

In vitro and *in vivo* study of the expression of the Syk/Ras/c-Fos pathway in chronic glomerulonephritis

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Abstract. Chronic glomerulonephritis (CGN) is the most common form of glomerular disease; however, its associated molecular mechanisms remain unclear. Spleen tyrosine kinase (Syk) is a key mediator of B-receptor signaling on the surface of inflammatory cells. The primary target for R406 is Syk. The aim of the present study was to investigate the molecular mechanisms involved in a rat model of CGN induced by adriamycin (ADR) and in the rat glomerular mesangial cell line, HBZY-1, stimulated by lipopolysaccharide (LPS). CGN was induced in the rat models by two intravenous injections of ADR into the tail: 3.5 mg/kg ADR was given on the first day and 3.0 mg/kg on the fourteenth day. HBZY-1 cells were incubated with 0.5 μ g/ml LPS for 48 h. The pathological alterations in the kidney tissues were observed by hematoxylin and eosin staining. The 24 h urinary protein, blood urea nitrogen (BUN) and creatinine levels were measured using an automatic biochemistry analyzer. The mRNA expression levels of Syk, Ras, mitogen activated protein kinase kinase (MEK), extracellular signal regulated kinase (ERK)1/2 and c-Fos was measured by reverse transcription-quantitative polymerase chain reaction. Subsequently, the protein levels of phosphorylated (p)-Syk, Ras, p-MEK1/2, p-ERK1/2 and c-Fos were measured by western blot analysis. In the model group, 24 h urinary protein, BUN and creatinine levels were increased when compared with the normal group ($P < 0.05$). In addition, compared with the normal group, the mRNA and protein levels of the Syk/Ras/c-Fos pathway components *in vitro* and *in vivo* were markedly increased, inhibiting the abnormal cell viability of mesangial cells. In conclusion, the

results of the present study suggested a potential role for the Syk/Ras/c-Fos signaling pathway in CGN, which indicated the necessity for further investigation at the clinical level.

Introduction

Chronic glomerulonephritis (CGN), the most common form of glomerular disease, accounts for ~20% of chronic kidney disease cases in many countries (1,2). CGN is associated with immune-mediated inflammatory diseases and is characterized by proteinuria, edema, hematuria and hypertension, which are accompanied by renal dysfunction which is a primary cause of end-stage of renal disease (ESRD) (3,4). Numerous pathogenic factors may promote the development of this disease; however, the molecular mechanisms remain unknown (5,6).

In the authors previous experiments, differentially regulated genes were screened and analyzed. The results revealed that Fos and spleen-associated tyrosine kinase (Syk) were potent hub genes and that CGN pathogenesis may be associated with the disordered inflammatory response in addition to abnormal metabolism (7). Therefore, it is important to explain the specific mechanism of Fos and Syk in CGN, which may contribute to understanding the pathogenesis of CGN and developing novel diagnostic markers.

The functions of B lymphocytes are adjusted by a number of signaling pathways, some of which involve the B-cell receptor (BCR) (8). Syk exhibits a central role in the activation of the BCR (9). The Fos gene family encode leucine zipper proteins that form the transcription factor complex activating protein (AP)-1, and can regulate the expression of tumor necrosis factor- α , interleukin (IL)-6 and IL-8 by phosphorylation of mitogen-activated protein kinase (MAPK) and the BCR signaling pathway, which participates in inflammation in CGN (10). The Syk/Ras/c-Fos signaling pathway has a critical role in B cells, including ontogeny, autoimmunity, immune response and immunoglobulin production.

By searching relevant literature, we found that LPS can be used as an inducer for cell viability of glomerular mesangial cells. And this is consistent with our CGN pathology (11,12).

In the present study, Adriamycin (ADR)-induced CGN rats and lipopolysaccharide (LPS)-stimulated HBZY-1 cells were used as experimental models to identify the differentially

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expressed mRNAs and proteins of the Syk/Ras/c-Fos signaling pathway, and elucidate the potential pathogenesis of CGN.

Materials and methods

Materials. ADR was obtained from Hisun Pfizer Pharmaceuticals Ltd. (cat. no. 15029611; Zhejiang, China). Sodium pentobarbital was obtained from Shanghai Chemical Reagent Company (cat. no. 127K1005; Shanghai, China). Total RNA from renal cortex tissues was extracted by TRIzol[®] reagent (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) following the manufacturer's protocol. Antibodies against phosphorylated (p)-Syk, Ras, p-MAPK extracellular signal regulated kinase (ERK; MEK)1/2, p-ERK1/2, c-Fos and β -actin were purchased from Abcam (Shanghai, China; cat. nos. ab79193, ab16907, ab194754, ab76299, ab209794, ab8226). The Syk/Ras/c-Fos pathway inhibitor R406 (inhibitor of Syk) was purchased from AbMole BioScience, (Shanghai, China). All the materials under current study were non-toxic to animals and cell cultures, including all biological and synthetical agents used for immunopharmacological studies.

Animals and cell cultures. The HBZY-1 cell line was obtained from the Cell Bank of Chinese Academy of Sciences (Shanghai, China) and incubated with Dulbecco's modified Eagle's/F-12 medium [10% (v/v) fetal calf serum and 1% (v/v) antibiotics mixture] in 95% air and 5% CO₂ at 37°C (13). Specific pathogen-free (SPF), male Sprague-Dawley (SD) rats (weighing 280-320 g, 9 weeks old) were provided by the Laboratory Animal Center of Anhui Medical University (Hefei, China). All rats were kept in standard cages under 40-60% humidity at 18-22°C with free access to food and water. All animal experiments were approved by the Committee on the Ethics of Animal Experiments of The First Affiliated Hospital of Anhui University of Chinese Medicine (Hefei, China). All surgeries were performed under sodium pentobarbital anesthesia, and all efforts were made to minimize suffering.

CGN rat model establishment and experimental protocols. Following acclimatization for 2 weeks, all animals were divided randomly into the control group and experimental model group (n=10 per group). CGN was induced in the rats by tail intravenous injection with ADR: 3.5 mg/kg ADR was given on the 1st day and 3.0 mg/kg on the 14th day (7,14), whereas the control group was administered a saline solution for comparison at the same time. On the 21st day, all rats were placed into metabolism cages and urine was collected over 24 h to determine the urinary protein levels. A successful model was considered to be indicated by a 24 h urinary protein quantitation of >50 mg/kg. The rats were anesthetized with intraperitoneal sodium pentobarbital (2 ml/kg), and serum samples were obtained from the abdominal aorta for measuring biochemical parameters. All urine and serum samples were stored at -70°C prior to analysis. Animals were then sacrificed and each kidney was retrieved to determine kidney viscera index, and then one half of each kidney was frozen in liquid nitrogen for RNA preparation and protein extraction, while the other half was fixed in 10% neutral formalin for histological evaluation.

Biochemical determination. The 24-h urinary protein, blood urea nitrogen (BUN) and creatinine (Crn) were measured using an automatic biochemistry analyzer.

Hematoxylin and eosin (HE) staining. Glomerular specimens were fixed in 10% neutral formalin, and 4- μ m-thick paraffin-embedded sections were stained with HE and observed microscopically.

HBZY-1 cell model establishment and experimental protocols. HBZY-1 cells were seeded into 6-well plates at a density of 3x10⁵ cells per well and allowed to grow until 70-80% confluent. The cells were divided into three groups: Normal control (normal HBZY-1 cells), an LPS model group (cells were incubated with 0.5 μ g/ml LPS for 48 h) and an LPS + R406 group (cells were incubated with 1.5 μ g/ml R406 for 48 h following model establishment). Each treatment and control were performed at least in triplicate.

mRNA extraction and reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA samples from glomerular specimens and HBZY-1 cells were extracted by TRIzol reagent according to the manufacturer's protocol. The total RNA was used as a template to synthesize first-strand cDNA using a ThermoScript RT-qPCR system (Thermo Fisher Scientific, Inc.) The primers for Syk, Ras, MEK1/2, ERK1/2, c-Fos and β -actin were synthesized by Thermo Fisher Scientific, Inc. RT-qPCR was completed in a final volume of 25 μ l and the following thermal cycling profile for SYBR Green PCR was used: 95°C for 5 min, followed by 40 cycles of 95°C for 10 sec and 60°C for 30 sec. To confirm that only one PCR product was amplified and detected, a dissociation curve analysis of amplification products was performed at the end of each PCR. The comparative C_q method (2^{- $\Delta\Delta$ C_q} method) was used to quantify the expression levels of the different genes (15). Primer sequences are listed in Table I.

Protein extraction and western blot analysis. Total protein samples were extracted from glomerular specimens and rat HBZY-1 cells using a Total Protein Extraction kit, according to the manufacturer's protocol. Protein concentrations were determined by BCA assay. An aliquot of 30 μ g of denatured protein from each sample was subjected to 10% SDS-PAGE, transferred onto a polyvinylidene difluoride membrane, and then incubated with 5% skimmed milk for 1 h. Primary antibodies against p-Syk (1:500 dilution; ab79193), Ras (1:25 dilution; ab16907), p-MEK1/2 (1:500 dilution; ab194754), p-ERK1/2 (1:5,000 dilution; ab76299), c-Fos (1:100 dilution; ab209794) and β -actin (1:500 dilution; ab8226; all from Abcam) were added and incubated at 4°C overnight. Following washing with TBST, the membranes were incubated with goat anti-rabbit or anti-mouse IgG secondary antibodies conjugated with horseradish peroxidase (1:5,000 dilution) for 1 h at 37°C. The blots were visualized using an ECL western blot detection system and scanned with a Gel Imaging System.

Statistical analysis. Data are presented as the mean \pm standard deviation. All data were analyzed using SPSS software, v.17.0 (SPSS, Inc., Chicago, IL, USA). Two groups were compared with t-test, and one-way analysis of variance with Tukey's post

Table I. Primer sequences.

Gene name	Forward and reverse sequences (5'-3')	Product length (bp)
β -actin	F: CAGCGGAACCGCTCATTGATGG R: TCACCCACACTGTGCCCAACGA	155
Syk	F: AGAGGGGAGCTCAGACATGA R: TCTTGTACACACCCTTGGCA	138
Ras	F: GAGTACAGTGCAATGAGGGAC R: CCTGAGCCTGTTTTGTGTCTAC	130
MEK1/2	F: GACGAGCAGCAGCGG R: CTTGAACACCACTCCACCATTG	126
ERK1/2	F: TCATAGGCATCCGAGACATC R: TGGTAGAGGAAGTAGCAGATG	129
c-Fos	F: TACTACCATTCCCCAGCCGA R: GCTGTCACCGTGCGGATAAA	113

β -actin was used as an internal control. F, forward; R, reverse; Syk, spleen associated tyrosine kinase; ERK, extracellular signal regulated kinase; MEK, mitogen activated protein kinase kinase.

Table II. Body weight, kidney viscera index, 24 h urine protein, blood urea nitrogen and Syk in the different groups.

Parameter	Normal	Model	P-value
Body weight (g)	315.15 \pm 23.61	281.91 \pm 44.95 ^a	0.046
Kidney viscera index (%)	0.64 \pm 0.04	0.90 \pm 0.17 ^b	<0.001
24 h urine protein (mg/24 h)	27.32 \pm 5.99	292.99 \pm 44.21 ^b	<0.001
BUN (mmol/l)	5.53 \pm 1.89	12.19 \pm 3.60 ^b	<0.001
Crn (μ mol/l)	38.58 \pm 6.65	65.75 \pm 13.78 ^b	<0.001

^aP<0.05 and ^bP<0.001 vs. normal (control) group. Syk, spleen tyrosine kinase; BUN, blood urea nitrogen; Crn, creatinine.

hoc test was used to determine the significance of three groups. P<0.05 was considered to indicate a statistically significant difference.

Results

Characteristics of experimental rats. Table II presents the laboratory data of the two groups of rats at the end of the experimental period. Compared with the normal group, body weights were significantly lower (P<0.05) and the kidney viscera index and 24 h urine protein were significantly increased (both P<0.01; Table II) in the model group. Furthermore, the levels of BUN and Crn in serum samples were significantly increased in the model group (both P<0.01; Table II), which was in accord with previous studies (16,17).

Histopathology. HE staining is presented in Fig. 1. Rats from the control group invariably exhibited normal glomerular structure and glomerular basement membrane thickness, clear Bowman's capsule structure and convoluted tubule structure, and opened capillary loops. However, in the model group, there were incrustations of the capillary loops and Bowman's capsule. In addition, degeneration of renal tubule epithelial cells, infiltration of inflammatory cells and casts (protein) in

the lumen were also observed, which was in agreement with the authors previous research and indicated that the CGN model was successfully established (6,14).

mRNA and protein expression of Syk/Ras/c-Fos signaling pathway components in the kidney of CGN rats. In order to evaluate Syk/Ras/c-Fos signaling pathway whether involved in CGN lesion, the key genes mRNA and protein expression level were detected in kidney of CGN rats (Figs. 2 and 3). According to western blot results, levels of p-Syk, Ras, p-MEK1/2, p-ERK1/2 and c-Fos were higher in CGN model group than in the control group (Fig. 3). Similar results were found in the relative mRNA levels of Syk, Ras, MEK1/2, ERK1/2 and c-Fos mRNA (Fig. 2).

mRNA and protein levels of Syk/Ras/c-Fos signaling pathway components in LPS-stimulated HBZY-1 cells. The literature shows that LPS can be used as an inducer to induce cell viability of mesangial cells. And this is consistent with our CGN pathology (11,12). So in this experiment, LPS-stimulated HBZY-1 cells were used as experimental models to elucidate the potential pathogenesis of CGN. The results revealed that Syk, Ras, MEK1/2, ERK1/2 and c-Fos mRNA and p-Syk, Ras, p-MEK1/2, p-ERK1/2 and c-Fos protein levels markedly increased in the LPS model group (Figs. 4 and 5). This may suggest that the key

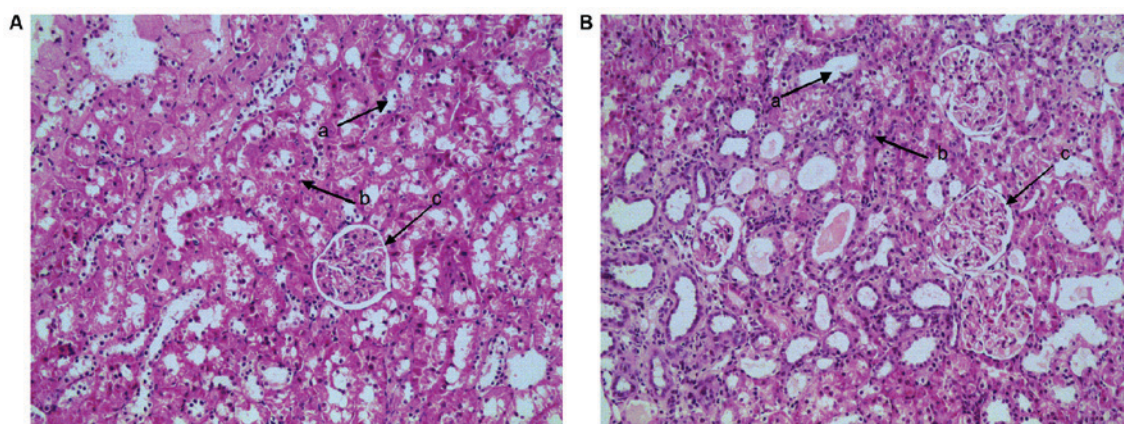


Figure 1. Pathological analysis of kidney tissues from adriamycin-treated and normal rats: (A) Normal and (B) model groups. In the model group, (a, arrows) casts (protein) in the lumen, (b, arrows) infiltration of inflammatory cells and (c, arrows) incrasation of the basal lamina were observed. Compared with the normal group, degeneration of renal tubule epithelial cells, infiltration of inflammatory cells and casts (protein) in the lumen were observed in the model group (magnification, x200).

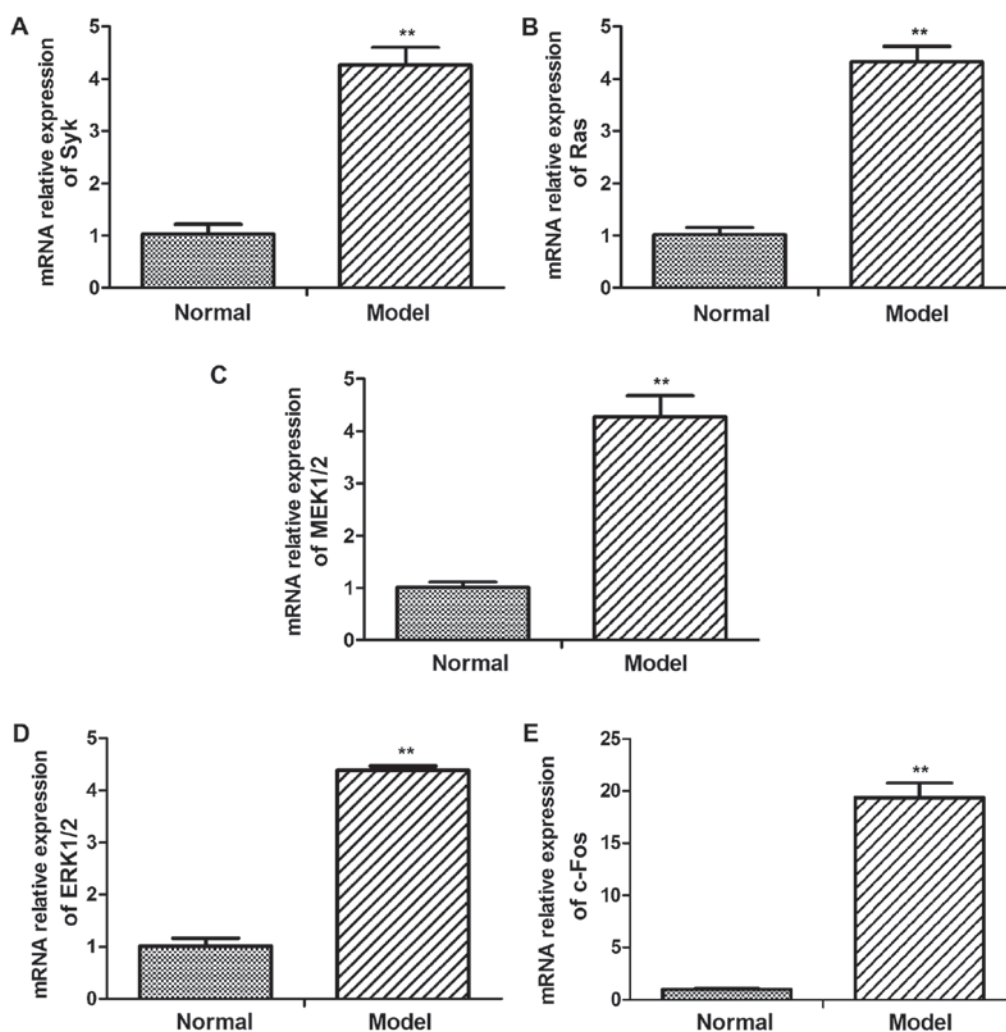


Figure 2. mRNA levels of Syk, Ras, MEK1/2, ERK1/2 and c-Fos in glomerular tissues of adriamycin-treated and normal rats. The mRNA expression levels of (A) Syk, (B) Ras, (C) MEK1/2, (D) ERK1/2 and (E) c-Fos were assessed using reverse transcription-quantitative polymerase chain reaction. Syk, Ras, MEK1/2, ERK1/2 and c-Fos mRNA levels were significantly increased in the model group. Data are presented as the mean \pm standard deviation of at least three independent experiments. ** $P < 0.01$ vs. normal (control) group. Syk, spleen associated tyrosine kinase; ERK, extracellular signal regulated kinase; MEK, mitogen activated protein kinase kinase.

genes mRNA and protein expression level increased evidently in Syk/Ras/c-Fos signaling pathway in LPS-stimulated HBZY-1

cells. Furthermore, R406 was revealed to inhibit the LPS-induced activation of the Syk/Ras/c-Fos signaling pathway.

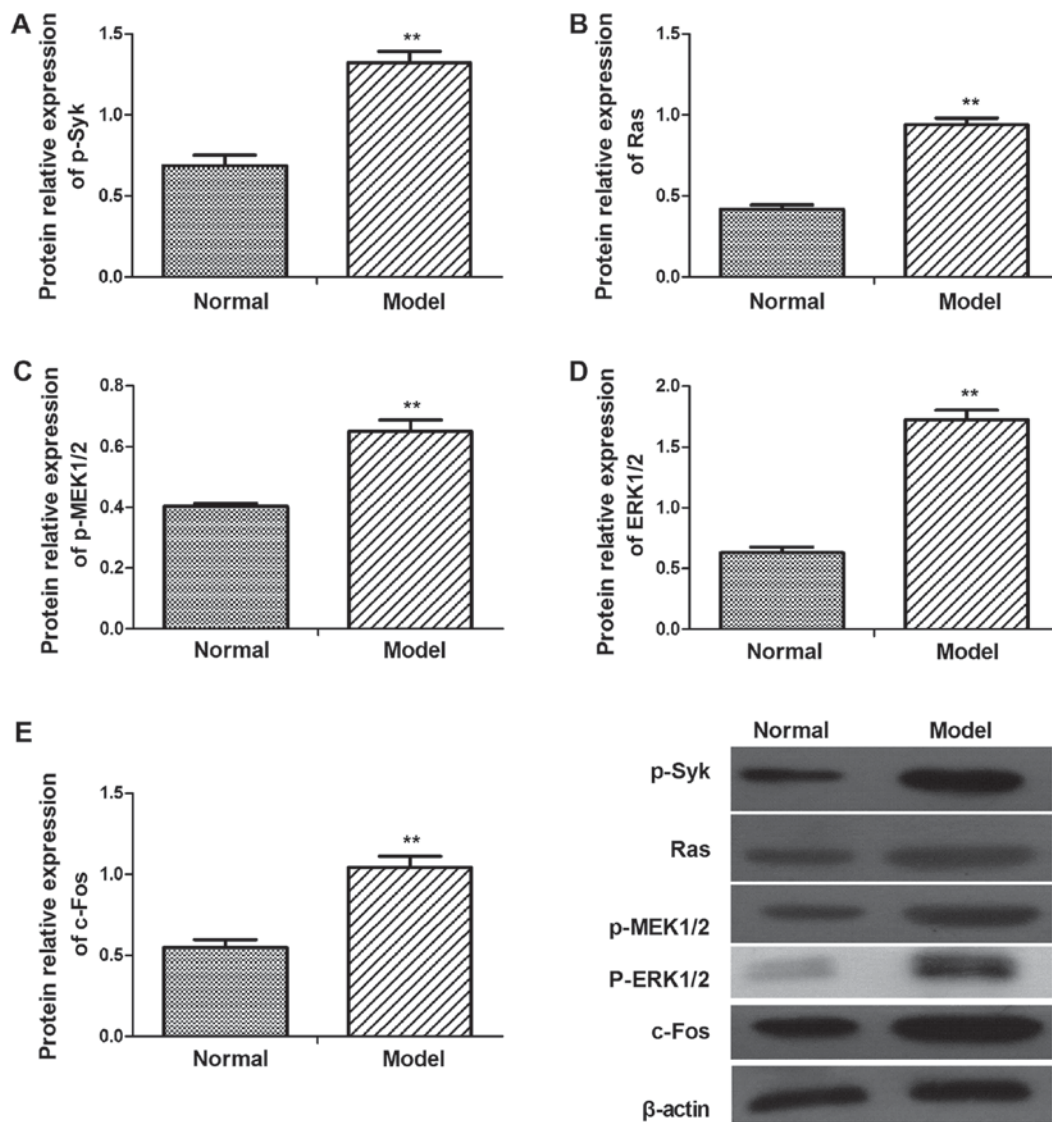


Figure 3. Protein levels of p-Syk, Ras, p-MEK1/2, p-ERK1/2 and c-Fos in the glomerular tissues of adriamycin-treated and normal rats. Protein expression levels of (A) p-Syk, (B) Ras, (C) p-MEK1/2, (D) p-ERK1/2 and (E) c-Fos were assessed by western blot analysis. p-Syk, Ras, p-MEK1/2, p-ERK1/2 and c-Fos protein levels were upregulated. Data are presented as the mean \pm standard deviation of at least three independent experiments. ** $P < 0.01$ vs. normal (control) group. Syk, spleen associated tyrosine kinase; MEK, mitogen activated protein kinase kinase; ERK, extracellular signal regulated kinase; p-, phosphorylated.

Discussion

CGN, which is associated with immune-mediated inflammatory diseases, frequently occurs during ESRD and seriously affects patient survival. Biological and clinical observations indicate that focal infection, caused by hematuria, proteinuria, arterial hypertension and edema, primarily manifests as glomerular injury (18). Autoimmunity, infection and the inflammatory response are known to be involved in the pathogenesis of CGN (19). However, despite ongoing investigation, the exact molecular mechanisms remain unclear.

In the current study, ADR-induced CGN rats and LPS-stimulated HBZY-1 cells were used to explore the molecular pathogenesis of CGN (7). The results indicated that the kidney viscera index and the 24 h urinary protein, BUN and Crn levels were significantly increased while body weight decreased. The Syk/Ras/c-Fos signaling pathway was activated both *in vitro* and *in vivo*. Therefore, it was hypothesized that activation of Syk/Ras/c-Fos signaling may be involved in

the inflammatory reaction and proteinuria during the process of CGN. For all that, The LPS as ADR-induced CGN may not be accurate, but it can be used as an inducer for glomerular cell viability and it as a limitation of the present study.

The establishment of appropriate models is critical for disease research. In the current study, the ADR-induced CGN rat model was selected as it has previously been demonstrated to be similar to human CGN progression (20). LPS was used in the *in vitro* studies, however not in the animal models. In the present study, ADR-induced rats developed expansion of the convoluted tubules, degeneration of renal tubule epithelial cells, infiltration of inflammatory cells, and casts (protein) in the lumen, which were consistent with results from a previous study (7). The present study focused on cell viability of the glomerular mesangial cells, and used the classical proliferation and inflammatory inducer LPS to simulate CGN in the cells. However, LPS is considered to be one of the strong stimulating factors for glomerular mesangial cells, it may be used as an inducer for glomerular cell viability.

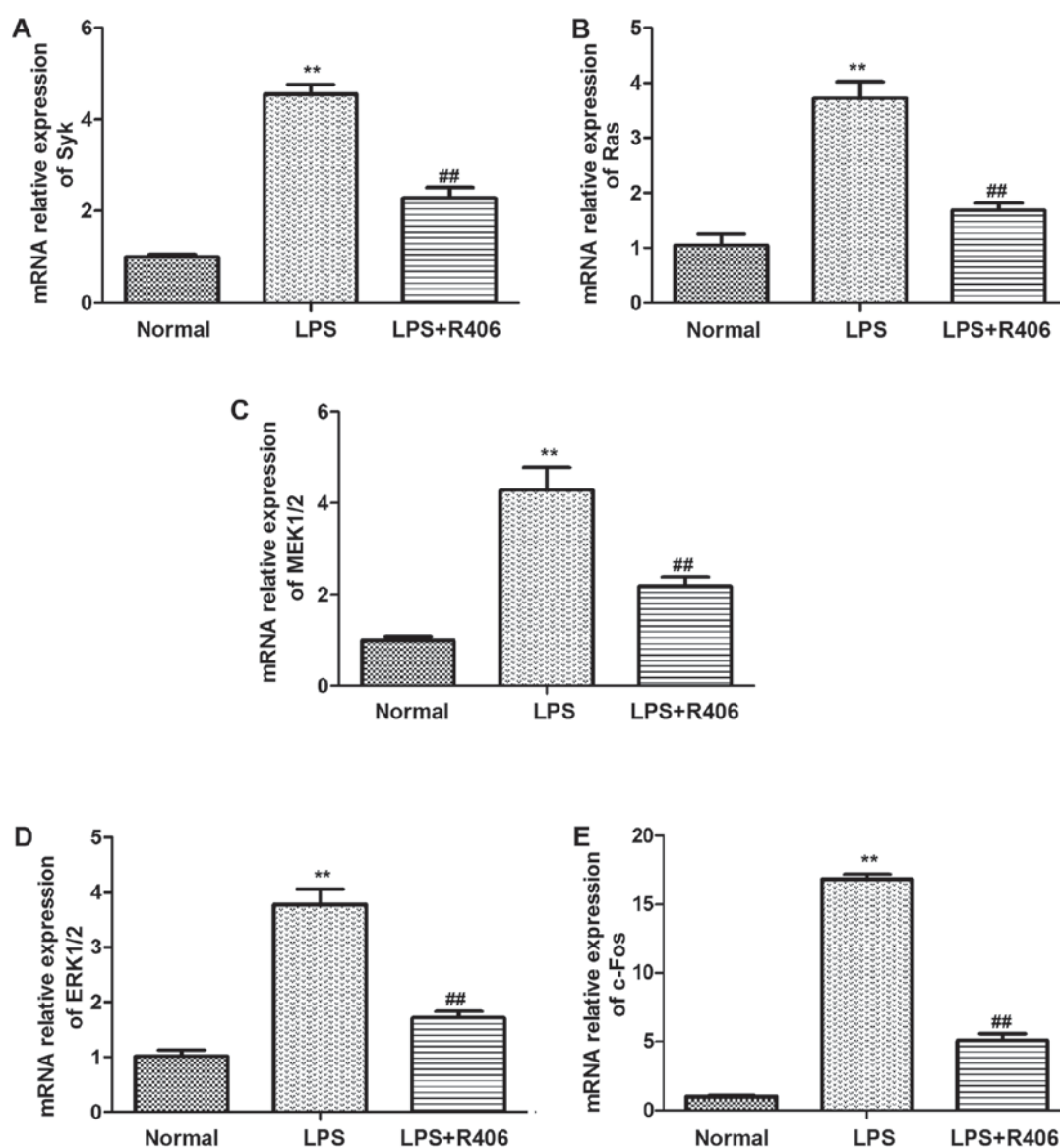


Figure 4. mRNA levels of (A) Syk, (B) Ras, (C) MEK1/2, (D) ERK1/2 and (E) c-Fos in HBZY-1 cells were determined by reverse transcription-quantitative polymerase chain reaction. Syk, Ras, MEK1/2, ERK1/2 and c-Fos mRNA levels significantly increased in the LPS model group. The inhibitor R406 inhibited the LPS-induced activation of the Syk/Ras/c-Fos signaling pathway. Data are presented as the mean \pm standard deviation of at least three independent experiments. ** $P < 0.01$ vs. normal group; ## $P < 0.01$ vs. LPS group. Syk, spleen associated tyrosine kinase; MEK, mitogen activated protein kinase kinase; ERK, extracellular signal regulated kinase; p-, phosphorylated; LPS, lipopolysaccharide.

Syk and c-Fos were demonstrated to be involved in the BCR signaling pathway. BCR signaling is a complex process that involves a number of kinases, phosphatases and adaptor proteins that transmit, modulate or terminate the signal (21). Once activated, Syk propagates the BCR signal through an important signaling intermediate associated with the phosphorylation of adapter proteins, including B-cell linker protein and phospholipase $C\gamma 2$ (22). The signaling cascade then proceeds to activate downstream signaling molecules that regulate the cellular response, including Ras GTPase-activating protein (Ras GAP). Ras GAP regulates Ras by converting the active GTP-bound form of Ras into the inactive GDP-bound form and may also function as an effector of Ras (23,24).

MAPKs are important mediators of the intracellular signal transduction pathways that are responsible for cell growth and differentiation (25). Ras may induce cell proliferation by activating the MAPK survival pathway and regulating the

expression of IL-8, IL-2 and IL-6. A previous study suggested that the expression of p-ERK is significantly increased in the anti-Thy1 nephritis group as compared with the sham group ($P < 0.01$), and it was suggested that kidney injury may be directly associated with the inactivation of the ERK signaling pathway, thereby inhibiting the abnormal cell viability of intravascular cells (26). In another study, the development of diabetic nephropathy is accelerated with a decrease in Raf kinase inhibitor protein and an increase in p-ERK1/2 (27).

Activated ERK1/2 is transferred from the cytoplasm to the nucleus, where it further mediates the transcriptional activation of c-Fos and c-Jun. c-Fos is an important member of the AP-1 transcription complex, which is involved in major cellular functions including proliferation, transformation, differentiation and apoptosis (28). Zu *et al* (29) concluded that saikosaponin-D inhibits the proliferation of glomerular mesangial cells and the synthesis of extracellular matrix

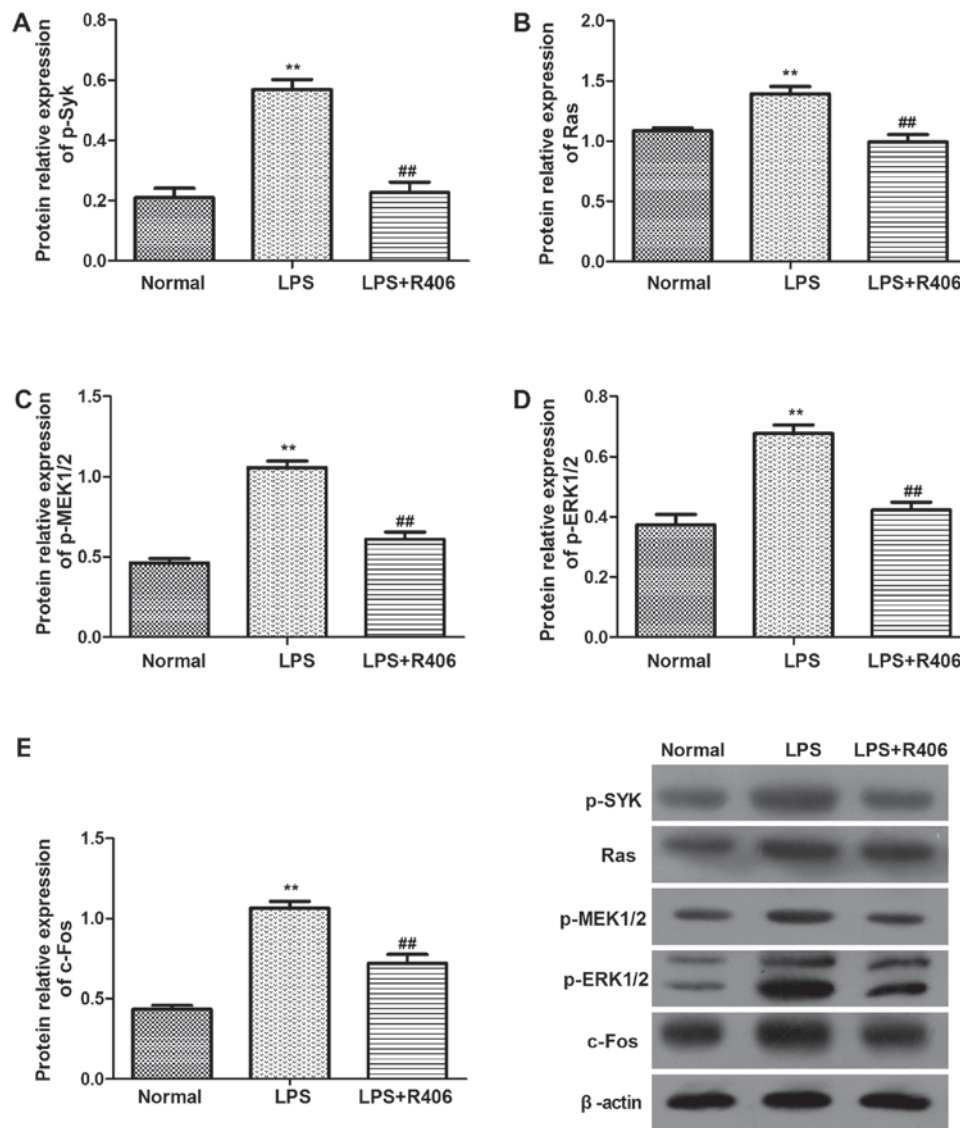


Figure 5. Protein levels of (A) p-Syk, (B) Ras, (C) p-MEK1/2, (D) p-ERK1/2 and (E) c-Fos in HBZY-1 cells were determined by western blotting. p-Syk, Ras, p-MEK1/2, p-ERK1/2 and c-Fos protein levels significantly increased in the LPS model group. The inhibitor R406 inhibited the LPS-induced activation of the Syk/Ras/c-Fos signaling pathway. Data are presented as the mean \pm standard deviation of at least three independent experiments. ** $P < 0.01$ vs. normal group; ## $P < 0.01$ vs. LPS group. Syk, spleen associated tyrosine kinase; MEK, mitogen activated protein kinase kinase; ERK, extracellular signal regulated kinase; p-, phosphorylated; LPS, lipopolysaccharide.

proteins through the downregulation of the cyclin dependent kinase 4, c-Jun and c-Fos genes. Therefore, members of the Fos gene family are known to be regulators of cell proliferation, differentiation, transformation and inflammation, which are involved in inflammation in CGN (10). In the present study, the expression levels of Ras, p-MEK, p-ERK1/2 and c-Fos were increased in the model group rats and HBZY-1 cells after LPS treatment compared with control group, which was in accordance with the literature (30).

Although the inhibitor R406 is used in cell experiments, there is no interference experiment with syk on animal models, is a great regret of our project and also the limitation of this experiment. In addition, we should increase the expression of Syk/Ras/c-fos by immunohistochemical staining, which will give us a direct and vivid expression.

In conclusion, the Syk/Ras/c-Fos signaling pathway was activated significantly in ADR-induced CGN rats and LPS-induced HBZY-1 cells. The results of the present study

provide novel insights suggesting that Syk/Ras/c-Fos signaling may be directly associated with CGN.

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Availability of data and materials

All data generated or analyzed during the present study are included in this published article.

Authors' contributions

JG, LW and HJ conceived and designed the study. YG, XW and SX performed the experiments. JS participated in the analysis and processing of animal experiments and data, and wrote the paper. HJ critically revised the manuscript for important intellectual content. All authors read and approved the manuscript.

Ethical approval and consent to participate

All animal experiments were approved by the Committee on the Ethics of Animal Experiments of The First Affiliated Hospital of Anhui University of Chinese Medicine (Hefei, China). All surgeries were performed under sodium pentobarbital anesthesia, and all efforts were made to minimize suffering.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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