inflammatory factors such as IL-6, therefore promoting the occurrence of inflammation. Research on the animal models [18] indicated that inhibiting p38MAPK/NF- $\kappa$ B pathway alleviated the renal injury and inflammatory response in mice with DN induced

by streptozotocin. In this study, gene and protein expression in the p38MAPK/NF- $\kappa$ B p65 pathway was increased in the kidneys of diabetic rat models, resulting in the activation of the p38MAPK/NF- $\kappa$ B pathway. The Ac2-26 significantly decreased the gene and protein expression of the p38MAPK/NF- $\kappa$ B p65 pathway, as well as exerted a protective effect by suppressing its activation, reduced renal inflammation, improved renal function and slowed down pathological changes of the kidney.

### **Limitations of this study**

The sample size of the study is small and should be expanded in further studies. Furthermore, mouse experiments cannot fully mimic the actual conditions of the human body. For the next step, cell assays should be carried out to further validate the inconsistent characteristics of the pathway since the cell signaling pathway is intricate.

### **CONCLUSION**

The mimetic peptide Ac2-26 of ANXA1 alleviates disorders of fasting glucose and lipid metabolism, improves renal function, ameliorates pathological changes of the kidneys, and reduces NF- $\kappa$ B p65, p38MAPK protein and mRNA expression in the diabetic mice model. Furthermore, Ac2-26 also inhibits the p38MAPK protein pathway that leads to renal inflammation in diabetic mice, playing a protective role. While Ac2-26 may be a new therapeutic target for diabetic renal injury, the mechanism by which it regulates the p38MAPK/NF- $\kappa$ B-p65 pathway needs further study.

### **DECLARATIONS**

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None provided.

#### ***Ethical approval***

None provided.

#### ***Availability of data and materials***

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

### Conflict of Interest

No conflict of interest associated with this work.

### Contribution of Authors

The authors declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by them.

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