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HBx-induced PLA_2R overexpression mediates podocyte pyroptosis through the ROS-NLRP3 signaling pathway

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ABSTRACT

Introduction: Hepatitis B virus-associated glomerulonephritis (HBV-GN) is one of the main types of secondary glomerular diseases, and podocyte injury is an important pathogenic mechanism of HBV-GN, participating in the occurrence and development of HBV-GN. However, the specific mechanism of podocyte injury remains to be studied.

Methods: Human renal podocytes cultured *in vitro* were divided into six groups. The podocyte morphology was observed under a transmission electron microscope, and the expression of M-type phospholipase A_2 receptor (M-PLA_2R) on the podocyte membrane was observed by indirect immunofluorescence staining under a fluorescence microscope. The pyroptosis rate and reactive oxygen species (ROS) of podocytes were assessed by FLICA/PI double staining and flow cytometry. Western blot (WB) and quantitative real-time PCR (qPCR) were used to determine the expression of PLA_2R, nucleotide-binding oligomerization domain-like receptor protein 3 (NLRP3), apoptosis-associated speck-like protein containing card (ASC), caspase-1, IL-1β, and IL-18.

Results: Hepatitis B virus X (HBx) transfected into human renal podocytes *in vitro* induced the overexpression of PLA_2R. Moreover, the overexpressed PLA_2R combined with secretory phospholipase A_2 group IB (sPLA_2-IB) aggravated podocyte injury and increased the pyroptosis rate. In addition, the expression of ROS, the NLRP3 inflammasome and downstream inflammatory factors was increased. In contrast, after inhibiting the expression of PLA_2R and ROS, podocyte damage was alleviated, and the pyroptosis rate and the expression of genes related to the ROS-NLRP3 signaling pathway were decreased.

Conclusion: HBx-induced PLA_2R overexpression on the podocyte membrane can significantly upregulate the ROS-NLRP3 signaling pathway, thereby mediating podocyte pyroptosis.

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Introduction

Chronic hepatitis B virus (HBV) infection exists in approximately 250 million people worldwide [1]. The HBV genome has four open reading frames, of which the X region encodes the X protein [2]. Hepatitis B virus-associated glomerulonephritis (HBV-GN) is one of the essential extrahepatic manifestations of hepatitis B virus infection. Among patients with chronic HBV infection in China, it is estimated that approximately 6.8-20% may progress to HBV-GN [3,4]. The pathogenesis of HBV-GN is mainly due to the deposition of HBV circulating immune complexes, direct HBV infection of renal cells, or HBV infection leading to human immune dysfunction [5]. However, the specific pathogenesis of hepatitis B virus-associated membranous nephropathy (HBV-MN) has not yet been elucidated.

M-type phospholipase A_2 receptor (M-PLA_2R) is a member of the C-type lectin superfamily mannose receptor family. Secretory phospholipase A_2 group IB (sPLA_2-IB) acts as a ligand for PLA_2R and can cause a variety of cellular effects [6]. Recent studies have found that M-PLA_2R is present on the podocyte membrane in secondary membranous nephropathy (SMN), especially HBV-MN [7–9]. Furthermore, we previously found that some HBV X (HBx) site mutations in some HBV-MN patients can lead to increased expression of PLA_2R on the surface of podocytes, resulting in kidney damage [10]. Because PLA_2R is not expressed on podocytes in rat or mouse models, we do not fully understand the specific mechanism of HBV-induced kidney injury through PLA_2R.

The NLRP3 inflammasome is comprised of nucleotide-binding oligomerization domain-like receptor

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protein 3 (NLRP3), apoptosis-associated speck-like protein containing card (ASC), and pro-caspase-1. Studies have shown that multiple pathways or signaling pathways can activate the NLRP3 inflammasome, in which the generation of reactive oxygen species (ROS) can mediate the activation of the NLRP3 inflammasome and the expression of downstream inflammatory pathways [11,12]. In addition, the combination of PLA_2R and its autoantibody can cause subepithelial podocyte damage, resulting in changes in the intracellular environment and increased ROS expression. Our previous study demonstrated that HBx could mediate podocyte pyroptosis through the ROS-NLRP3 signaling pathway in HBV-GN [13]. However, how HBx activates the ROS-NLRP3 signaling pathway remains to be explored.

Therefore, this study investigated whether HBx-induced PLA_2R overexpression on the podocyte membrane could mediate podocyte pyroptosis by targeting the ROS-NLRP3 signaling pathway.

Materials and methods

Cell treatments and plasmid transfection

A human renal podocyte cell line (cat# GD-C8618339, IPHASE, Beijing, China) was conditionally immortalized by introducing a temperature-sensitive SV40-T antigen by transfection. These cells proliferate at a permissive temperature. When the podocytes reached approximately 70–80% confluence at 33 °C, the podocytes were transferred to 37 °C for differentiation in medium without insulin, transferrin or selenium (ITS) for approximately seven days. The growth medium for human podocytes consisted of RPMI 1640 medium (HyClone, USA) supplemented with 10% fetal calf serum (Gibco, USA), 13 penicillin–streptomycin, 1 mM L-glutamine and 13 ITS (Invitrogen, Grand Island, NY, USA) at a permissive temperature.

HBx, PLA_2R-siRNA and ROS-siRNA were synthesized by GenePharma (Shanghai, China). For cell transfection, renal podocyte cells were seeded the day before transfection in 500 μl of media per well so that cells would be at a density of 30-50% at the time of transfection. Twelve pmol siRNA was diluted with 50 μl serum-free medium. Two microliters of Rfect (Baidai, Jiangsu, China) was diluted with 50 μl of serum-free medium. These were each mixed gently and incubated at room temperature for 5 min, after which the siRNA dilutions were mixed with the Rfect dilutions (100 μl total vulume) gently and incubated at room temperature for 20 min. The mixture was added to the cultured cells, with 100 μl of the mixture added to the culture wells, which contained 0.5 mL of cultured cells. The plate was gently shaken and mixed, followed by incubation at 37 °C for 48 h for assessment of the effect of gene repression.

The human renal podocyte line was thawed, and subculture expansion was conducted. After the cells were deemd to be in good condition and the number of cells was sufficient, the following groups were set up: (1) NC + $sPLA_2$-IB, (2) empty plasmid + $sPLA_2$-IB, (3) HBx, (4) HBx + $sPLA_2$-IB, (5) HBx + $sPLA_2$-IB + PLA_2R-siRNA, and (6) HBx + $sPLA_2$-IB + ROS-siRNA. A 10^{-5} mol/L concentration of $sPLA_2$-IB (MedChemExpress, NJ, USA) was used for 24 h.

Transmission electron microscopy

Slides with cells from the different experimental groups were prepared. After transfection and drug treatment, the culture medium was discarded, and electron microscope fixative was added to fix the cells at 4 °C for 4 h. The cells were centrifuged at 300 × g for 10 min. They were coated with 1% agarose and washed three times with 0.1 M phosphate buffer for 15 min each, followed by fixation with 1% osmic acid·0.1 M phosphate buffer for 2 h at room temperature (20 °C). The cells were rinsed three times with 0.1 M phosphate buffer for 15 min each and then dehydrated for 15 min each time. The cells were then treated with 1:1 acetone:812 embedding medium for 2–4 h, 1:2 acetone:812 embedding medium infiltration overnight, and pure 812 embedding medium for 5–8 h. After the polymerization was completed, the cells were dissolved in a 40% hydrofluoric acid solution for 15 min and then polymerized again. After cutting into 60-80 nm slices, uranium–lead double staining (2% uranyl acetate saturated alcohol solution and lead citrate, stained for 15 min each) was performed. The slides were dried at room temperature overnight and observed by a Zeiss Sigma electron microscope (Sigma500, Oberkochen, Germany), and the images were collected for analysis.

Immunofluorescence staining

First, a circle was drawn in the middle of the coverslip where the cells were evenly distributed. The cells in the circle were evenly covered with 3% BSA dropwise and sealed at room temperature for 30 min. A 1:100 dilution of PLA_2R primary antibody (Abcam, Cambridge, UK) was added dropwise to the cell well and incubated overnight at 4 °C. The cell plate was washed three times with shaking on a destaining shaker for 5 min. A 1:300 dilution of CY3-conjugated goat anti-rabbit secondary antibody (Abcam, Cambridge, UK) was added dropwise in the circle to cover the tissue and incubated at room

temperature for 50 min. The slides were placed in PBS (pH 7.4) and washed three times with shaking on a destaining shaker, 5 min each time. DAPI (Vector Labs, Peterborough, UK) staining solution was added dropwise to the circle and incubated for 10 min at room temperature in the dark. The slides were washed three times for 5 min each time and then mounted with antifluorescence quenching mounting medium. Sections were observed under a fluorescence microscope (OlympusCKX53, Tokyo, Japan), and images were collected.

Flow cytometry

The culture medium was discarded, and the cells were repeatedly pipetted with 0.01 M PBS. First, 1 mL of PBS was added to resuspend the cells, and then the probes were added at an initial concentration of 10 μM. A portion of cells with only PBS and no probe added was used as a negative control tube. An aliquot of the cell suspension to which the probe had been added was collected, and at the same time, 50 μM reactive oxygen species was added to induce the cells. The cells were incubated at 37 °C for 60 min, and then the single-cell suspension was collected after incubation (probe labeling), centrifuged at 1000 rpm/min for 5 min, removed the supernatant, and washed the cell pellet twice with PBS by centrifugation. The cells were observed under a fluorescence microscope (OlympusCKX53, Tokyo, Japan), and images were collected.

Western blot

First, the total cell protein extract was prepared. The protein concentration was determined using a BCA kit for protein concentration determination (Beyotime Biotechnology, Shanghai, China), after which the protein extract was fully denatured with 5× protein loading buffer. Subsequently, electrophoresis was performed using an SDS–PAGE gel preparation kit (Servicebio, Wuhan, China). The proteins on the separating gel were transferred to a PVDF membrane (Millipore, MA, USA). The membrane was then incubated with 5% skim milk and destained at room temperature for 60 min. The membranes were mixed with primary antibodies against PLA_2R (ABclonal Technology, Boston, MA, USA, 1:1000, Cat. No.: A10068), NLRP3 (Proteintech, Chicago, USA, 1:1000, Cat. No.: 19771-1-AP), ASC (ABclonal Technology, Boston, MA, USA, 1:1000, Cat. No.: sc-22514-R), caspase-1 (Proteintech, Chicago, IL, USA, 1:5000, Cat. No.: 22915-1-AP), IL-1β (ABclonal Technology, Boston, MA, USA, 1:5000, Cat. No.: A1112), IL-18 (ABclonal Technology, Boston, MA, USA, 1:5000, Cat. No.: A1115), or GAPDH (Proteintech, Chicago, IL, USA, 1:50000, Cat. No: 60004-1-lg) and incubated overnight at 4 °C. After three washes with PBST, the membranes were incubated with fluorescently conjugated goat anti-mouse secondary antibody (ABclonal Technology, Boston, MA, USA, 1:10,000) for 1 h. The target bands were analyzed using Photoshop 2021 and ImageJ software processing systems.

RT-PCR

Total RNA was extracted using TRIzol Reagent (Invitrogen, CA, USA). A reverse transcription reaction solution consisting of 2 μL of 5× Evo M-MLV RT Master Mix and RNase-free water was prepared, mixed with an appropriate amount of total RNA, and then reverse-transcribed to obtain cDNA. Quantitative PCR (qPCR) analysis was performed directly using SYBR® Green Premix Pro Taq HS qPCR Kit AG11701 (Accurate Biology, Changsha, China). The data were analyzed using the ΔΔCT method (Table 1).

Flow cytometry pyroptosis assay

Adherent cells were digested with 0.25% trypsin (Boster Biological Technology, CA, USA). Pyroptosis Wash Buffer was diluted 1:10 with double-distilled water. FLICA was diluted with 50 μL of DMSO, and 200 μL of PBS was added to dilute FLICA 1:5. Diluted FLICA was added to each group of cells at a ratio of 1:30 and incubated for 1 h. The cells were washed three times and analyzed by flow cytometry (Agilent, CA, USA).

Data analysis

All experiments were performed at least three times. The data are expressed as the mean ± SD. Prism 9.0 software was used to analyze the data and generate relevant data charts. For comparisons between two groups, an unpaired t test was performed; for comparisons among multiple groups, one-way analysis of variance (ANOVA) was performed. $p < .05$ was considered to indicate statistical significance.

Table 1. Primers used in qRT–PCR.

Gene	Forward primer	Reverse primer
NLRP3	GACCATCCTCGGCTGT	CACGATCCAGCAGACCA
ASC	ATGGACGCCTTGGACCT	CAGCACGTTAGCGGTGAG
caspase-1	TCTGCTCTTCCACACCAGATA	CCACATCACAGGAACAGGCATAT
IL-1β	ATCAGCACCTCTCAAGCAG	AGTCCACATTCAGCACAGG
IL-18	TGGCTGTAACTATCTCTGTGAAGT	TCCTGGGACACTTCTCTGAAAG
PLA2R	AAGAGGGATGGGAGAGACA	GGTTACAAGTGCAGGAGGA
GADPH	AGAAGGCTGGGGCTCATTTG	AGGGGCCATCCACAGTCTTC

Results

HBx induces PLA$_2$R overexpression

To investigate the effect of HBx on PLA$_2$R expression on the podocyte membrane, we used qPCR and Western blotting (WB) to assess the mRNA and protein expression levels of PLA$_2$R. Compared with the NC + sPLA2-IB group, the expression of PLA$_2$R mRNA on the podocyte membrane significantly increased after transfecting the HBx plasmid into cultured human renal podocytes (4.0826 ± 0.1660 vs. 1.0000 ± 0.2130, $p < .01$) (shown in Figure 1(A)). At the same time, we also found that the expression level of PLA$_2$R protein in the HBx group was significantly higher than that in the NC + sPLA$_2$-IB group by WB (0.971688 ± 0.000231 vs. 0.84958 ± 0.001256, $p < .01$) (shown in Figure 1(B)). However, in the PLA$_2$R-knockdown group, the expression of PLA$_2$R mRNA on the human renal podocyte membrane was significantly lower than that in the HBx group (1.0917 ± 0.1900 vs. 4.0826 ± 0.1660, $p < .01$) (shown in Figure 1(A)). We found the same results at the protein expression level (0.8828460 ± 002531 vs. 0.971688 ± 0.000231, $p < .01$) (shown in Figure 1(B)). At the same time, we observed the expression of PLA$_2$R by immunofluorescence staining under an immunofluorescence microscope (shown in Figure 1(C)). The above results indicated that HBx could induce an increase in the expression of PLA$_2$R on the podocyte membrane.

Overexpressed PLA$_2$R aggravates podocyte pyroptosis

To investigate the influence of overexpressed PLA$_2$R on podocyte injury, we used flow cytometry to measure the pyroptosis rate (shown in Figure 2(A)). We found that compared with the NC + sPLA$_2$-IB group, the pyroptosis rate of cultured human renal podocytes was higher in the HBx + sPLA$_2$-IB group ($p < .01$), while

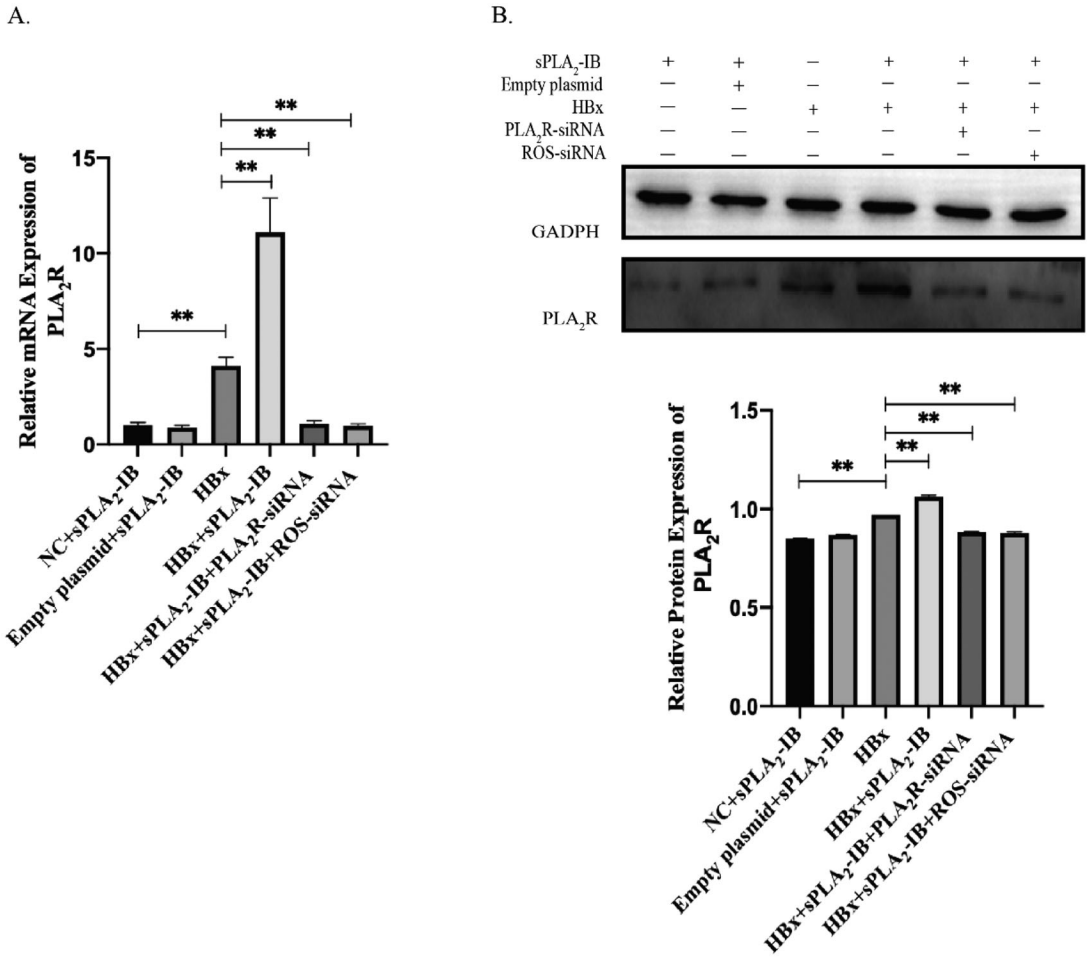

Figure 1. HBx induces PLA$_2$R overexpression. (A) The expression level of PLA$_2$R mRNA in each group was determined by qPCR ($n = 3$). (B) The protein expression level of PLA$_2$R in each group was determined by WB ($n = 3$). (C) The expression of PLA$_2$R was observed by fluorescence microscopy at a magnification of $\times 200$. $^{**}p < .01$. The data are presented as the mean ± SD. NC: negative control; siRNA: small interfering RNA; ROS: reactive oxygen species; mRNA: messenger RNA; sPLA$_2$-IB: secretory phospholipase A$_2$ group IB, a ligand for PLA$_2$R.

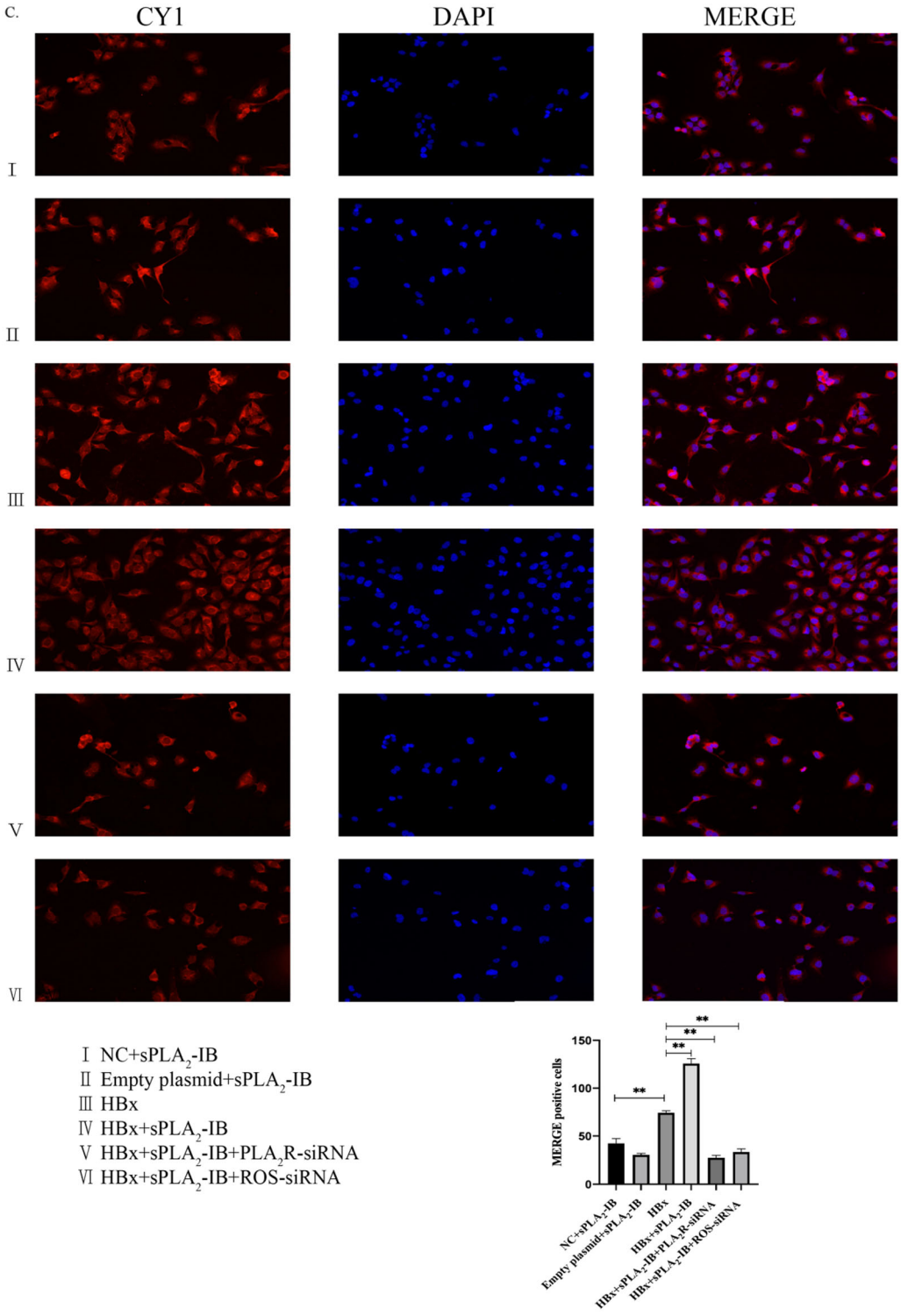

Figure 1. Continued

when PLA_2R was knocked down, the pyroptosis rate decreased ($p < .01$). After transfection of the HBx plasmid *in vitro*, the expression of PLA_2R on the podocyte membrane increased. Its combination with $sPLA_2$-IB aggravated the level of podocyte injury, while inhibiting the expression of PLA_2R on the podocyte membrane could reduce podocyte injury. In addition, using electron microscopy to observe the podocyte morphology in each group, we found that in the podocytes in the HBx + $sPLA_2$-IB group, the number of autophagolysosomes increased, the cell membrane was damaged, and the intracellular matrix was dissolved, indicating that podocyte injury was aggravated. After PLA_2R expression was inhibited, the number of autophagolysosomes decreased, and the damage was lessened (shown in Figure 2(B)). These results indicated that HBx-induced PLA_2R overexpression could exacerbate podocyte pyroptosis.

Overexpressed PLA_2R activates the NLRP3 inflammasome in podocytes

To investigate the role of the NLRP3 inflammasome in podocyte injury induced by PLA_2R overexpression, we used qPCR and WB to measure the mRNA and protein expression levels of the NLRP3 inflammasome and its downstream inflammatory factors. (shown in Figure 3(A,B)). Compared with the NC + $sPLA_2$-IB group, the expression of intracellular inflammasome pathway-related genes such as NLRP3, ASC, caspase-1, IL-1β, and IL-18 in the HBx + $sPLA_2$-IB group was simultaneously upregulated ($p < .01$). However, after the knockdown of PLA_2R, the upregulation of the above substances was inhibited. The above results indicated that the NLRP3 inflammasome and its downstream inflammatory factors were essential in podocyte injury induced by PLA_2R overexpression.

PLA_2R mediates podocyte pyroptosis through the oxidative stress pathway

To further verify the mechanism of overexpressed PLA_2R in podocyte pyroptosis, we measured ROS expression levels by flow cytometry (shown in Figure 4(A)). The average fluorescence intensity of ROS in the HBx + $sPLA_2$-IB group was more substantial than that in the NC + $sPLA_2$-IB group ($p < .01$), and the expression intensity of ROS decreased in the HBx + $sPLA_2$-IB + PLA_2R-siRNA group ($p < .01$). Furthermore, compared with the HBx + $sPLA_2$-IB group, ROS expression intensity showed a downward trend after knocking down ROS ($p < .01$). In addition, we used a FLICA/PI double-stained cell pyroptosis assay and flow cytometry to measure the podocyte pyroptosis rate (shown in Figure 4(B)). After downregulation of ROS, the podocyte pyroptosis rate decreased in the HBx + $sPLA_2$-IB group ($p < .01$). Similarly, after inhibiting the expression of ROS, the number of autophagolysosomes in podocytes was reduced, as observed in electron microscope, and the cell membrane was relatively intact, indicating that the damage was lessened (shown in Figure 4(C)). The above results suggested that HBx-induced PLA_2R overexpression caused podocyte pyroptosis by activating the oxidative stress pathway.

The inflammatory response is reduced after inhibition of ROS generation

To determine the mechanism of ROS in PLA_2R-mediated inflammatory reactions, we used qPCR and WB to analyze the expression levels of NLRP3 inflammasome pathway-related genes (shown in Figure 5(A,B)). Compared with the HBx + $sPLA_2$-IB group, the mRNA and protein expression levels of inflammasome pathway-related genes significantly decreased after inhibiting the expression of ROS ($p < .05$). These results illustrated that the ROS-NLRP3 signaling pathway played an essential role in PLA_2R-mediated cell damage.

Discussion

In the present study, we explored the specific mechanism of podocyte pyroptosis mediated by HBx-induced overexpression of M-type PLA_2R on the podocyte surface, fully confirming the pathological role of overexpressed PLA_2R upon binding to its ligands and demonstrating that the ROS-NLRP3 signaling pathway is closely related to the development of HBV-MN.

Chronic HBV infection is a worldwide public health problem. Studies have shown a close relationship between HBV infection and region [14]. China is a highly endemic area for HBV infection, and there are currently 86 million patients with chronic HBV infection [15]. HBx is an essential transcription factor in HBV replication, directly promoting the replication process and leading to apoptosis [16,17]. HBx also plays a vital role in the pathogenesis and development of liver diseases. In a previous study, Xie et al. [18] found that HBx could promote the pyroptosis of liver cells under oxidative stress through NLRP3, which provides a specific theoretical basis for our research.

HBV-MN is the primary pathological type of HBV-GN [19]. Although Combes et al. [20] first reported HBV-GN

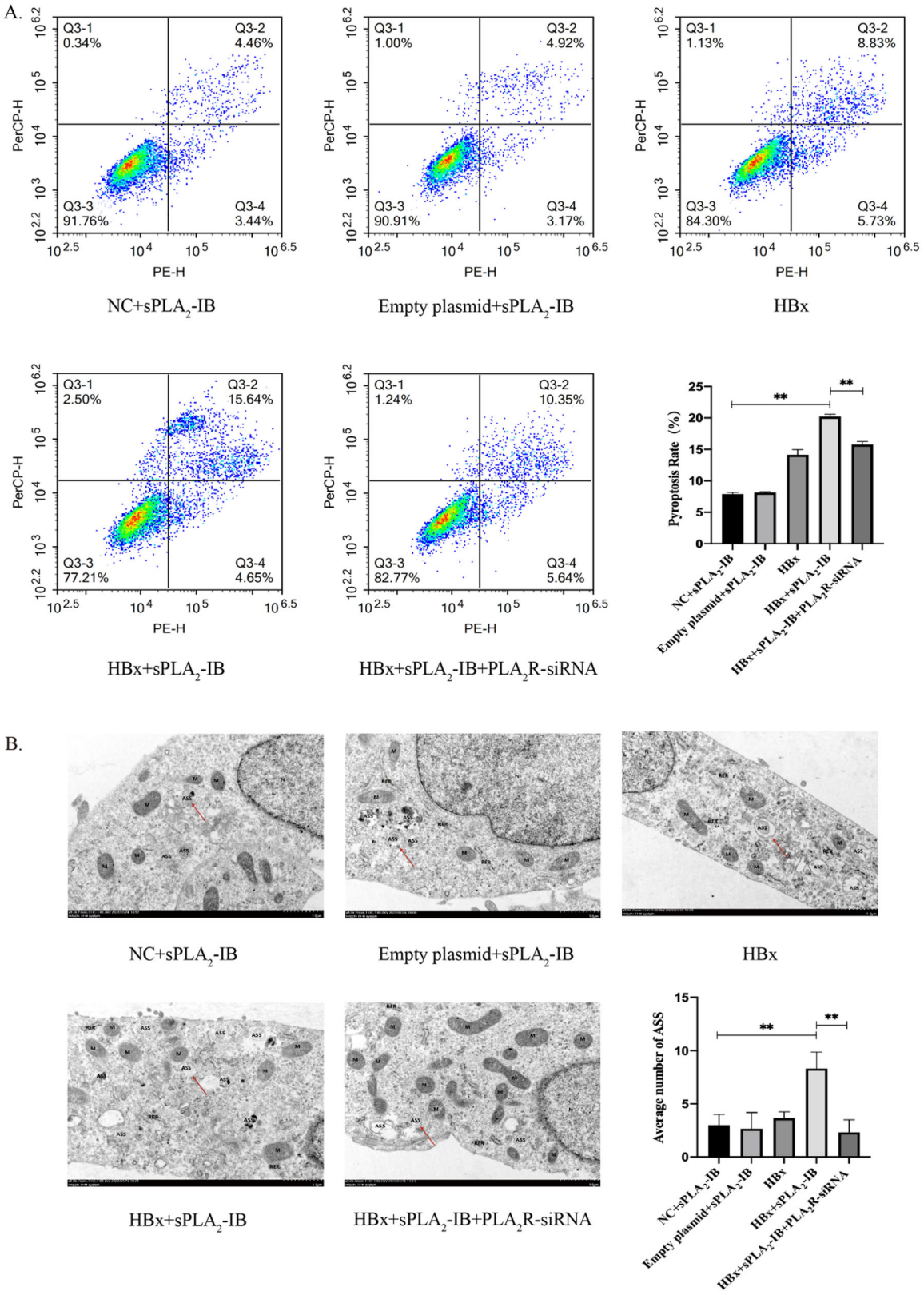

Figure 2. Overexpressed PLA_2R aggravates podocyte pyroptosis. (A) The pyroptosis level of podocytes in different groups was determined by FLICA/PI double staining and flow cytometry ($n = 3$). (B) Changes in podocytes in each group were observed under an electron microscope, and the arrows indicate autophagolysosomes. $^{**}p < .01$. The data are presented as the mean ± SD. NC: negative control; siRNA: small interfering RNA; aas: autophagolysosomes; $sPLA_2$-IB: secretory phospholipase A_2 group IB, a ligand for PLA_2R.

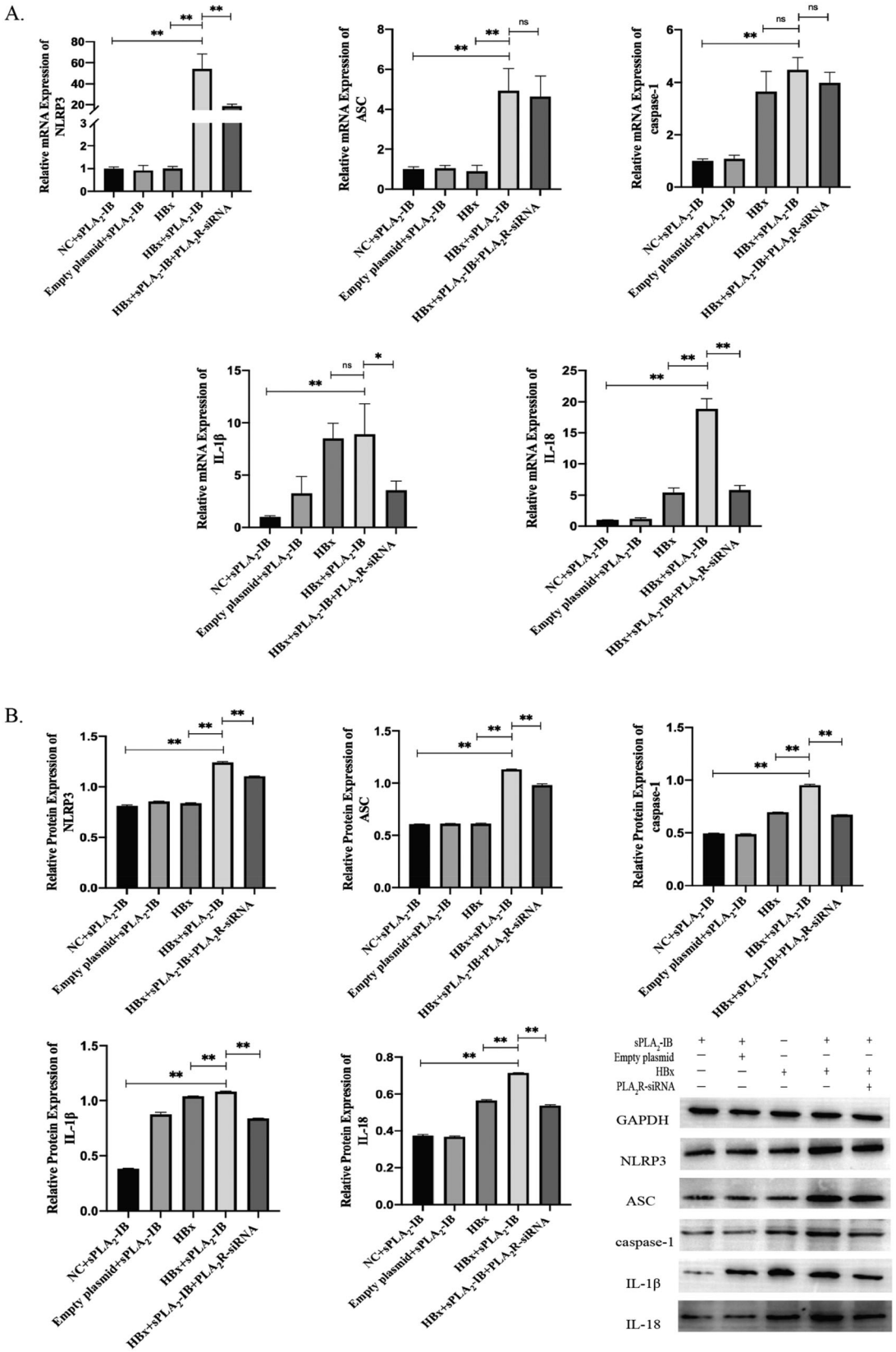

Figure 3. Overexpressed PLA_2R activates the NLRP3 inflammasome in podocytes. (A) The mRNA expression levels of the NLRP3 inflammasome and downstream inflammatory factors in each group were determined by qPCR ($n = 3$). (B) The protein expression levels of the NLRP3 inflammasome and downstream inflammatory factors in each group were determined by WB ($n = 3$). ^{ns}P indicates no significance, $^{*}p < .05$, $^{**}p < .01$. The data are presented as the mean ± SD. NC: negative control; siRNA: small interfering RNA; mRNA: messenger RNA; $sPLA_2$-IB: secretory phospholipase A_2 group IB, a ligand for PLA_2R.

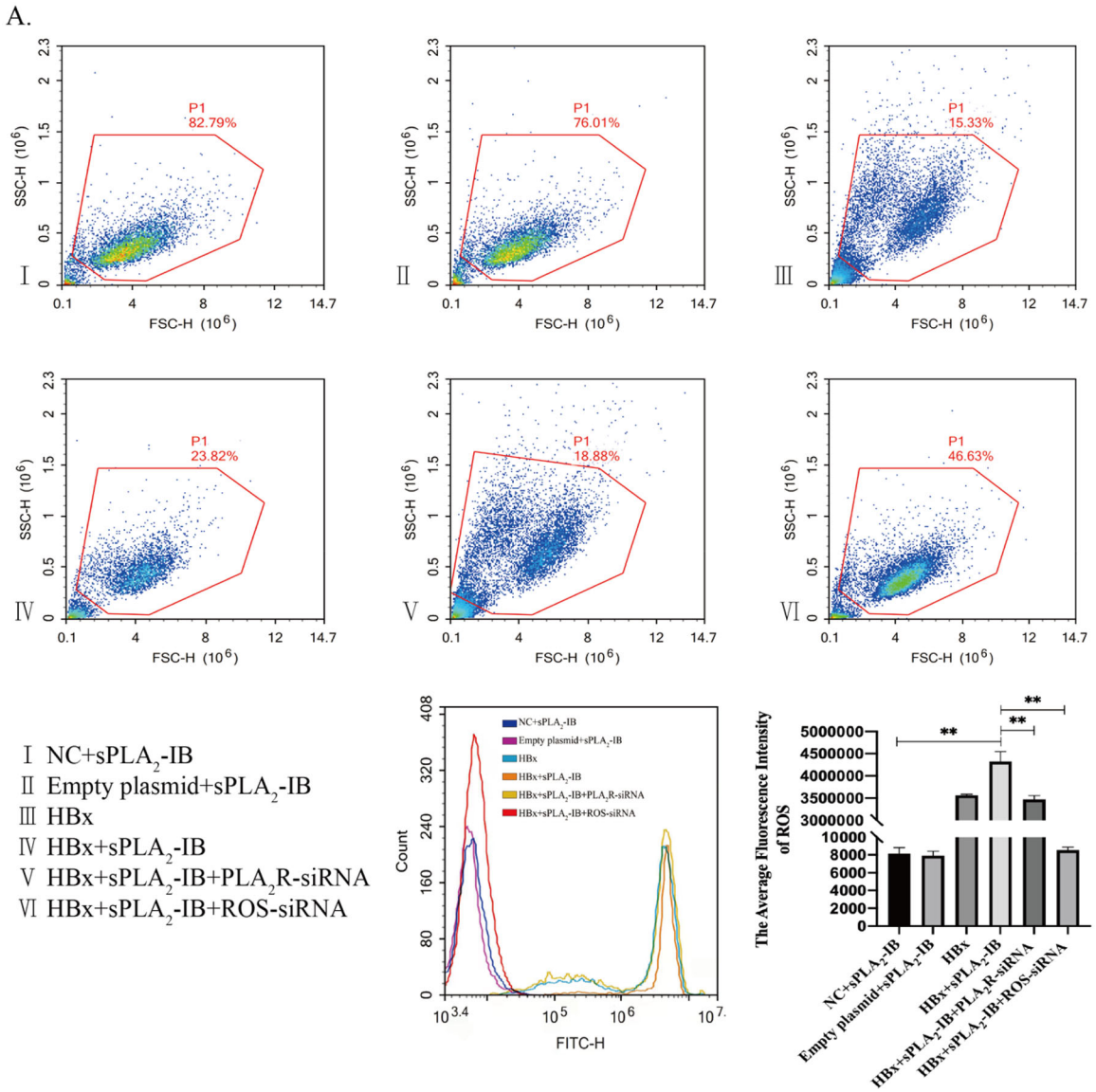

Figure 4. PLA_2R mediates podocyte pyroptosis through the oxidative stress pathway. (A) The average fluorescence intensity of ROS expression in each group was determined by flow cytometry ($n = 3$). (B) The pyroptosis level of podocytes between different groups was determined by FLICA/PI double staining and flow cytometry ($n = 3$). (C) Changes in podocytes in each group were observed under an electron microscope, and the arrows indicate autophagolysosomes. $^{**}p < .01$. The data are presented as the mean ± SD. NC: negative control; siRNA: small interfering RNA; aas: autophagolysosomes; ROS: reactive oxygen species; $sPLA_2$-IB: secretory phospholipase A_2 group IB, a ligand for PLA_2R.

in 1971, there are still few studies on the potential impact of HBx protein on renal cell damage and dysfunction during chronic HBV infection. Podocyte injury is one of the main pathological changes in HBV-GN. Podocytes are the core components of the glomerular filtration barrier. It can secrete laminin β and type IV collagen to form the basement membrane barrier, establishing and maintaining glomerular selective barrier properties [21]. Podocyte damage can lead to proteinuria, which can exacerbate the progression of kidney disease. Our previous study found that HBx could mediate podocyte pyroptosis by regulating the expression of the ROS-NLRP3 signaling pathway. Consistent with this, previous studies have also found that HBx can regulate signal transducer and activator of transcription 3 (STAT3)-related signaling pathways, lysine-specific demethylase 6B (KDM6B), and α3β1 integrin expression-induced podocyte apoptosis [22–24]. In addition to causing renal podocyte injury, HBx can also induce renal tubular epithelial cell damage by regulating the expression of TREM2/NF-κB, NF-κB/DR4, and CD4+ T cells, thereby aggravating the progression of related renal diseases [25–27]. In this study, we used a FLICA/PI double-stained cell pyroptosis assay and

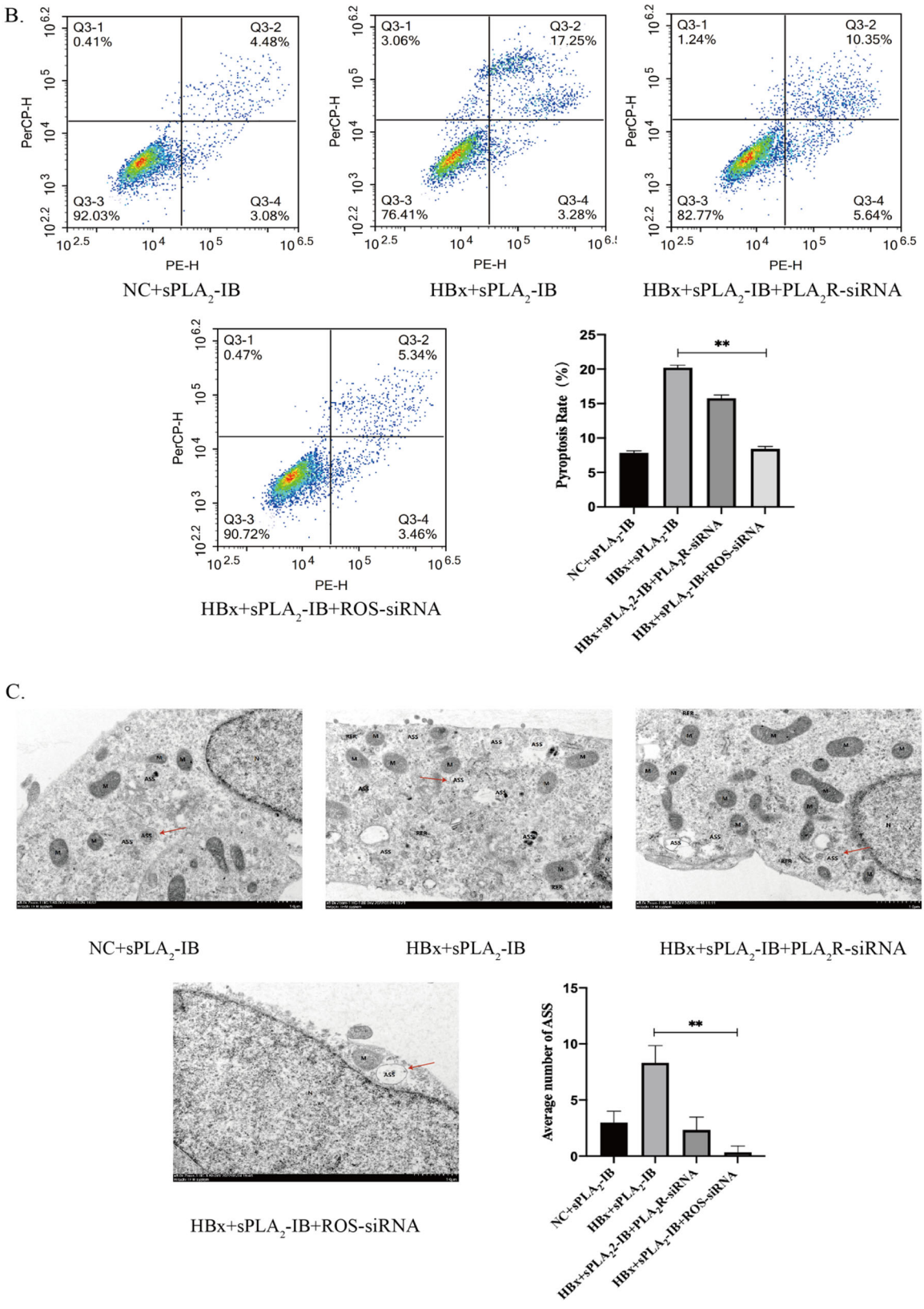

Figure 4. Continued.

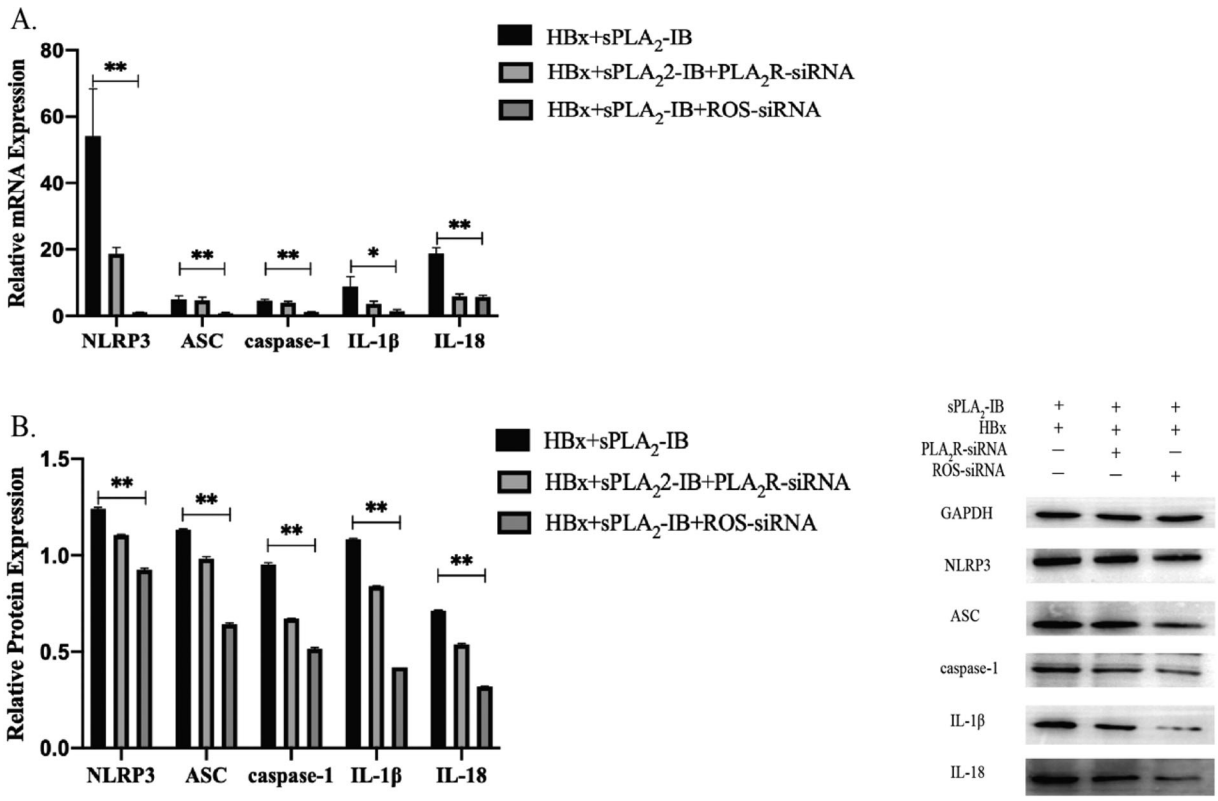

Figure 5. The inflammatory response is reduced after inhibition of ROS generation. (A) The mRNA expression levels of inflammasome pathway-related genes in each group were determined by qPCR ($n = 3$). (B) The protein expression levels of inflammasome pathway-related genes in each group were determined by WB ($n = 3$). $^{*}p < .05$, $^{**}p < .01$. The data are presented as the mean ± SD. siRNA: small interfering RNA; ROS: reactive oxygen species; sPLA$_2$-IB: secretory phospholipase A$_2$ group IB, a ligand for PLA$_2$R.

flow cytometry to analyze the podocyte pyroptosis rate, observed the changes in podocytes under an electron microscope, and found that after HBx-induced PLA$_2$R overexpression, the podocyte pyroptosis rate was more severe than that in the control group. Apparent features of cell damage were observed through electron microscopy. After inhibiting the expression of PLA$_2$R, pyroptosis was alleviated, and podocyte injury was improved. Our study once again demonstrated the impact of HBx expression on renal cell damage and dysfunction and identified for the first time that PLA$_2$R could act as a causative antigen targeting HBV-GN.

M-type PLA$_2$R is expressed on the surface of human renal podocytes and can mediate cellular responses by interacting with sPLA$_2$-IB through its C-type lectin-like domain (CTLD) extracellular pore. Since Beck et al. [6] discovered in 2009 that PLA$_2$R is a specific antigen expressed on the podocyte membrane of idiopathic membranous nephropathy (IMN), it has been widely used in the clinical diagnosis of IMN. In recent years, we found that the mechanisms of PLA$_2$R-induced membranous nephropathy (PLA$_2$R-related MN) pathogenesis may include the immune response, genetic susceptibility, and environmental factors [28–31]. The most crucial pathogenic mechanism is that after the circulating anti-PLA$_2$R antibody in serum binds to podocyte PLA$_2$R, the formation of *in situ* complex immune depositions activates the complement system. It mediates membrane attack complexes, leading to subepithelial podocyte damage. Proteinuria is caused by glomerular basement membrane damage, spike formation, foot process fusion, and apoptosis [32]. In addition to assisting in diagnosing PLA$_2$R-related MN, PLA$_2$R can also be used to evaluate the activity and severity of membranous nephropathy. Studies have found that the degree of proteinuria in MN patients increases with serum anti-PLA$_2$R antibody titers [33,34]. Similarly, we can use the expression of renal PLA$_2$R and serum anti-PLA$_2$R antibody titers to predict the prognosis of MN patients in the clinic [35]. In this study, we used WB, qPCR, and immunofluorescence staining techniques to find that the expression of PLA$_2$R on the surface of human renal podocytes was significantly increased after transfection with HBx. *In vitro* experiments again proved that PLA$_2$R is expressed on the surface of podocytes of IMN and the surface of podocytes of SMN. Thus, HBx can regulate the expression of PLA$_2$R on the surface of human renal podocytes.

Due to the lack of endogenous glomerular PLA_2R expression in rats and mice, an animal model of PLA_2R-dependent membranous nephropathy cannot be established. Therefore, the specific mechanism of PLA_2R-induced podocyte injury cannot be verified in animal experiments. Furthermore, the study of the *in vitro* mechanism of PLA_2R expression in the pathogenesis or progression of kidney disease is also rare. In the existing *in vitro* studies, it has been proved that PLA_2R can mediate podocyte injury by regulating the p38 MAPK/mTOR/ULK1^{ser757} and PI3K/AKT/mTOR signaling pathway after binding to its ligand at an appropriate concentration [36,37]. In this study, we found for the first time that PLA_2R overexpression could mediate podocyte pyroptosis through the ROS-NLRP3 signaling pathway. We used WB, qPCR, and flow cytometry to measure the expression of PLA_2R, ROS, the NLRP3 inflammasome, and downstream inflammatory factors and found that podocyte pyroptosis was aggravated in the HBx + $sPLA_2$-IB group, PLA_2R expression was significantly increased, and the expression of ROS, the NLRP3 inflammasome, and downstream inflammatory factors was also increased significantly compared with the normal group. After inhibiting the expression of PLA_2R, the pyroptosis of podocytes was alleviated, and the expression of related genes was also decreased. It is worth noting that after inhibiting ROS expression, the expression of PLA_2R also showed a significant downward trend, which may be related to the structural changes of PLA_2R or the expression of hidden epitopes caused by oxidative stress (shown in Figure 1(A–C)) [38].

This study focused on how HBx-induced PLA_2R overexpression leads to pyroptosis. As a newly elucidated form of cell death, pyroptosis has been demonstrated in some pathological changes in the kidney. Pyroptosis can be divided into two pathways. The classical pathway is initiated when the elements from the exterior environment stimulate cells, inducing pattern recognition receptors in some cells to promote the transcription and production of inflammatory factors, such as nucleotide-binding oligomerization domain-like receptor protein 3 (NLRP3). NLRP3 can recruit the precursor of caspase-1 through apoptosis-associated speck-like protein containing card (ASC). This triggers the activation of caspase-1 and induces the maturation and release of downstream IL-1β and IL-18, resulting in renal podocyte pyroptosis [39]. In addition to the above pathways, caspase-4/11 and Gasdermin D have been shown to induce podocyte pyroptosis through noncanonical pathways in a mouse model of diabetic nephropathy [40]. In addition to causing podocyte pyroptosis, the ROS-NLRP3 signaling pathway also plays an essential role in other renal pathological changes. In a mouse model of ischemia–reperfusion injury, the ROS/NLRP3 axis can aggravate renal inflammatory injury and promote the transition from acute kidney injury to chronic renal failure [41]. In diabetic nephropathy, high glucose can also induce increased expression of the ROS/NLRP3 axis to mediate autophagy and aggravate the progression of diabetic nephropathy [42]. Similarly, the ROS-NLRP3 signaling pathway in lupus nephritis can also mediate cell damage and aggravate proteinuria [43]. In this study, the expression changes in the ROS-NLRP3 signaling pathway were determined by WB, qPCR, and flow cytometry, and it was proven that HBx induced PLA_2R overexpression to mediate podocyte pyroptosis by activating the ROS-NLRP3 signaling pathway.

Although this study proves that HBx-induced PLA_2R overexpression can mediate podocyte pyroptosis by activating the ROS-NLRP3 signaling pathway, it has certain limitations. For example, the relevant experimental analysis was carried out only in the cultured human renal podocyte line. HBV-GN model mice were not established for verification. Catherine et al. first constructed a transgenic mouse line expressing full-length PLA_2R in podocytes in 2020, which provided technical support for further exploration of the pathogenesis of PLA_2R-related nephropathy in the future [44]. Although there are imperfections in this study, it can still provide some new evidence on the pathogenesis of HBV-GN.

In conclusion, our study demonstrated for the first time that HBx-induced overexpression of M-type PLA_2R on podocyte membrane is closely related to the mechanism of HBV infection-induced renal podocyte injury by activating the ROS-NLRP3 signaling pathway. The PLA_2R and ROS-NLRP3 signaling pathways, as new targets for preventing podocyte pyroptosis, are significant for the treatment and prevention of HBV-GN.

Ethics approval and consent to participate

The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Author contributions

Moxuan Feng and Yani Yu designed the experimental idea and wrote the main manuscript. Yueqi Chen and Xiaoqian Yang performed the experiments and collected the relevant data. Baoshuang Li finished the formal analysis and investigation. Wei Jiang prepared Figures 1–5 and approved the

final version of the manuscript. All authors reviewed the manuscript.

Disclosure statement

No potential conflict of interest was reported by the author(s).

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Data availability statement

All data generated or analyzed during this study are included in this article. Further enquiries can be directed to the corresponding author.

References

[1] Nguyen MH, Wong G, Gane E, et al. Hepatitis B virus: advances in prevention, diagnosis, and therapy. Clin Microbiol Rev. 2020:33(2):19–46.
[2] Cheng ST, Hu JL, Ren JH, et al. Dicoumarol, an NQO1 inhibitor, blocks cccDNA transcription by promoting degradation of HBx. J Hepatol. 2021;74(3):522–534.
[3] Zhang Y, Fang L, Ma X. [Hepatitis B virus infection and pathogenesis of glomerulonephritis]. Zhonghua Bing Li Xue Za Zhi. 1995;24(6):341–344.
[4] Zhao W, Ma Y, Wang M, et al. Expression of Foxp3 in renal tissue of patients with HBV-associated glomerulonephritis and their clinical and pathological characteristics. Exp Ther Med. 2017;14(5):4928–4934.
[5] Ren J, Wang L, Chen Z, et al. Gene expression profile of transgenic mouse kidney reveals pathogenesis of hepatitis B virus associated nephropathy. J Med Virol. 2006;78(5):551–560.
[6] Beck LH Jr, Bonegio RG, Lambeau G, et al. M-type phospholipase A2 receptor as target antigen in idiopathic membranous nephropathy. N Engl J Med. 2009;361(1):11–21.
[7] Xie Q, Li Y, Xue J, et al. Renal phospholipase A2 receptor in hepatitis B virus-associated membranous nephropathy. Am J Nephrol. 2015;41(4-5):345–353.
[8] Xu X, Zhu X, Yuan S[, et al. Role of M-type phospholipase A2 receptor and its antibody in hepatitis B virus-associated membranous nephropathy. Zhong Nan Da Xue Bao Yi Xue Ban. 2016;41(10):1064–1068.
[9] Nikolopoulou A, Teixeira C, Cook HT, et al. Membranous nephropathy associated with viral infection. Clin Kidney J. 2021;14(3):876–883.
[10] Dong H, Xu Y, Xu T, et al. The Role of HBx gene mutations in PLA 2R positive hepatitis-B-associated membranous nephropathy. Biomed Environ Sci. 2020;33(4):269–272.
[11] Zhang MY, Jiang YX, Yang YC, et al. Cigarette smoke extract induces pyroptosis in human bronchial epithelial cells through the ROS/NLRP3/caspase-1 pathway. Life Sci. 2021;269:119090.
[12] Gao Y, Ma Y, Xie D, et al. ManNAc protects against podocyte pyroptosis via inhibiting mitochondrial damage and ROS/NLRP3 signaling pathway in diabetic kidney injury model. Int Immunopharmacol. 2022;107:108711.
[13] Yu Y, Dong H, Sun J, et al. Hepatitis B virus X mediates podocyte pyroptosis by regulating the ROS/NLRP3 signaling pathway in hepatitis B virus-associated glomerulonephritis. Iran J Basic Med Sci. 2022;25(1):103–109.
[14] Liu J, Liang W, Jing W, et al. Countdown to 2030: eliminating hepatitis B disease, China. Bull World Health Organ. 2019;97(3):230–238.
[15] Polaris Observatory C. Global prevalence, treatment, and prevention of hepatitis B virus infection in 2016: a modelling study. Lancet Gastroenterol Hepatol. 2018;3:383–403.
[16] Cui H, Li QL, Chen J, et al. Hepatitis B virus X protein modifies invasion, proliferation and the inflammatory response in an HTR-8/SVneo cell model. Oncol Rep. 2015;34:2090–2098.
[17] Shan H, Wang B, Zhang X, et al. CUL4B facilitates HBV replication by promoting HBx stabilization. Cancer Biol Med. 2021;19(1):120–131.
[18] Xie WH, Ding J, Xie XX, et al. Hepatitis B virus X protein promotes liver cell pyroptosis under oxidative stress through NLRP3 inflammasome activation. Inflamm Res. 2020;69(7):683–696.
[19] Kupin WL. Viral-associated GN: hepatitis B and other viral infections. Clin J Am Soc Nephrol. 2017;12(9):1529–1533.
[20] Combes B, Shorey J, Barrera A, et al. Glomerulonephritis with deposition of Australia antigen-antibody complexes in glomerular basement membrane. Lancet. 1971;2(7718):234–237.
[21] Abrahamson DR, Hudson BG, Stroganova L, et al. Cellular origins of type IV collagen networks in developing glomeruli. J Am Soc Nephrol. 2009;20(7):1471–1479.
[22] Yang YT, Du Y, Yuan WJ, et al. [Role of histone demethylase KDM6B in HBx-mediated podocyte-macrophage transdifferentiation]. Zhonghua Yi Xue Za Zhi. 2021;101(12):866–871.
[23] He P, Liu D, Zhang B, et al. Hepatitis B virus X protein reduces podocyte adhesion via downregulation of alpha3beta1 integrin. Cell Physiol Biochem. 2017;41(2):689–700.
[24] Lei XY, Chen XX, Sun YH, et al. Hepatitis B virus X protein decreases nephrin expression and induces podocyte apoptosis via activating STAT3. Exp Ther Med. 2019;17(5):4223–4229.
[25] Zhang Y, Wu Q, Zhong L, et al. Echinacoside promotes the proliferation of human renal tubular epithelial cells by blocking the HBX/TREM2mediated NFkappaB signalling pathway. Mol Med Rep. 2020;22:1137–1144.
[26] Yang Y, Wang X, Zhang Y, et al. Hepatitis B virus X protein and proinflammatory cytokines synergize to enhance TRAIL-induced apoptosis of renal tubular

cells by upregulation of DR4. Int J Biochem Cell Biol. 2018;97:62–72.
[27] Wang X, Wang L, Zhu N, et al. Hepatitis B virus X protein modulates renal tubular epithelial cell-induced T-cell and macrophage responses. Immunol Cell Biol. 2016;94(3):266–273.
[28] Skoberne A, Behnert A, Teng B, et al. Serum with phospholipase A2 receptor autoantibodies interferes with podocyte adhesion to collagen. Eur J Clin Invest. 2014;44(8):753–765.
[29] Xie J, Liu L, Mladkova N, et al. The genetic architecture of membranous nephropathy and its potential to improve non-invasive diagnosis. Nat Commun. 2020; 11(1):1600.
[30] Xu X, Nie S, Ding H, et al. Environmental pollution and kidney diseases. Nat Rev Nephrol. 2018;14(5):313–324.
[31] Xu X, Wang G, Chen N, et al. Long-Term exposure to air pollution and increased risk of membranous nephropathy in China. J Am Soc Nephrol. 2016;27:3739–3746.
[32] Glassock RJ. The pathogenesis of membranous nephropathy: evolution and revolution. Curr Opin Nephrol Hypertens. 2012;21(3):235–242.
[33] Chi JN, Lai TS, Wu CF, et al. The relationship of anti-phospholipase A2 receptor antibody and C5a complement with disease activity and short-term outcome in idiopathic membranous nephropathy. J Formos Med Assoc. 2019;118(5):898–906.
[34] Li C, Li P, Guo W, et al. The optimal anti-phospholipase A2 receptor cutoff for the diagnosis of idiopathic membranous nephropathy: a single-center retrospective study. Korean J Intern Med. 2021;37(1):154–166.
[35] Guo N, Cao Y, Dai H, et al. Anti-phospholipase A2 receptor (anti-PLA2R) antibody in diagnosis and treatment of idiopathic membranous nephropathy: a single-center observational study in China. Med Sci Monit. 2019;25:9364–9368.
[36] Yang L, Wu Y, Lin S, et al. sPLA2-IB and PLA2R mediate insufficient autophagy and contribute to podocyte injury in idiopathic membranous nephropathy by activation of the p38MAPK/mTOR/ULK1(ser757) signaling pathway. FASEB J. 2020:1–16.
[37] Chiou TT, Chau YY, Chen JB, et al. Rapamycin attenuates PLA2R activation-mediated podocyte apoptosis via the PI3K/AKT/mTOR pathway. Biomed Pharmacother. 2021;144:112349.
[38] Ponticelli C, Glassock RJ. Glomerular diseases: membranous nephropathy–a modern view. Clin J Am Soc Nephrol. 2014;9(3):609–616.
[39] Shi J, Zhao Y, Wang K, et al. Cleavage of GSDMD by inflammatory caspases determines pyroptotic cell death. Nature. 2015;526(7575):660–665.
[40] Cheng Q, Pan J, Zhou ZL, et al. Caspase-11/4 and gasdermin D-mediated pyroptosis contributes to podocyte injury in mouse diabetic nephropathy. Acta Pharmacol Sin. 2021;42(6):954–963.
[41] Zheng T, Tan Y, Qiu J, et al. Alternative polyadenylation trans-factor FIP1 exacerbates UUO/IRI-induced kidney injury and contributes to AKI-CKD transition via ROS-NLRP3 axis. Cell Death Dis. 2021;12(6):512.
[42] Gao C, Chen J, Fan F, et al. RIPK2-Mediated autophagy and negatively regulated ROS-NLRP3 inflammasome signaling in GMCs stimulated with high glucose. Mediators Inflamm. 2019;2019:6207563. 2019:
[43] Fu R, Guo C, Wang S, et al. Podocyte activation of NLRP3 inflammasomes contributes to the development of proteinuria in lupus nephritis. Arthritis Rheumatol. 2017;69:1636–1646.
[44] Meyer-Schwesinger C, Tomas NM, Dehde S, et al. A novel mouse model of phospholipase A2 receptor 1-associated membranous nephropathy mimics podocyte injury in patients. Kidney Int. 2020;97(5):913–919.