Inhibition of Calcium$^{2+}$/Calmodulin-dependent Protein Kinase Type IV Ameliorates Experimental Nephrotic Syndrome

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Abstract

Objective Evidence has demonstrated that Ca$^{2+}$/calmodulin-dependent protein kinase type IV (CaMKIV) contributes to altered cytokine production by promoting the production of inflammatory cytokines. This study aimed to explore the protective role and underlying mechanisms of CaMKIV inhibition in experimental nephrotic syndrome.

Methods BALB/c mice received single intravenous injections of adriamycin (10 mg/kg) then were sacrificed at two, four and six weeks. In the second study, treatment with KN-93, a CaMKIV inhibitor, or vehicle administered via intraperitoneal injection was started five days after adriamycin injection. Functional and pathologic parameters, the presence of inflammatory infiltration and the expressions of pro-inflammatory cytokines were assessed.

Results The CaMKIV protein expression levels were upregulated in the mice with adriamycin nephropathy, which was significantly inhibited by KN-93 (p<0.01). As compared with the vehicle-treated controls, KN-93 treatment resulted in marked suppression of proteinuria and serum creatinine at week 6 (p<0.01), but not at two weeks after induction of the disease. KN-93 inhibited glomerulosclerosis and the development of tubulointerstitial lesions. The renal alpha-smooth muscle actin ($\alpha$-SMA) expression was also significantly suppressed by KN-93 treatment at week 6 (p<0.01). Moreover, KN-93 inhibited the renal monocyte chemoattractant protein-1 (MCP-1) expression, paralleled by a reduction in the interstitial infiltration of macrophages and T-cells (p<0.01).

Conclusion Our findings suggest that activation of CaMKIV signaling is involved in the progression of glomerular diseases with a proteinuric state. Our data therefore justify the development of small molecule CaMKIV inhibitors for the treatment of clinical nephrotic syndrome.

Key words: calcium$^{2+}$/calmodulin-dependent protein kinase type IV, KN-93, nephrotic syndrome, proteinuria

Introduction

Nephrotic syndrome (glomerular dysfunction), the common denominator in a variety of kidney diseases such as diabetes mellitus, focal segmental glomerulosclerosis and systemic lupus erythematosus, involves the massive loss of protein in the urine, termed proteinuria (1). Kidney diseases that lead to nephrotic syndrome are major causes of morbidity and mortality affecting human beings (2). From a clinical perspective, nephrotic syndrome is a condition that is difficult to treat, and there is an urgent need to develop novel strategies to prevent the development of severe and progressive glomerular damage (3). CaMKIV is essential for mesangial cell proliferation and has been implicated in the pathophysiology of renal disease (4). Of interest, blockage of CaMKIV has been shown to ameliorate proteinuria and suppress interferon signaling in experimental mice (5). Proteinuria is now recognized to be a risk factor in the progression of chronic renal diseases (6).
However, it is not known whether CaMKIV plays a role in the progression of glomerular diseases with persistent proteinuria. KN-93 is a methoxybenzene-sulfonamide that is an effective inhibitor of CaMKIV (5). Therefore, we investigated whether the inhibition of CaMKIV can improve the disease pathology in an experimental model of glomerulosclerosis.

Materials and Methods

Mice

Male BALB/c mice were obtained from our Animal Care Center. The experiments were approved by the Ethical Committee of the University and performed in accordance with the NIH’s Guiding Principles in the Care and Use of Laboratory Animals.

Experimental design

Single tail vein injections of adriamycin (10 mg/kg) (Sigma-Aldrich, China) were used to induce a model of progressive glomerulosclerosis and tubulointerstitial inflammation (7). An equal volume of vehicle (saline) was administered to control animals. The mice were housed in metabolic cages for 24-hour urine collection at different time points. Groups of eight mice were sacrificed at weeks 2, 4 and 6. In the second set of experiments, mice subjected to adriamycin injection were treated with intraperitoneal injection of either vehicle (DMSO) or a selective CaMKIV inhibitor KN-93 (EMD Bioscience) dissolved in 5% DMSO at a dose of 0.24 mg/kg (body weight) three times a week starting five days after adriamycin intervention. Mice with adriamycin nephropathy treated with KN-93 were compared with those treated with vehicle according to the same protocol as that used in the KN-93-treated group. Groups of eight mice were sacrificed at weeks 2 and 6. Eight normal mice treated with vehicle (DMSO) were used as normal controls and killed at week 6. To avoid interference with DMSO control, those treated with vehicle according to the same protocol as that used in the KN-93-treated group. Groups of eight mice were sacrificed at weeks 2 and 6.

Histological analysis

The kidneys were removed, fixed in 10% buffered formalin and embedded in paraffin. Renal sections (4 μm) were stained with Hematoxylin-Eosin (HE) or Periodic Acid Schiff (PAS) for light microscopic investigation. We evaluated and semiquantified the pathology of the kidney disease in the glomerular and tubulo-interstitial areas according to previously described scoring methods (8, 9). Focal glomerulosclerosis was defined as being positive when mesangial expansion caused by increases in PAS-positive material, adhesion formation and tuft collapse were present in one segment using the following scoring system: 0, 0%; 1, 1-25%; 2, 26-50%; 3, 51-75%; 4, 76-100%. The final average score in each animal was obtained by dividing the sum of the scores by the number of glomeruli examined. At least 50 glomeruli were assessed in each animal.

Immunohistochemistry

For macrophage detection, slides were incubated with F4/80 (anti-mouse CD68 antibody, Serotec, UK). T-cells were stained using CD3 without hematoxylin. Macrophage and T-cell infiltrates in the interstitium were assessed by counting the number of F4/80- and CD3-positive cells, respectively, in 30 non-overlapping cortical interstitial fields (>400). The mean number of positive cells per field was calculated for each animal by dividing the sum of positive cells by the number of high-power fields (hpfs) examined. The numbers of glomerular macrophages and T-cells were not scored since only a few of these cells were present. Alpha-smooth muscle actin (α-SMA) was stained with mouse anti-human-α-SMA antibodies cross-reacting with mouse (Dako). The slides were counterstained with PAS without hematoxylin. A histological assessment was performed by two independent observers using a blinded method.

Real-time quantitative polymerase chain reaction (qPCR)

Total RNA in the kidneys was extracted using Trizol Reagent then reverse-transcribed into cDNA using the Revert Aid Kit (Invitrogen, USA). cDNA was amplified using random primers obtained from an equal amount of RNA. The specific primers were as follows: CaMKIV, forward: 5'-CTC TCACACCCCAGACATCATAAAA-3', reverse: 5'-CTCACTGT AGATATCCCTTCTCCA-3'; α-SMA, forward: 5'-CCCAGAC ATCAGGGGATAATGG-3', reverse: 5'-TCTATCCGATACCT CAGCGTCA-3'; monocyte chemoattractant protein-1 (MCP-1), forward: 5'-TTAAAAACCCTGGATCGGAAACAAA-3', reverse: 5'-GCATTAGCTTCAGATTTACGGGT-3', TGF-B1, forward: 5'-ACCTGCAAGACACTGCAATGG-3', reverse: 5'-GTTTTCTCATAGATGGCGT-3', 18s rRNA forward: 5'-AA CCCGTTGAACCCCAT-3', reverse: 5'-CCATCCAAT CGG TAGTAGCC-3'.

Western blotting

The total proteins of the renal tissue samples were prepared according to standard procedures and quantified using the bicinchoninic acid method (Pierce, USA). Denatured proteins (30 μg per lane) were loaded onto a 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel. After electrophoresis, the proteins were transferred onto a polyvinylidene difluoride membrane (Millipore, USA) via electroelution. The membrane was incubated with primary antibodies against CaMKIV (1:1,000; Abcam, USA) overnight at 4°C. Antibodies against 18s rRNA (1: 5,000, Santa Cruz, USA) were used as an internal standard and incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG (1:10,000; Pierce, USA) for one hour at 4°C.
Table. General Features during the Development of Adriamycin-induced Nephropathy

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Vehicle-injected mice at week 6</th>
<th>Adriamycin-injected mice week 2</th>
<th>Adriamycin-injected mice week 4</th>
<th>Adriamycin-injected mice week 6</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum albumin, g/L</td>
<td>47.9±5.2</td>
<td>40.3±7.2*</td>
<td>36.9±4.5**</td>
<td>30.2±6.2*</td>
</tr>
<tr>
<td>Serum cholesterol, mmol/L</td>
<td>2.6±0.4</td>
<td>7.6±1.4**</td>
<td>8.4±1.1**</td>
<td>5.6±1.2**</td>
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<tr>
<td>Serum creatinine, μmol/L</td>
<td>47.4±2.6</td>
<td>49.4±5.3</td>
<td>55.7±3.7**</td>
<td>65.4±5.6**</td>
</tr>
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Data are presented as mean±SD for each group of 8 animals. * p<0.05, ** p<0.01, compared to the saline-injected mice.

Figure 1. Proteinuria and histology in adriamycin-injected mice. (A) 24-hour urinary protein excretion was corrected according to the urinary creatinine excretion in groups of eight normal (◊) and adriamycin-injected mice (■) at different time points. *p<0.05; ** p<0.01 vs. normal control. Significantly increased urine protein excretion in mice with adriamycin nephropathy. The data are presented as the mean±SEM (n=8 per group), *p< 0.05 vs. normal control. (B) Histology in the mice with adriamycin nephropathy compared with the normal controls. (C) KN-93 treatment significantly reduced the CaMKIV protein expression according to a Western blot analysis. Shown are CaMKIV and 18sRNA Western blot examples and the summary of the CaMKIV protein band intensities (expressed as ratio to 18sRNA, mean±SE, n=3 experiments). *p<0.05 compared with the vehicle.

Statistical analysis

The values are expressed as the mean ± SEM or mean ± SD. Comparisons were made with a one-way analysis of variance using the SPSS16.0 software package. p<0.05 was considered to be statistically significant.
Results

Adriamycin-induced nephrotic syndrome

The adriamycin-treated mice developed proteinuria, hypalbuminemia, hypercholesterolemia and increased serum creatinine indicative of nephropathy (Table). Prominent proteinuria occurred on day 5, peaked at week 3 and persisted throughout the study (Fig. 1A, Table). The mice were killed two weeks after adriamycin treatment, and the histology was evaluated. A morphological analysis of the adriamycin-treated mice showed focal podocyte proliferation with epithelial-to-fibroepithelial adhesions in the Bowman’s capsule and frequent podocytic hypertrophy as well as rare segmental sclerosis. The glomeruli appeared slightly swollen with Bowman spaces focally containing proteinaceous material. The tubules were dilated, contained variable numbers of hyaline casts and exhibited widespread degeneration and focal epithelial necrosis (Fig. 1B).

Inhibition of the CaMKIV expression in adriamycin-induced nephropathy by KN-93

KN-93 treatment significantly reduced the expression of CaMKIV in this model (Fig. 1C). To avoid interference with DMSO contained in the vehicle, CaMKIV activation was compared between the saline-treated and vehicle-treated mice. There were no significant differences in the activation of CaMKIV between the saline-treated and vehicle-treated mice (p>0.05). Additionally, there were no significant differences in the activation of CaMKIV between the normal mice injected with saline and the mice injected with vehicle (DMSO) (p>0.05).

KN-93 treatment reduced proteinuria and improved the renal function

Mice with established adriamycin-induced nephropathy treated with KN-93 exhibited reduced albuminuria at week 2; however, the difference did not reach statistical significance (Fig. 2a). There were no significant differences in the levels of serum albumin or cholesterol between the two groups at week 2 (p>0.05) (Fig. 2b, c). In contrast, by week 6, the amount of proteinuria in the KN-93-treated mice was significantly lower than that observed in the vehicle-treated controls (p<0.05). The serum cholesterol levels were also significantly lower and the serum albumin levels were significantly increased in the KN-93-treated mice compared to those observed in the vehicle-treated controls (p<0.05), which is a reflection of reduced protein loss in the urine. Furthermore, the KN-93-treated mice exhibited significantly lower serum creatinine levels compared to the vehicle-treated mice at week 6 (p<0.01) (Fig. 2d).
**Figure 3.** PAS and immunohistochemical staining of formalin-fixed sections of kidney tissue obtained from normal control mice (a, d, g, j) or adriamycin nephropathy mice treated with either vehicle only (b, e, h, k) or KN-93 (c, f, i, l) at week 6 (×400). a-c: PAS staining. d-f: Immunohistochemical staining of α-SMA in both the glomerulus and the tubulointerstitium (brown). g-i: Immunohistochemical staining of F4/80+ macrophages in the interstitium (brown). j-l: Immunohistochemical staining of CD3+ T-cells in the interstitium (brown).

**KN-93 attenuated glomerulosclerosis and tubulointerstitial lesions**

Glomerulosclerosis and tubulointerstitial lesions induced by adriamycin began to appear two weeks after injection and became very prominent at week 6 (Fig. 3b) in contrast to that observed in the normal controls (Fig. 3a). Treatment with KN-93 starting on day 5 did not alter the extent of glomerular and tubulointerstitial lesions observed at week 2. However, the glomerulosclerosis and tubulointerstitial lesions were significantly improved in the KN-93-treated mice at week 6 (Fig. 3c). The scores for glomerulosclerosis and tubulointerstitial injury were significantly lower in the KN-93-treated mice with adriamycin-induced nephropathy than
Figure 4. KN-93 treatment reduced renal histologic lesions and the α-SMA expression in mice with adriamycin nephropathy. Glomerulosclerosis (a), tubulointerstitial lesions (b) as well as the glomerular (c) and interstitial (d) expressions of α-SMA were quantitated in the kidneys of mice with adriamycin nephropathy treated with vehicle only (solid bar) or KN-93 (hatched bar) starting on day 5 until sacrifice at week 2 or week 6. Normal mice treated with vehicle for six weeks were used as normal controls (open bar). **p<0.01; compared to vehicle-treated mice; *p<0.01, compared to the normal control.

 KN-93 treatment suppressed the expression of α-SMA

In the normal mice kidneys, the expression of α-SMA was limited to arterial smooth muscle cells (Fig. 3d). In the mice with adriamycin-induced nephropathy, there was extensive glomerular and tubulointerstitial α-SMA staining by week 6 (Fig. 3e). The expression of α-SMA was significantly reduced in the KN-93-treated mice at week 6 compared to that observed in the vehicle-treated controls (p<0.01) (Figs. 3f, 4c, d).

 KN-93 inhibited interstitial macrophage and T-cell accumulation

The number of macrophages in the mice with adriamycin nephropathy significantly decreased by KN-93 treatment both at week 2 (6.1±0.7 cells/hpf vs. 8.1±1.1 cells/hpf, p<0.01) and week 6 (3.6±0.7 cells/hpf vs. 6.5±1.0 cells/hpf, p<0.01) compared to that observed in the vehicle-treated mice (Fig. 3g-i). The interstitial T-cell infiltration also significantly decreased by KN-93 treatment both at week 2 (4.2±1.2 cells/hpf vs. 5.5±1.9 cells/hpf, p<0.01) and week 6 (7.5±1.4 cells/hpf vs. 11.3±2.8 cells/hpf, p<0.01) (Fig. 3j-l).

Effects of KN-93 treatment on the expressions of TGF-β and MCP-1

The renal expression of TGF-β1 mRNA was significantly suppressed by KN-93 treatment at week 6 (2.8±1.4 vs. 1.3±1.0, p<0.01). The expression of MCP-1, an IFN-α-inducible gene, was also examined. Real-time RT-PCR showed that the MCP-1 mRNA expression was significantly upregulated in the mice with adriamycin nephropathy and significantly inhibited by KN-93 treatment at week 6 (3.6±1.9 vs. 1.7±1.0, p<0.01).

Discussion

In this study, CaMKIV signaling was activated in an experimental glomerulosclerosis model, accompanied with pro-
gressive focal segmental glomerulosclerosis (FSGS), tubulointerstitial damage and inflammatory infiltration, suggesting its involvement in the progression of chronic renal diseases. To further confirm the role of this pathway in disease progression, we treated nephritic mice with KN-93, a selective inhibitor of CaMKIV. KN-93 significantly reduced the amount of proteinuria, attenuated histological lesions, improved the renal function and suppressed inflammatory infiltration. Our results provide direct evidence that in vivo inhibition of the CaMKIV pathway slows the progression of glomerular diseases with a proteinuric state. In addition, we showed that 5% DMSO (in saline) itself does not cause renal damage compared with saline treatment (data not shown).

To determine the role of CaMKIV signaling during the progressive stage of chronic proteinuric renal diseases, we did not begin treating the mice with KN-93 until day 5 when proteinuria developed. Nine-day KN-93 treatment did not reduce the amount of albuminuria two weeks after induction of adriamycin nephropathy in spite of significant inhibition of interstitial mononuclear infiltration. However, at week 6, the proteinuria and histologic lesions were significantly reduced by KN-93 treatment. The occurrence of decreased inflammatory infiltration prior to reduction in proteinuria suggests that reduced proteinuria is most likely not a direct consequence of CaMKIV inhibition, but rather could be associated with the inhibition of inflammatory infiltration.

Inflammatory infiltration was prominent and associated with an increased renal expression of MCP-1 in this model. A previous report showed that proximal tubular cells exposed to protein exhibit increased production of chemokines and cytokines (10). Anti-MCP-1 gene therapy significantly reduces the severity of interstitial inflammation and fibrosis in animals with overload proteinuria (11). The depletion of CD8+ T-cells and macrophages and blocking the activation of T-cells protects against renal functional or structural damage in murine adriamycin-induced nephropathy models (12, 13). In this study, the inflammatory infiltration in the kidneys was significantly suppressed by KN-93 treatment. Our results suggest that KN-93 exerts a protective effect on adriamycin nephropathy via the inhibition of inflammatory accumulation and activation.

The expression of α-SMA by glomerular mesangial cells is correlated with subsequent glomerulosclerosis (14), and the tubular α-SMA expression is associated with tubular epithelial-myofibroblast trans differentiation (15). In this study, the de novo α-SMA expression in the glomerulus and tubulointerstitium was inhibited by KN-93 treatment, indicating that CaMKIV signaling participates in the development of renal fibrosis. The exact factors triggering the activation of CaMKIV in this model are unclear. Progressive renal damage is unlikely to be caused by the toxic effects of the chronic accumulation of adriamycin, which is rapidly cleared from the blood and the kidneys. More studies are needed to explore the underlying mechanisms.

In conclusion, the inhibition of CaMKIV signaling with KN-93 substantially reduced interstitial mononuclear infiltration and subsequent renal fibrosis in a murine model of FSGS. CaMKIV signaling may therefore represent a promising therapeutic target for chronic proteinuric nephropathies that progress to ESRD.

The authors state that they have no Conflict of Interest (COI).

References