



# Rituximab regulates the expression of the Raf kinase inhibitor protein via NF- $\kappa$ B in renal tissue of rats with diabetic nephropathy

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**ABSTRACT.** This study aimed to investigate the expression levels of the Raf kinase inhibitor protein (RKIP) and NF- $\kappa$ B in renal tissues of diabetic nephropathy (DN) rats, and to determine the underlying molecular targets of rituximab (RTX), with the goal of developing new clinical treatment selection for DN. Sprague-Dawley rats were randomly divided into a normal group (N), a DN group (M), and an RTX treatment group (D). Blood glucose and 24-h urine protein levels of rats were determined. The expression levels of RKIP and NF- $\kappa$ B in glomerular tissues were determined by immunohistochemistry staining and Western blotting. Comparisons between the M and N groups revealed that the concentrations of blood glucose and 24-h urine protein were significantly increased by DN ( $P < 0.01$ ), and the expression levels of RKIP and NF- $\kappa$ B

were significantly decreased and increased ( $P < 0.05$ ), respectively. In the D group, the expression levels of RKIP and NF- $\kappa$ B were, respectively, upregulated and downregulated by RTX, and the concentrations of 24-h urine protein were also decreased by RTX. These results suggest that expression levels of RKIP might be regulated by RTX via NF- $\kappa$ B. This pathway could play an important role in the development and pathogenesis of DN. Therefore, RTX could be selected for clinical treatment of DN.

**Key words:** Diabetic nephropathy; Rituximab; RKIP; NF- $\kappa$ B

## INTRODUCTION

The number of cases of diabetes mellitus (DM) has exceeded 170 million globally, as improvement in the lifespan of DM patients has increased in recent years (KDOQI, 2007). Approximately 30-40% of patients with either type 1 or 2 DM ( $T_1$  DM,  $T_2$  DM) will develop diabetic nephropathy (DN) after several years (Kaul et al., 2010). Currently, specific therapies against DN are lacking, and the prognosis of DN is always poor. Therefore, further investigation into the molecular pathological mechanisms of DN and identifying new therapies against DN are crucial for postponing its further development. Intracellular signal transduction, immune response, metabolic disorders, and renin-angiotensin system disorder are all known to be generally associated with DN pathogenesis (Kaul et al., 2010). NF- $\kappa$ B is one of the transcription factors that mediates signal transduction processes, and plays an important role in the immune response and in the cell growth. Results of our previous studies and those of other authors (Lamhamedi-Cherradi et al., 2003; Li et al., 2006) have suggested that the inhibition of NF- $\kappa$ B activation could delay the development of DN (Li et al., 2006). The Raf kinase inhibitor protein (RKIP), which is expressed in various tissues and cell types, participates in the modulation and control of intracellular G protein-coupled receptor signaling pathways and in the NF- $\kappa$ B signal channel. Recent studies have suggested that RKIP likely plays an important role in DM pathways with respect to protein kinase C (Keller et al., 2004; Thongboonkerd and Klein, 2004). Rituximab (RTX), a monoclonal antibody, is used to treat autoimmune thrombocytopenic purpura, systemic lupus erythematosus, rheumatoid arthritis, and other immune diseases (Kadikoy et al., 2012). Jazirehi et al. (2004) demonstrated that RTX could promote RKIP expression in a non-Hodgkin's lymphoma cell line, which increased sensitivity to the drug. Therefore, the present study investigated the role of RKIP in the pathological mechanism of DN and the mechanism of RTX in postponing DN development. These results will provide a theoretical foundation for developing new clinical treatments for DN.

## MATERIAL AND METHODS

### Animals

Sixty 6-week-old male Sprague-Dawley rats were obtained from the Experimental Animal Center of Guiyang Medical College (Guizhou, China). All protocols were approved by the Animal Care Committee of Guiyang Medical College.

## General experimental protocol and DM induction

Rats were randomly divided into three groups: normal control group (N; N = 20), DN group (M; N = 20), and RTX treatment group (D; N = 20). In the M and D groups, diabetes was induced by intraperitoneal injection of streptozotocin (STZ) combined with Freund's complete adjuvant and a high-fat diet. Rats that had blood glucose levels >16.65 mM twice within 4 weeks after STZ injection were considered to be diabetic (Flynn et al., 2012). STZ-injected mice with urine protein (UP) levels greater than or equal to 30 mg or more than 10 times that of the N group were considered to have DN (Soler et al., 2012). Rats in the D group were injected with 58 mg/kg RTX, diluted by saline to 1 mg/mL, in the tail vein once a week for a total of 4 weeks.

## Tissue treatments

Peripheral blood was monitored after the DM model was established. Rats were sacrificed after 2 weeks ( $T_1$  stage) or 4 weeks ( $T_2$  stage) by RTX treatment. Urine was collected to compare UP levels among all groups. Right kidneys from the D group were harvested immediately and protein was extracted from the tissue. Left kidneys were fixed in 10% neutral buffer formaldehyde solution for subsequent hematoxylin and eosin staining, periodic acid-Schiff staining, and immunohistochemistry.

## Immunohistochemistry and analysis

An immunohistochemical kit was used to detect RKIP (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) and NF- $\kappa$ B (Santa Cruz Biotechnology, Inc.) (Li et al., 2006). Immunohistochemical staining was quantified using an HPIAS-2000 HD color pathological image analysis system. A total of four slides were randomly selected from each sample and observed under three fields of view (40X objective magnification) with 100 cells per view. The positive rate was determined by the following formula: positive rate (%) = [positive cell number / (positive cell number + negative cell number)] x 100. The positive rate of each group was compared (Fiore et al., 2012).

## Western blotting

Total proteins were isolated from kidney tissues. Equal amounts of proteins (60  $\mu$ g) were separated by electrophoresis and transferred to polyvinylidene fluoride membranes. Each membrane was incubated overnight at 4°C with the specific primary antibodies: RKIP. The membranes were then incubated at 37°C with horseradish peroxidase-linked secondary antibody (Zhong Shan, Beijing, China). After washing with Tris-buffered saline with Tween 20, immunoreactive proteins were visualized and exposed. Using the expression of GAPDH as reference, protein bands were quantified with the QuantitOne 4.52 in Bio-Rad image software. The ratio of RKIP to GAPDH was calculated and this represented the intensity of the immunoreaction.

## Statistical analysis

Data are reported as means  $\pm$  SD. Statistical comparisons of means between two

groups with normally distributed variables were accomplished using the Student *t*-test. Comparisons of variables among the three groups were performed by one-way analysis of variance. Comparisons of variables that showed variance homogeneity were performed with the S-N-K test. Comparisons of variables with heterogeneity of variance were performed by the Tamhane test. Correlation analysis was performed with Pearson's analysis. P values less than 0.05 were considered to be significant. All analyses were carried out with the SPSS 18.0 software.

## RESULTS

### Levels of blood glucose (BG) and UP over 24 h

As shown in Table 1, compared with those of the N group, the BG and UP content of the M group significantly increased ( $P < 0.01$ ), while the UP content of the D group markedly decreased. No significant differences in BG levels were observed.

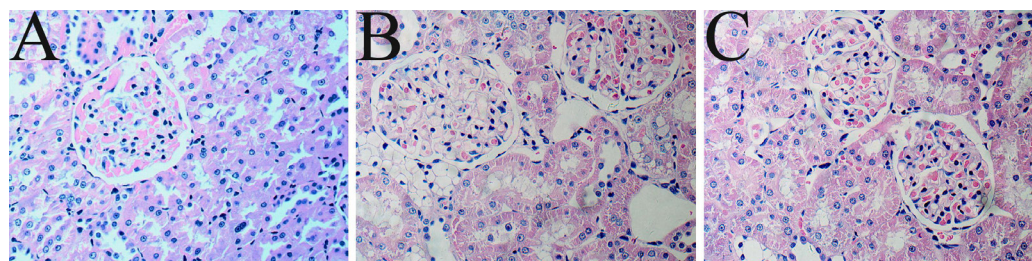
**Table 1.** Levels of blood glucose and urine proteins in 24 h (means  $\pm$  SD).

Groups	Time	Number	Blood glucose level (mM)	Urine protein level (mg/day)
N	T <sub>1</sub>	10	5.5 $\pm$ 0.5	8.7 $\pm$ 3.2
	T <sub>2</sub>	10	5.6 $\pm$ 0.5	9.6 $\pm$ 1.0
M	T <sub>1</sub>	10	23.2 $\pm$ 2.5*	59.8 $\pm$ 15.3*
	T <sub>2</sub>	10	24.0 $\pm$ 2.0*	61.6 $\pm$ 11.4*
D	T <sub>1</sub>	10	22.8 $\pm$ 1.7*	52.1 $\pm$ 9.4*▲
	T <sub>2</sub>	10	23.4 $\pm$ 2.3*	50.1 $\pm$ 15.1*▲

N = normal group; M = diabetic nephropathy group; D = rituximab-treated group. \* $P < 0.01$  vs N group; ▲ $P < 0.05$  vs M group.

### Pathological analyses

At the T<sub>1</sub> stage, renal lesions of DN of the M group showed glomerular hypertrophy, renal tubule vacuolar degeneration and expansion, general thickening of the glomerular basement membrane, and an increase in the mesangial matrix. Other lesions included renal arterioles hyalinosis, occlusion, and a large amount of inflammatory cell infiltration in the renal interstitium. The basement membrane of the glomerular capillary thickened in the D group, whereas the lesion characteristics mentioned above showed improvement: thickening of the basement membrane of renal arterioles was mild, no vacuoles, and little inflammatory cell infiltration (Figure 1A-C).



**Figure 1.** Pathological morphology of kidney tissue of rats in each group. **A.** N group [hematoxylin and eosin (HE), 400X]. **B.** M group (HE, 400X). **C.** D group (HE, 400X). For group abbreviations, see legend to Table 1.

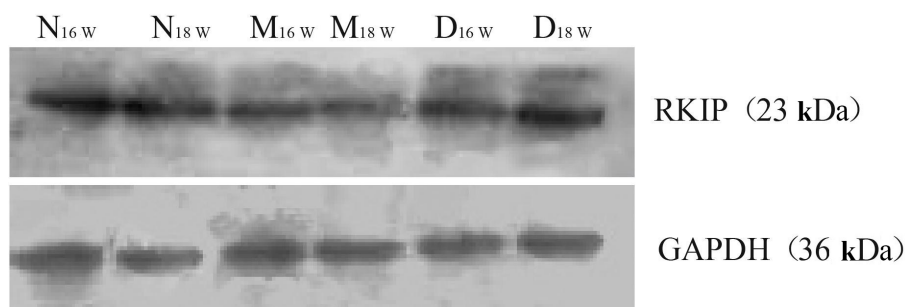
### Expression of RKIP in renal tissues

Results of the Western blot analysis (Table 2 and Figure 2) showed that the expression levels of RKIP were downregulated in M and D groups at the T<sub>1</sub> and T<sub>2</sub> stages. Moreover, the expression levels of RKIP were increased in the D group compared to those in the M group (Figure 3A-F). RKIP was observed as brown-yellow particles, and its expression occurred mainly in the cytoplasm and in the plasma membrane, with some expression in the nucleus. In the N group, expression of RKIP in renal tissues at the T<sub>1</sub> and T<sub>2</sub> stages was seen as brown-yellow in the cytoplasm, whereas in the M group, a remarkable decrease in positive staining was observed in the glomerulus and tubules, particularly at the T<sub>2</sub> stage.

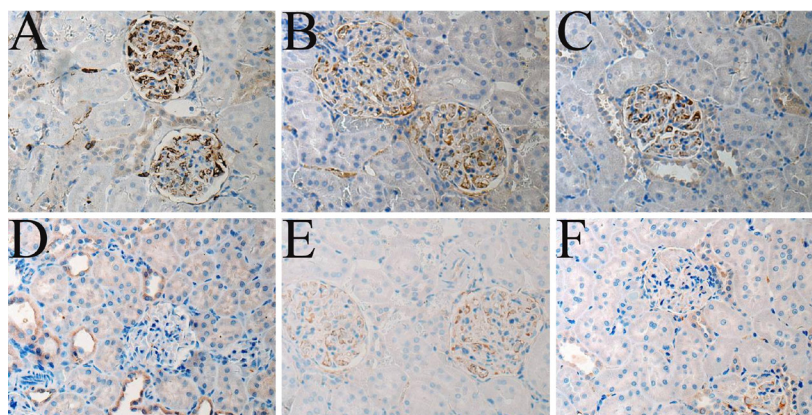
**Table 2.** Expression of RKIP in 16 and 18 weeks from the Western blot analyses (means  $\pm$  SD).

Times (weeks)	16 weeks			18 weeks		
	N	M	D	N	M	D
RKIP/GAPDH	0.9 $\pm$ 0.1	0.4 $\pm$ 0.1*	0.7 $\pm$ 0.2**	1.0 $\pm$ 0.2	0.5 $\pm$ 0.1*	0.7 $\pm$ 0.1**

For group abbreviations, see legend to Table 1. \*P < 0.05 vs N group; \*\*P < 0.05 vs M group.



**Figure 2.** Expression levels of RKIP in 16 and 18 weeks (W) of N, M, and D groups by Western blotting. For group abbreviations, see legend to Table 1.



**Figure 3.** Immunohistochemical staining of RKIP in renal tissue. **A.** N group = T<sub>1</sub> stage [hematoxylin and eosin (HE), 400X]. **B.** N group = T<sub>2</sub> stage (HE, 400X). **C.** M group = T<sub>1</sub> stage (HE, 400X). **D.** M group = T<sub>2</sub> stage (HE, 400X). **E.** D group = T<sub>1</sub> stage (HE, 400X). **F.** D group = T<sub>2</sub> stage (HE, 400X). For group abbreviations, see legend to Table 1.



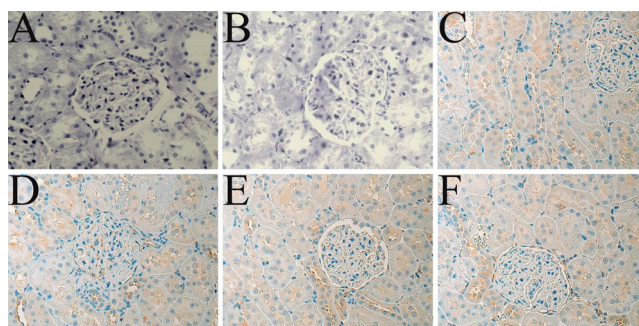
## Expression of NF- $\kappa$ B in renal tissue

There was no expression of NF- $\kappa$ B in renal tissues of the N group. By contrast, in the M group, a brown NF- $\kappa$ B-positive substance was observed in glomerular mesangial cells, proximal tubular epithelial cells, and in the cytoplasm of lymphocytes and renal interstitium-infiltrated macrophages. Furthermore, the expression of NF- $\kappa$ B was higher at the T<sub>2</sub> stage than at the T<sub>1</sub> stage in the M group. In the D group, the expression of NF- $\kappa$ B was reduced relative to that of the M group at the same stage ( $P < 0.05$ ) (Table 3, Figure 4A-F).

**Table 3.** Expressions of NF- $\kappa$ B in T<sub>1</sub> and T<sub>2</sub> stages of different groups (means  $\pm$  SD).

Groups	Time (weeks)	Number	NF- $\kappa$ B (%)
N	T <sub>1</sub>	10	6.3 $\pm$ 1.4
	T <sub>2</sub>	10	6.4 $\pm$ 1.7
M	T <sub>1</sub>	10	40.7 $\pm$ 3.5*
	T <sub>2</sub>	10	46.2 $\pm$ 4.2* <sup>Δ</sup>
T	T <sub>1</sub>	10	33.3 $\pm$ 2.6* <sup>Δ</sup>
	T <sub>2</sub>	10	34.1 $\pm$ 3.4* <sup>Δ</sup>

\* $P < 0.05$  vs N group, <sup>Δ</sup> $P < 0.05$  vs M group in T<sub>2</sub> stage. For group abbreviations, see legend to Table 1.



**Figure 4.** Immunohistochemical staining of NF- $\kappa$ B in renal tissue. **A.** N group = T<sub>1</sub> stage [hematoxylin and eosin (HE), 400X]. **B.** N group = T<sub>2</sub> stage (HE, 400X). **C.** M group = T<sub>1</sub> stage (HE, 400X). **D.** M group = T<sub>2</sub> stage (HE, 400X). **E.** D group = T<sub>1</sub> stage (HE, 400X). **F.** D group = T<sub>2</sub> stage (HE, 400X). For group abbreviations, see legend to Table 1.

## Correlation analyses

Correlation analyses revealed a negative relationship between NF- $\kappa$ B and RKIP at both the T<sub>1</sub> and T<sub>2</sub> stages in the M group (Pearson's coefficient ( $r$ ) = -0.71 for the T<sub>1</sub> stage and  $r$  = -0.67 for the T<sub>2</sub> stage).

## DISCUSSION

DN is one of the most important chronic complications of DM, and is the main contributor of renal failure that occurs at the end stage of diabetes. Wu et al. (2012) proposed that DN was an inflammatory disease caused by metabolic disorder, and inflammation was likely to be the main contributor to progression of the disease. Recently, some researchers have further suggested that the downregulation of RKIP expression levels might be related with

hepatic fibrosis and tumor formation, which have been reported in prostate, lung cancer, liver, and thyroid tumors among others (Zeng et al., 2008; Dangi-Garimella et al., 2009). Not only were expression levels of RKIP higher in normal tissue than in tumor tissue, expression levels were higher in primary tumors compared to those in metastatic tumors. Furthermore, high expression levels or deletion of RKIP resulted in powerful tumor invasion and poor prognoses (Beshir et al., 2010). Collectively, these results suggest that RKIP could be a suppression gene for tumor metastasis. In the present study, the expression levels of RKIP showed remarkable downregulated positive staining in the glomerulus and tubules of the M group, especially at the T<sub>2</sub> stage (Figure 3). In the T<sub>1</sub> stage, renal lesions of DN of the M group demonstrated DN characteristics, including glomerular hypertrophy, renal tubule vacuolar degeneration and expansion, glomerular basement membrane thickening, increased mesangial matrix, renal arterioles hyalinosis and occlusion, and a large amount of inflammatory cell infiltration in the renal interstitium (Figure 3). NF- $\kappa$ B proteins were expressed in glomerular mesangial cells, proximal tubular epithelial cells, and in the cytoplasm of lymphocytes and macrophages that infiltrated the renal interstitium of the M group (Figure 4). In the M group, expression of NF- $\kappa$ B was higher in the T<sub>2</sub> stage than in the T<sub>1</sub> stage ( $P < 0.05$ ), supporting the notion that NF- $\kappa$ B plays a critical role in the occurrence and development of DN (Luis-Rodríguez et al., 2012). Furthermore, the expression levels of NF- $\kappa$ B were negatively correlated with those of RKIP. Several mechanisms might be invoked to explain how RKIP could regulate the occurrence and development of DN, including RKIP coupling with NF- $\kappa$ B-inducing kinase, TGF- $\beta$ -activated kinase 1, I- $\kappa$ B kinase, and I- $\kappa$ B kinase  $\beta$ , which are all involved in inhibition of NF- $\kappa$ B signal transduction (Yeung et al., 2001; Corbit et al., 2003; Lorenz et al., 2003; Houben et al., 2007; Matallanas et al., 2011; Fujimori et al., 2012). Downregulation of RKIP might trigger the NF- $\kappa$ B pathway in DN, which then activates the I- $\kappa$ B kinase. I- $\kappa$ B kinase phosphorylates NF- $\kappa$ B-bound I- $\kappa$ Bs, resulting in the translocation of NF- $\kappa$ B dimers to the nucleus. In the nucleus, NF- $\kappa$ B performs distinct regulatory functions, which might contribute to tumorigenesis (Karin et al., 2002). Therefore, it could be inferred that downregulated RKIP and the subsequent activation of NF- $\kappa$ B might be associated with DN development.

RTX is the first chimeric murine-human monoclonal antibody against CD20 that was approved by the Food and Drug Administration for the treatment of CD20<sup>+</sup> B cell lymphoma (Brezinschek et al., 2012). Several studies have found that T-lymphocyte-mediated DM involves a B-lymphocyte component, suggesting that B lymphocytes could contribute to the pathogenesis of T<sub>1</sub> DM. B lymphocytes can be selectively depleted with RTX, which would decrease the immune-mediated destruction of beta cells leading to preserved beta-cell function in patients with T<sub>1</sub> DM (Pescovitz et al., 2009). The present study showed that RTX had a beneficial effect on beta cell function in diabetes and decreased UP levels within 24 h, indicating that RTX could be used in clinical treatment of DN. Moreover, RKIP could be molecular target of RTX. Considering these results and those of previous studies, it could be concluded that RTX regulates the expression of RKIP via NF- $\kappa$ B in renal tissues of rats with DN. Therefore, anti-B lymphocyte therapy with RTX would likely prevent or delay DN, suggesting that RTX should be seriously considered for clinical treatments of DN.

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