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Norcantharidin Ameliorates Proteinuria, Associated Tubulointerstitial Inflammation and Fibrosis in Protein Overload Nephropathy

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Key Words
Norcantharidin · Proteinuria · Interstitial inflammation · Fibrosis · Nuclear factor-κB · Connective tissue growth factor

Abstract
Norcantharidin (NCTD), the demethylated analog of cantharidin isolated from *Mylabris*, is an anticancer drug routinely used against various human cancers in China. The aims of this study are to learn if NCTD has a protective action against severe proteinuria and consequent interstitial inflammation and fibrosis, and if the inhibition of nuclear factor-κB (NF-κB) and connective tissue growth factor (CTGF) by NCTD might be involved. Male Sprague-Dawley rats with protein overload nephropathy induced by intraperitoneally injected bovine serum albumin were used as a model. The histopathological examination of kidney tissue in the 9th week by light microscopy and scanning electron microscopy revealed that inflammatory cells had extensively infiltrated into the tubulointerstitial areas with interstitial fibrosis. The administration of NCTD at 0.1 mg/kg/day to the bovine-serum-albumin-injected animal models effectively reduced the proteinuria, and prevented the proteinuria-induced interstitial inflammation and fibrosis. Expressions of the NF-κB p65 subunit and CTGF, detected by immunohistochemistry, Western blotting and reverse-transcription polymerase chain reaction, were upregulated in protein overload nephropathy and were attenuated by NCTD. Inhibition of the expressions of the NF-κB p65 subunit and CTGF was one beneficial effect of NCTD. These results suggest that in addition to the antiproteinuric action of NCTD, due to its anti-inflammatory and antifibrotic effects as shown in the present study, it may become a therapeutic agent for proteinuria and its associated chronic inflammatory and fibrotic nephropathy.

Introduction

Tubulointerstitial lesions may determine the progression of chronic kidney disease independent of the type of the initial insult. Recent studies suggested that proteins filtered through the glomerular capillary, previously considered as a marker of the severity of renal lesions, have an intrinsic toxicity on the proximal tubular cells [1]. Proteinuria provokes a multitude of inflammatory and fibrogenic mediators, which contributes to the progression of renal disease by exacerbating tubulointerstitial damage [2]. There is an urgent need to identify therapeutic agents for limiting proteinuria and preventing the consequential

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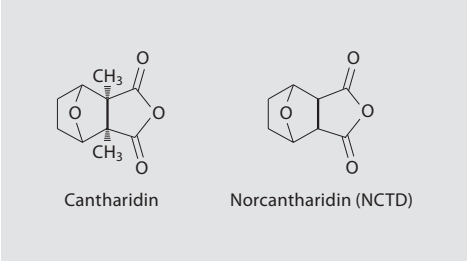

Fig. 1. Structures of cantharidin and NCTD.

renal inflammation or fibrosis more effectively. A specific antagonism of multiple injurious pathways might help to arrest or even reverse the progression of renal damage. Current protocols directed against nephroprogression focus on the inhibition of proteinuria, such as avoiding high dietary protein intake, stringent blood pressure control, restricting dietary sodium, the combined use of renin-angiotensin-aldosterone blockers, effective diuretic therapy, weight loss etc. [3]. However, despite these therapies, renal dysfunction continues to progress in the majority of patients. It is almost certain that simultaneously targeting different components of the proteinuric cascade would be an effective therapeutic approach.

Cantharidin used as an antitumor agent is the active constituent of *Mylabris* and is produced by different species of blister beetles. However, severe nephrotoxicity has been observed. Norcantharidin (NCTD), a synthetic demethylated analog of cantharidin (fig. 1), is now used in China as a routine anticancer drug against different kinds of digestive-tract cancers. The reason for its use is that the 2 methyl groups of cantharidin are not the main functional groups for its antitumor activity, and there are reduced nephrotoxic side effects with the demethylated analog [4, 5] . The molecular formula of NCTD is $C_8H_8O_4$ and molecular weight is 168.15. It has been demonstrated that NCTD was excreted mainly by the urinary route and seldom in feces [6]. Presently, the principal actions of NCTD emphasized the antiproliferative effect on some cancer cells in vitro by retarding progression through the cell cycle and inducing apoptosis [7]. NCTD was also found to stimulate the bone marrow production of white cells and to be a highly selective catalytic site-directed inhibitor of protein phosphatase (PP) 2B [8, 9]. It is known that the immunosuppressant drugs FK506 and cyclosporin A bind to the immunophilins, and these complexes selectively inhibit PP2B, leading to the suppression of T cell proliferation. Presently, FK506 and cyclosporin A are widely used as effective therapies for clinical proteinuric nephropathy. We therefore hypothesized that NCTD might attenuate proteinuria and its associated tubulointerstital lesion.

Materials and Methods

Experimental Protein Overload Nephropathy Model

The right kidney was removed from male Sprague-Dawley rats weighing from 150 to 200 g with negative tests of urinary protein and glucose. Protein overload nephropathy was induced as described previously [10]. After a week's rest, the uninephrectomized rats received intraperitoneal injections of bovine serum albumin (BSA). The BSA (Proliant Co., USA) was dissolved in saline (0.33 mg/ml). The dosage of BSA was 1 ml on the first day and was increased gradually to 3 ml, 5 days later. Rats with a 24-hour proteinuria of greater than 8 mg were used in this study and were injected daily with 5 g/kg BSA until its completion. The NCTD therapy began on the day when the 24-hour proteinuria of the rats was greater than 8 mg. The rats with protein overload nephropathy were randomly divided into 2 groups that were either treated or untreated with NCTD (0.1 mg/kg daily). An additional set of uninephrectomized rats which were given saline, served as the control group (n = 9 in each group). The rats were housed in a temperature controlled room at 22°C with ad libitum access to commercial standard rat food and water during the entire study. The rats were sacrificed in the 9th week of the experiment. The animal care protocols in this study were approved in advance by an appropriate animal care and use committee.

Urine and Blood Measurements

The rats were placed in metabolic cages with free access to water. Twenty-four-hour urine samples were collected for urinary protein quantitation using the Bio-Rad method after 1, 5 and 9 weeks of treatment. Blood samples were obtained from the abdominal aorta at sacrifice in the 9th week and were used for measuring routine blood, liver function including albumin, globin and alanine transaminase, renal function including blood urea nitrogen and creatinine clearance rate.

Renal Histology

A portion of the left kidney was removed from the animal and fixed with 4% paraformaldehyde, then embedded in paraffin for light-microscopic evaluation. The paraffin sections were stained with hematoxylin-eosin, periodic acid-Schiff reagent (PAS), Masson's trichrome, and periodic acid-silver methenamine (PASM). In all proteinuric rats, a mononuclear infiltrate was present in the renal interstitium, as evidenced by morphometric analysis of PAS-stained sections. Interstitial fibrosis was assessed in sections of kidneys stained by the PASM stain. The proportion of the cortex that was infiltrated by inflammatory cells or fibrous tissue was evaluated according to previously published methods [11]. Sections were coded by an investigator, then digitally photographed and analyzed by another blinded investigator. Photographs of each section were taken using a stereomicroscope fitted with a digital camera (CX41 light microscopy, Olympus, Japan).

Using 2 PAS- and 2 PASM-stained sections from each animal, 6 random low-power (×200 magnification) photographs were taken of each section (representing approximately 10% of the cortex) and morphometrically analyzed using point counting. To this end, a standardized grid of 70 points was superimposed on each photograph. The number of points over glomeruli, tubular epithelia or interstitial tissues was determined and used to assess the proportion of the cortex occupied by each structure.

In addition, tubulointerstitial injuries, seen as tubular dilation and/or atrophy, interstitial fibrosis and inflammatory cell infiltration, were graded by the following semiquantitative score: 0, no changes; 1, focal changes involving 25% of the sample; 2, changes affecting 25–50%; 3, changes involving 50–75%; 4, lesions affecting 75% or more. These examinations evaluated the areas overlying the tubular basement membrane and interstitial space, but avoided the glomeruli and large vessels. Twenty cortical tubulointerstitial fields were randomly selected and assessed by an observer who was blinded to the experimental groups, and the mean values were calculated for each specimen.

Scanning Electron Microscopy

A portion of the left kidney from 3 rats in each of the groups was fixed with ice-cold 3.75% glutaraldehyde in phosphate buffer (pH 7.4) at 4°C overnight, and postfixed with 1% osmium tetroxide in phosphate buffer at 4°C for 2 h, then dehydrated in a graded ethanol series (15, 35, 70, 95 and 100%) for 10 min in each step, and embedded in an Epon mixture. Ultrathin sections were cut with a glass knife, stained with a lead citrate-uranyl acetate solution and observed with an electron microscope.

Immunohistochemistry

Paraffin-embedded tissue sections were dewaxed, hydrated, treated with 5 mmol/l levamisole for blocking endogenous alkaline phosphatase and incubated with blocking serum for 30 min at room temperature to reduce nonspecific background staining. Sections were rehydrated in PBS-0.1% BSA for 15 min before the addition of the appropriate blocking serum for an additional 15 min. Sections were incubated with polyclonal mouse anti-rat nuclear factor κB (NF-κB) p65 1:100 (Abcam, UK) and rabbit anti-rat connective tissue growth factor (CTGF) 1:100 (Abcam, UK) overnight at 4°C. After rinsing, the sections were incubated with biotinylated goat anti-rabbit IgG (Abcam, UK) and processed using an alkaline phosphatase-streptavidin-biotin immunoperoxidase method (Maixin Biotechnological Company, China). The tissue sections were counterstained with hematoxylin. Negative controls for specific labeling were performed in parallel by replacing the primary antibody with a normal rabbit serum. Renal cortex sections were digitally imaged and quantitatively examined using the computer-assisted image analysis software (CX41 light microscopy, Olympus). For each renal cortex section, NF-κB p65 and CTGF expressions were analyzed in 10 nonoverlapping random fields viewed at a magnification of ×200 and expressed as the percentage of area occupied by NF-κB p65 and CTGF staining, and graded according to a threshold that was arbitrarily considered as positive. In addition, NF-κB p65 staining was localized within the cell cytoplasm and/or nucleus, and the nuclear localization of NF-κB p65 was categorized as positive expression. The rate of the nuclear localization of p65 was calculated according to the recommendation of Nakayama et al. [12] by counting positive NF-κB staining nuclei of the total cells and calculating the percentage. An independent observer evaluated the immunostaining results in a blinded manner.

Western Blotting Analysis

Renal cortex samples were homogenized in 1 ml of lysis buffer (50 mmol/l Hepes, pH 7.5, 150 mmol/l NaCl, 1.5 mmol/l $MgCl_2$, 1 mmol/l ethylene glycol tetraacetic acid, 10% glycerol, 1% Triton X-100, 1 g/ml aprotinin, 1 g/ml leupeptin, 1 mmol/l phenylmethyl sulfonyl fluoride, 0.1 mmol/l sodium orthovanadate) at 4°C. After centrifugation, soluble lysates (60–80 μg) were loaded in each lane and separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membranes. Membranes were blocked with 5% nonfat dry milk in Tris-buffered saline/0.5% Tween-20 for 1 h, washed with Tris-buffered saline/Tween-20, and incubated with rabbit polyclonal CTGF antibody (1:2,000; Abcam, UK). Blots were rinsed with Tris-buffered saline/Tween-20 and subsequently incubated with horseradish-peroxidase-conjugated anti-rabbit IgG (1:10,000; Abcam, UK). After washing with Tris-buffered saline/Tween-20, the blots were developed with the enhanced chemiluminescence method (Amersham, UK). The intensity of the identified bands was quantified by densitometry. Results were expressed as arbitrary densitometric units.

Measurement of Renal CTGF and NF-κB p65 mRNA Levels by Reverse-Transcription Polymerase Chain Reaction

Total cellular RNA was extracted using the Trizol reagent kit (Invitrogen, USA) according to the manufacturer's instructions. RNA samples were quantified with a spectrophotometer, then were reverse transcribed using a commercial kit (Fermentas Company, Lithuania). NF-κB p65 primers (Ying Jun Biotechnological Limited Company, China) were chosen to yield an expected product of 198 bp. The sense primer sequence was 5′-GCGGCCAAGCTTAAGATCTGCCGAGTAAAC-3′, and the antisense primer sequence was 5′-GCTGCTCTAGAGAACACAATGGCCACTTGCCG-3′. CTGF primers (Ying Jun Biotechnological Limited Company) were chosen to yield an expected product of 383 bp. The sense primer sequence was 5′-CTAAGACCTGTGGAATGGGC-3′, and the antisense primer sequence was 5′-CTCAAAGATGTCATTGCCCCC-3′. The housekeeping gene β-actin (593 bp) was co-amplified with CTGF, and NF-κB as an internal control and specific primers were designed according to the published sequence. The sense primer sequence was 5′-TGGGTCAGAAGGACT CCTATG-3′ and the antisense primer sequence was 5′-CAGGCAGCTCATAGCTC TTCT-3′. Polymerase chain reaction (PCR) conditions for CTGF were 30 cycles with denaturation at 95°C (40 s), annealing at 55°C (40 s), and extension at 72°C (50 s). The PCR conditions for NF-κB were 30 cycles with denaturation at 95°C (40 s), annealing at 62°C (40 s) and extension at 72°C (50 s). Aliquots of the PCR products were run on 2.5% agarose gels. The relative quantities of CTGF and NF-κB p65 mRNA in the kidney cortex were estimated as the ratio of band density of CTGF and NF-κB versus that of β-actin.

Statistical Methods

Data are expressed as the mean ± SEM. Groups were compared using an ANOVA and Student's t test. $p < 0.05$ was considered to be statistically significant.

Table 1. NCTD ameliorated the 24-hour urine protein excretion

Group	Proteinuria, mg/24 h		
	1 week	5 weeks	9 weeks
Saline	4.2 ± 0.45	8.9 ± 1.4	13.1 ± 1.7
BSA	17.2 ± 1.8[a]	247.7 ± 37.5[a]	114.9 ± 21.4[b]
NCTD	20.9 ± 4.7[a]	87.1 ± 24.5[c]	41.6 ± 10.1[b]

[a] $p < 0.01$ and [b] $p < 0.05$ compared with the saline group, [c] $p < 0.05$ compared with the BSA group.

Table 2. Effects of NCTD on routine blood elements in rats with protein overload nephropathy

Group	White blood cells $\times 10^9$/l	Hemoglobin g/l	Platelets $\times 10^9$/l
Saline	8.5 ± 1.2	133.1 ± 8.0	502.5 ± 106.2
BSA	8.6 ± 1.1	132.3 ± 13.7	877.2 ± 77.5
NCTD	8.8 ± 0.9	119.1 ± 20.1	792.4 ± 68.5

Table 3. Effects of NCTD on liver and renal functions in rats with protein overload nephropathy

Group	ALT, U/l	TP, g/l	Alb, g/l	BUN, mmol/l	C_{cr}, ml/min
Saline	46.5 ± 6.27	66.8 ± 7.5	20.7 ± 2.6	10.1 ± 1.2	0.67 ± 0.11
BSA	44.2 ± 4.8	71.5 ± 7.1	18.7 ± 3.2	14.3 ± 1.0[a]	0.12 ± 0.02[b]
NCTD	38.9 ± 4.3	66.4 ± 10.2	20.0 ± 3.0	12.2 ± 0.8[d]	0.77 ± 0.20[c]

[a] $p < 0.01$ and [b] $p < 0.05$ compared with the saline group, [c] $p < 0.01$ and [d] $p < 0.05$ compared with the BSA group. ALT = Alanine transaminase; TP = total protein; Alb = albumin; BUN = blood urea nitrogen; C_{cr} = creatinine clearance rate.

Results

Effect of NCTD on Proteinuria in Rats with Protein Overload Nephropathy

Severe proteinuria was induced in the rats by BSA injection, which peaked during the 5th week after the BSA injection and gradually decreased until the 9th week. NCTD decreased the proteinuria by an additional 65% at weeks 5 and 9 (table 1).

Effect of NCTD Treatment on Routine Blood and Serum Biochemistry in Rats with Protein Overload Nephropathy

The routine blood, liver and renal functions were checked in all the groups of rats. The rats who had been administered BSA had proteinuric nephropathy, increased serum blood urea nitrogen and decreased creatinine clearance rate. NCTD treatment significantly ameliorated the renal function impairment. In contrast, no differences were observed in the white blood cells, the red blood cells, the platelets and liver functions (including serum albumin, globin and alanine transaminase) between the BSA, NCTD treated or the saline control groups. These results suggest that renal function in the model was slightly impaired by BSA administration, and that NCTD can protect renal function and is non-toxic to the bone marrow, liver and kidney of rats (tables 2 and 3).

Effect of NCTD on Proteinuria-Associated Interstitial Inflammation and Fibrosis

No significant renal morphological lesions were seen by light microscopy and scanning electron microscopy in the rats of the saline control group. However, animals receiving BSA developed a marked tubular atrophy, interstitial infiltration of inflammatory cells, and extensive fibrosis. In the preliminary experiment, we found that the tubulointerstitial injury was severer in the rats who had the BSA intraperitoneal injection for 9 weeks than those who had it for 5 weeks, although the maximum increase in proteinuria was seen in the 5th week and gradually decreased over time until the 9th week, and therefore we chose the BSA administration for 9 weeks in the following experiment. We can see that treatment of the rat models with NCTD led to a significant reduction of tubulointerstitial monocyte/macrophage infiltration and markedly ameliorated the severity of the tubulointerstitial fibrosis (fig. 2–4). By analysis, the scores of tubulointerstitial injury in saline, BSA and NCTD groups were

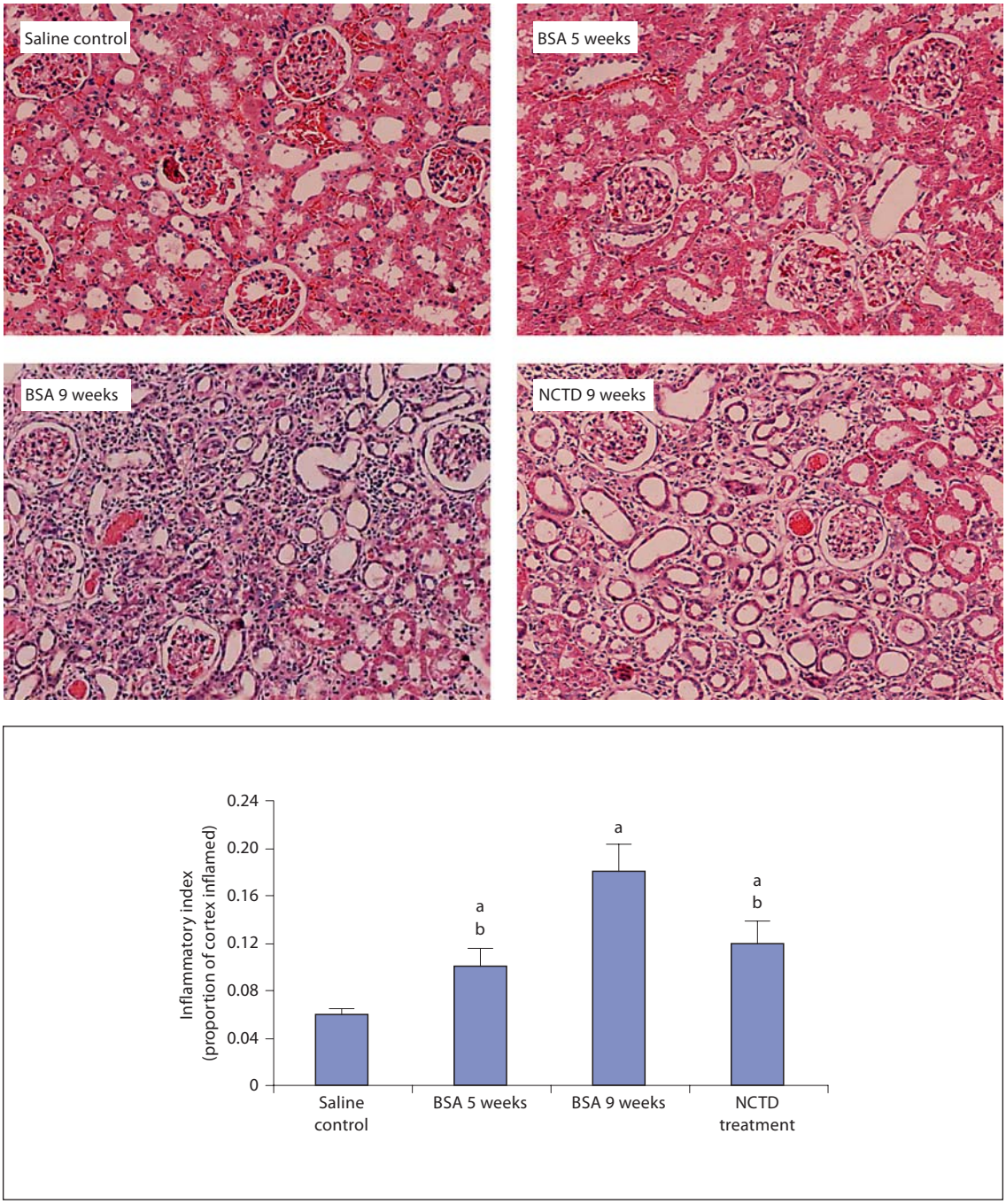

Fig. 2. NCTD ameliorates interstitial inflammation that develops in proteinuric animals. There was no infiltration of inflammatory cells in renal interstitium in saline control. Mild interstitial inflammatory infiltrate was shown in BSA administration for 5 weeks. There was marked tubulointerstitial inflammation in BSA-injected rats after 9 weeks. Treatment of BSA rats with NCTD significantly inhibited the tubulointerstitial inflammation. Morphometric analysis of tissue demonstrates that proteinuria-induced interstitial inflammation was ameliorated by NCTD treatment. [a] $p < 0.01$ versus saline control; [b] $p < 0.01$ versus BSA injection for 9 weeks. PAS. ×200.

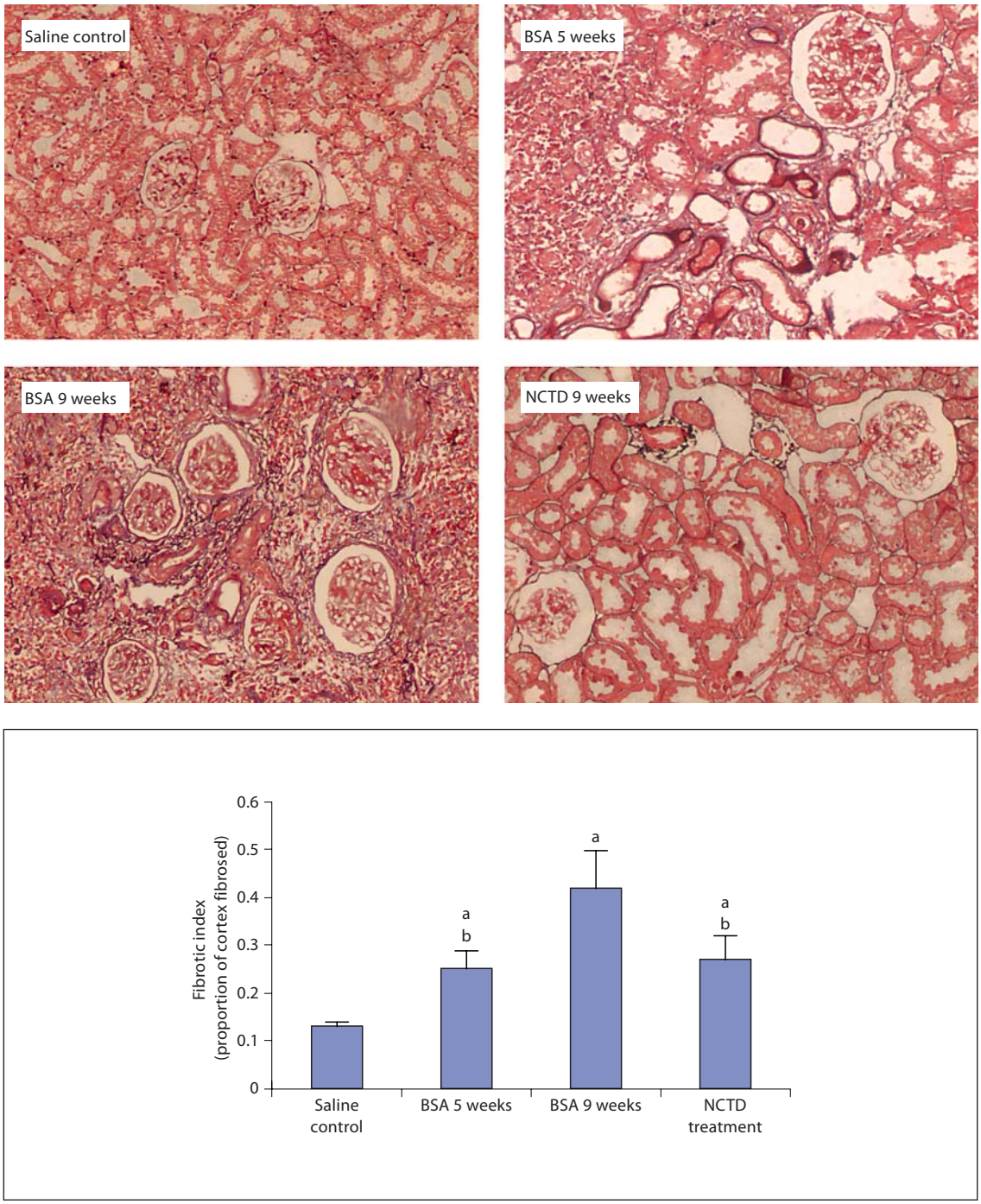

Fig. 3. NCTD lessens interstitial fibrosis in proteinuric animals. There was no cortical interstitial fibrosis or tubular atrophy in controls. BSA administration was associated with an increase in interstitial fibrosis and atrophic tubules and this kind of change worsened over time. There was more marked tubulointerstitial fibrosis in BSA-injected rats after 9 weeks than that in BSA rats injected for 5 weeks. Treatment of BSA rats with NCTD was associated with a reduction in both interstitial fibrosis and tubular atrophy. Morphometric analysis of the tissue demonstrates that proteinuria-induced interstitial fibrosis was ameliorated by NCTD treatment. [a] $p < 0.01$ versus saline control; [b] $p < 0.01$ versus BSA injection for 9 weeks. PASM. ×200.

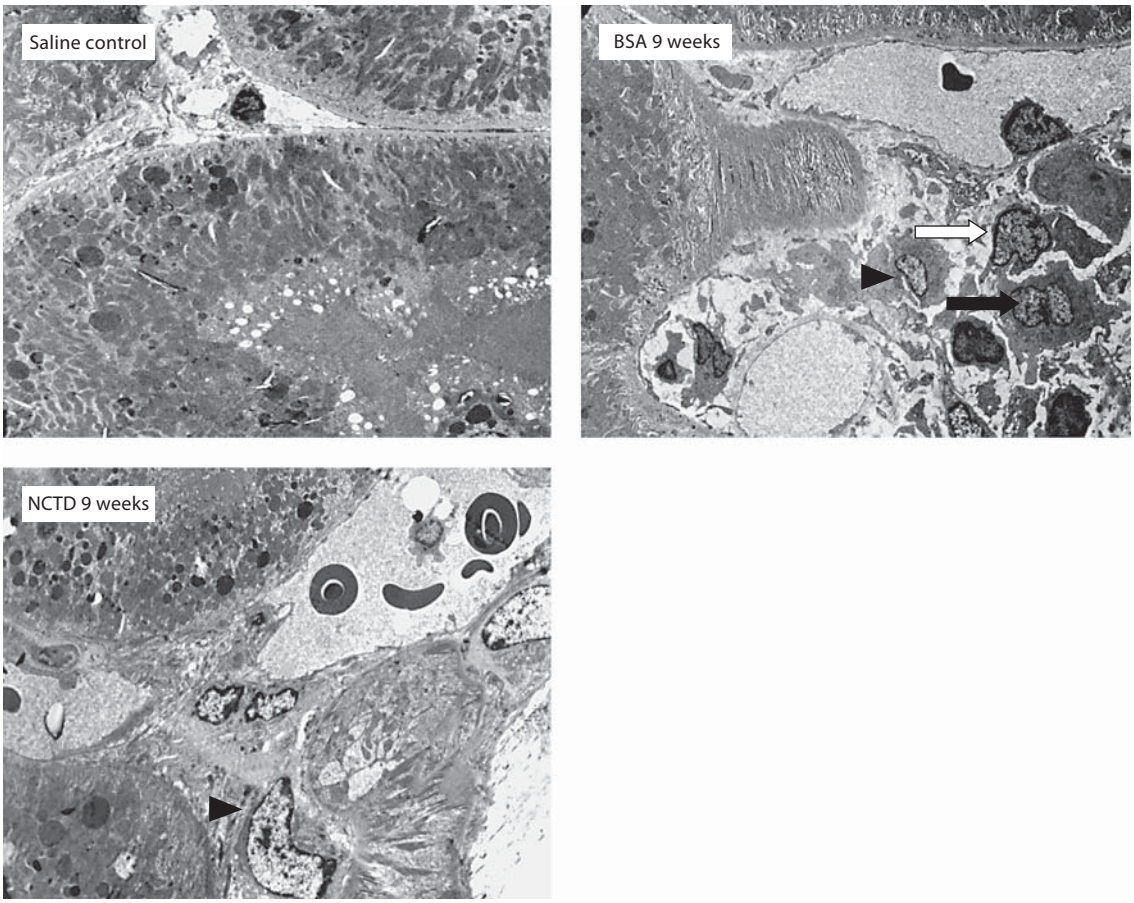

Fig. 4. Interstitial ultrastructure of the kidney in proteinuric animals treated with NCTD. Tubular epithelial cell and interstitium were normal in the saline control group. There were lots of inflammatory cells including macrophages (white arrow), fibroblasts (arrowhead) and lymphocytes (black arrow) in interstitium with the extracellular matrix increasing in the group of BSA injections for 9 weeks. NCTD recovered the shape of the tubular epithelial cells and significantly lessened the inflammatory cells and fibroblasts in the interstitium (n = 3 in each group). ×5,000.

0.75 ± 0.29, 3.12 ± 0.86 and 1.88 ± 0.52, respectively ($p < 0.05$). NCTD attenuated both inflammation and fibrosis in proteinuria nephropathy.

Effect of NCTD on NF-κB p65 Subunit Expression in Rats with Protein Overload Nephropathy

Immunohistochemical analyses revealed that the NF-κB p65 subunit deposition was mainly found in the cytoplasm of the kidneys obtained from the saline control rats, with slight expression in the nucleus of the proximal tubules and to a lesser extent in the glomeruli, which indicates that NF-κB was present in its inactive form. The administration of BSA to rats increased the nuclear NF-κB p65 expression in tubules, glomeruli and lymphocytes/monocytes in the interstitium, suggesting translocation of NF-κB to the nucleus. The number of NF-κB p65 nuclear localizations in renal sections obtained from the rats treated with NCTD was significantly lower than that in the BSA group (fig. 5). Semiquantitative analyses of the reverse-transcription (RT) PCR revealed that the level of the NF-κB p65 subunit mRNA expression was significantly inhibited by NCTD therapy (fig. 6). The concomitantly decreased progression of interstitial inflammation was associated with a decreased positive expression area of NF-κB p65, NF-κB-p65-positive cells and expression of NF-κB p65 subunit mRNA in rats that were treated with NCTD ($r = 0.69$, 0.64 and 0.80, respectively, $p < 0.05$). These results showed that NCTD could significantly inhibit NF-κB p65 expression and interstitial inflammation induced by NF-κB.

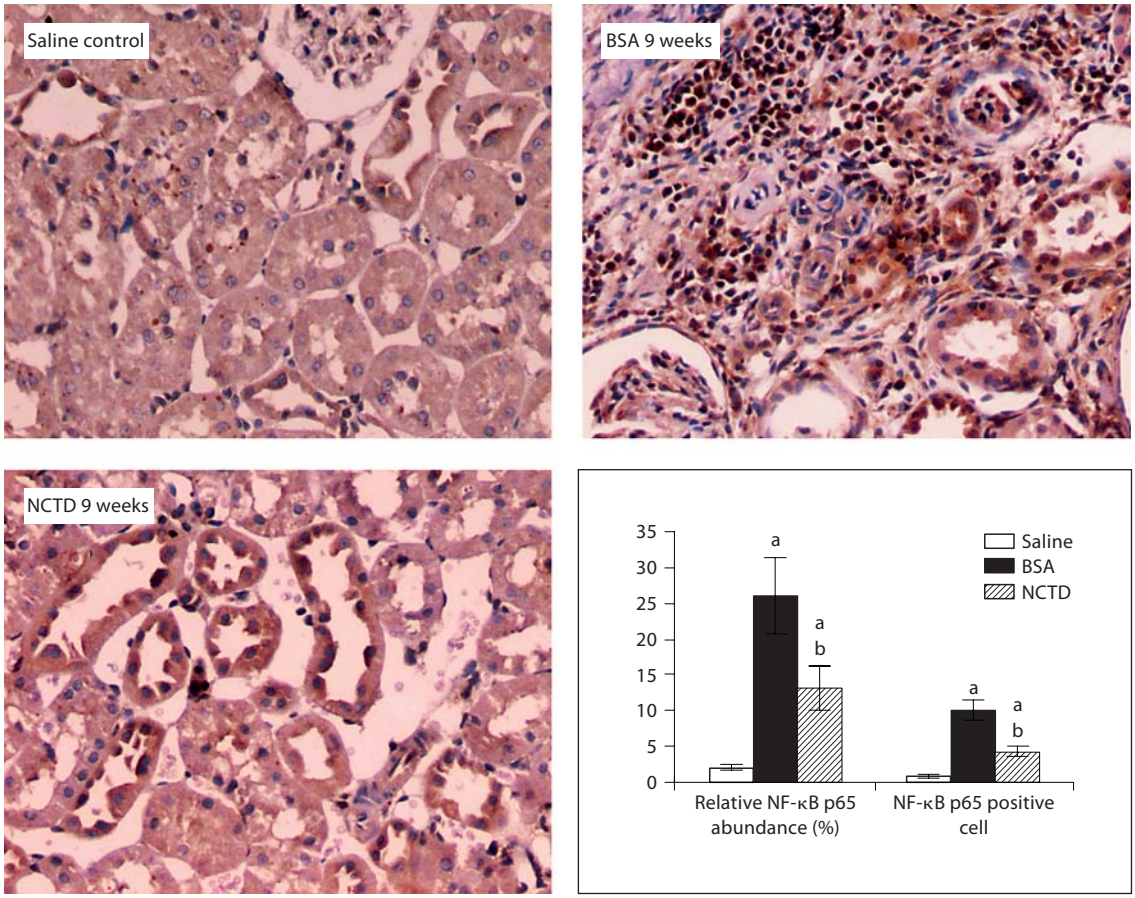

Fig. 5. NCTD inhibits NF-κB p65 subunit distribution in protein overload nephropathy. In saline control rats, there is slight NF-κB p65 subunit staining in the cytoplasm of proximal tubules and to a lesser extent in glomeruli, whereas the BSA group is associated with a dramatic increase in the NF-κB p65 subunit staining and the nuclear localization of NF-κB p65. NCTD treatment was associated with a reduction in the extent of NF-κB p65 subunit deposition (n = 9 in each group). [a] $p < 0.01$ versus control; [b] $p < 0.05$ versus BSA group. ×200.

Effect of NCTD on CTGF Expression in Rats with Protein Overload Nephropathy

Immunohistochemical analyses revealed that there was no CTGF staining in the glomeruli and tubules in rats treated with the saline, but it was significantly higher in the BSA group. Staining was mainly in the tubular epithelial cells, mesangial cells and interstitial infiltrating cells, and, to a lesser extent, in the glomeruli (fig. 7). Western blotting results showed that CTGF expression was markedly elevated in the BSA group when compared to the saline control group (fig. 8). Semiquantitative analyses of the RT-PCR revealed that the relative quantity of CTGF mRNA was significantly elevated by 2.1-fold in BSA-injected kidneys as compared to the control kidneys. NCTD treatment markedly inhibited CTGF mRNA expression by 1.8-fold (n = 9) compared with the BSA group (fig. 9). Related observations revealed that tubulointerstitial fibrosis was associated with the induction of a positive expression area of CTGF abundance, CTGF protein expression and CTGF mRNA expression ($r = 0.68$, 0.70 and 0.67, respectively, $p < 0.05$). These increases were suppressed by NCTD treatment, indicating that NCTD efficiently reduced CTGF expression and tubulointerstitial fibrosis induced by CTGF.

Discussion

Persistent proteinuria is a major risk factor for the development of tubulointerstitial damage. Therapeutic measures that reduce proteinuria will slow or halt the progression of proteinuric nephropathies [13]. Inhibiting

the angiotensin-converting enzyme or blocking the angiotensin receptors were found to consistently reduce proteinuria and prevent severe structural injury, including interstitial lesions [14–17]. However, angiotensin-converting enzyme inhibitors and angiotensin receptor blockers mostly retard, rather than stabilize, renal function deterioration in various nephropathies, therefore the cessation of renal disease progression in the long term is not expected to be achieved with these 2 types of medication. Another proteinuric therapeutic treatment is the administration of various steroids or cytotoxic drugs, such as cyclophosphamide, cyclosporine A, chlorambucil and mycophenolate mofetil. But there is evidence that glucocorticoids do not alter the expression of some growth factors, such as transforming growth factor-β1 and CTGF, known to influence the development or progression of renal diseases, and exacerbate proteinuria in rats having mesangial proliferative glomerulonephritis [18]. Cytotoxic drugs are ineffective in the treatment of nonimmunologically mediated renal disease with progressive renal impairment, with accompanying undesirable side effects. Efforts to control interstitial inflammation and fibrosis in proteinuria with steroids and immunosuppressive agents have been disappointing.

Therefore, despite the considerable advances in unraveling the pathogenesis of proteinuria, no particular treatment for the proteinuria and consequential tubulointerstitial damage is available as the universally acceptable standard. Obviously, there is an urgent need to develop new therapeutic agents for the treatment of proteinuria and consequent tubulointerstitial damage. Some Chinese herbal medicines including *Astragalus*, *Ligusticum*, triptolide and rhubarb, possess a range of pharmacological properties for retarding progressive chronic kidney disease. The effects of these herbals are multifunctional and multitargeted, and often are associated with a reduction in proteinuria, inflammation and inhibition of transforming growth factor-β overproduction [19]. *Mylabris*, the dried body of the Chinese blister beetle, has been used as a medicine for over 2,000 years. Its active constituent, cantharidin, has antitumor properties and causes leukocytosis. However, besides interference with mitochondrial respiration, severe nephrotoxicity has been observed. NCTD, the demethylated form of cantharidin, with less kidney toxicity and metabolic disturbances, is now used in China as a routine anticancer drug rather than cantharidin, because the 2 methyl groups of cantharidin are not the main functional groups for antitumor activity [20, 21]. It was reported that NCTD inhibits the proliferation and growth of a variety of human tumor cell lines

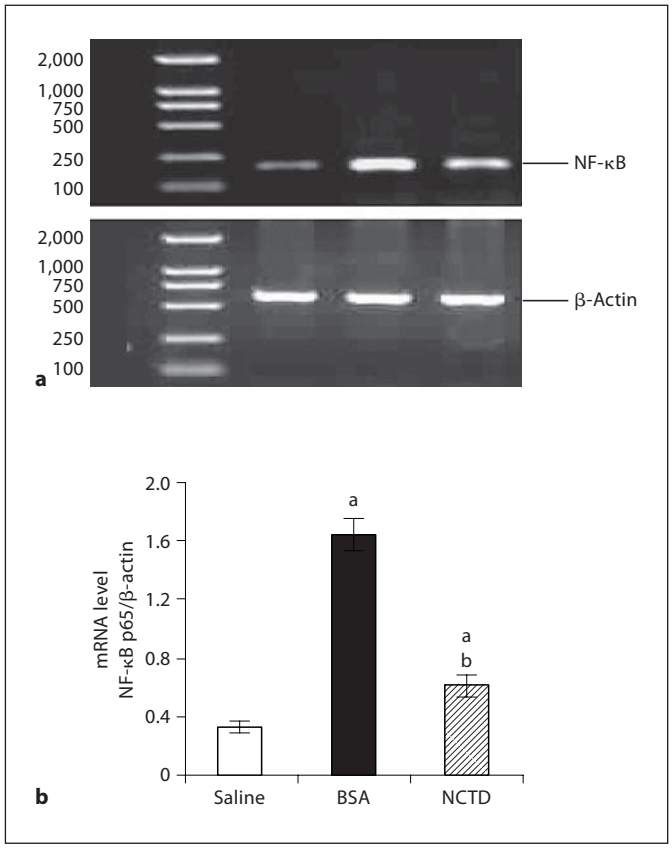

Fig. 6. NCTD inhibits the level of NF-κB p65 mRNA expression in protein overload nephropathy (n = 9 in each group). A representative RT-PCR experiment in different animals from the 3 groups (**a**) and data of densitometric analysis expressed as arbitrary units of mean SEM from each group (**b**). [a] $p < 0.01$ versus control; [b] $p < 0.01$ versus BSA group.

in vitro, and is used to treat human cancers with stimulation of the bone marrow and increase in the peripheral leukocyte count [7, 22–24]. NCTD appears to be an attractive potential therapeutic agent in cancer chemotherapy in western countries. However, there are no reports describing the properties of NCTD administration for alleviating proteinuria and tubulointerstitial damage.

The first objective of this study was to explore the effect of NCTD on the proteinuria and the progression of interstitial injury induced by proteinuria. Protein overload nephropathy is considered an appropriate experimental model to investigate the relationship between proteinuria and interstitial damage. The BSA overload model is frequently used because it is highly reproducible and can induce heterologous as well as autologous proteinuria [25]. In the present study, the protein overload

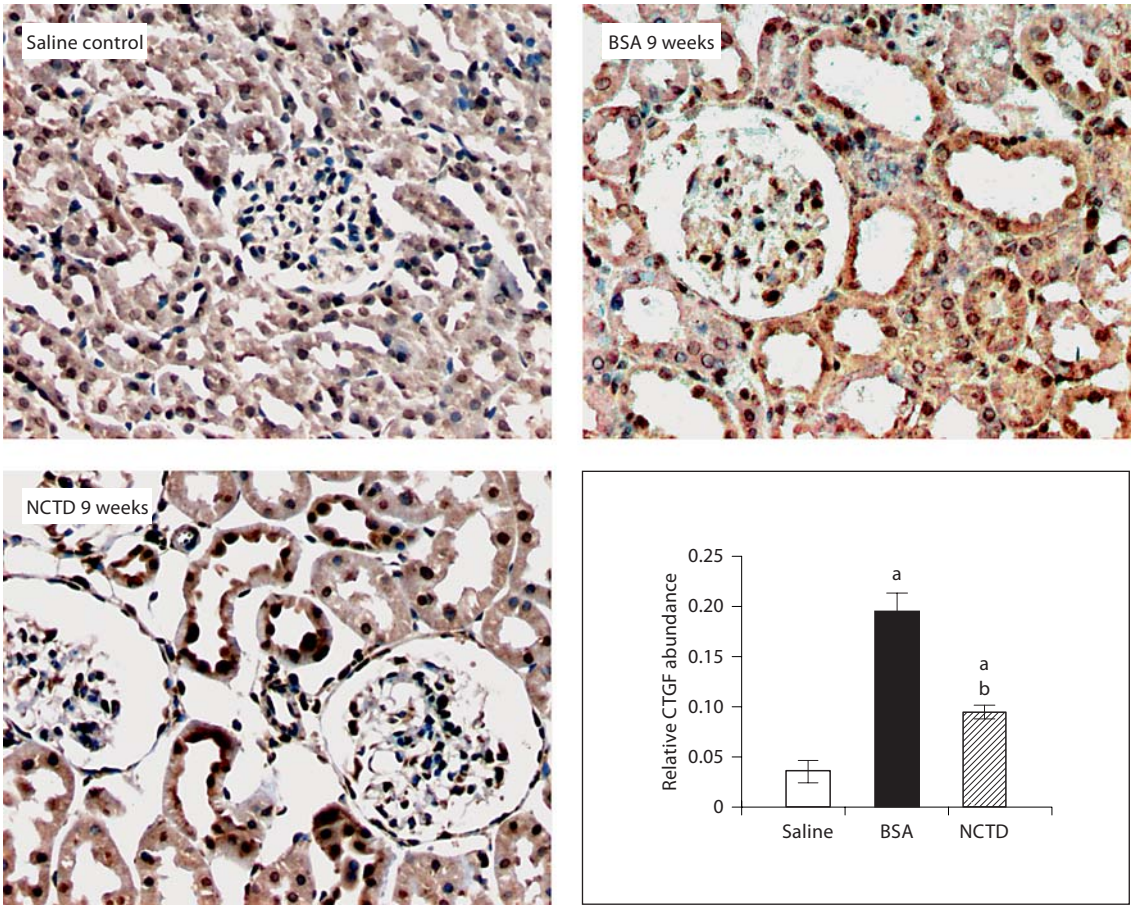

Fig. 7. NCTD inhibits CTGF distribution in protein overload nephropathy. In saline control rats, there is slight elevation but in the BSA group there is a dramatic increase in CTGF staining. NCTD treatment was associated with a reduction in the extent of CTGF deposition (n = 9 in each group). [a] $p < 0.01$ versus control; [b] $p < 0.05$ versus BSA group. ×200.

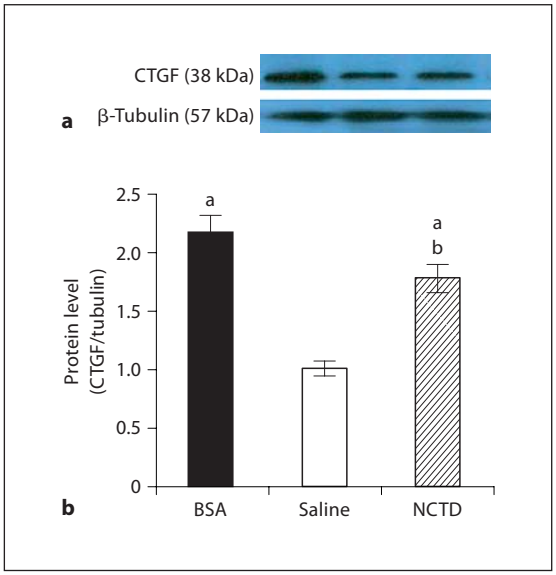

Fig. 8. NCTD downregulates CTGF protein production in protein overload nephropathy (n = 9 in each group). A representative western blot of CTGF and β-tubulin were used as a loading control (**a**). Results of total CTGF production obtained from densitometric analysis, and expressed as the ratio CTGF/β-tubulin of n-fold over control (**b**). [a] $p < 0.01$ versus control; [b] $p < 0.05$ versus BSA group.

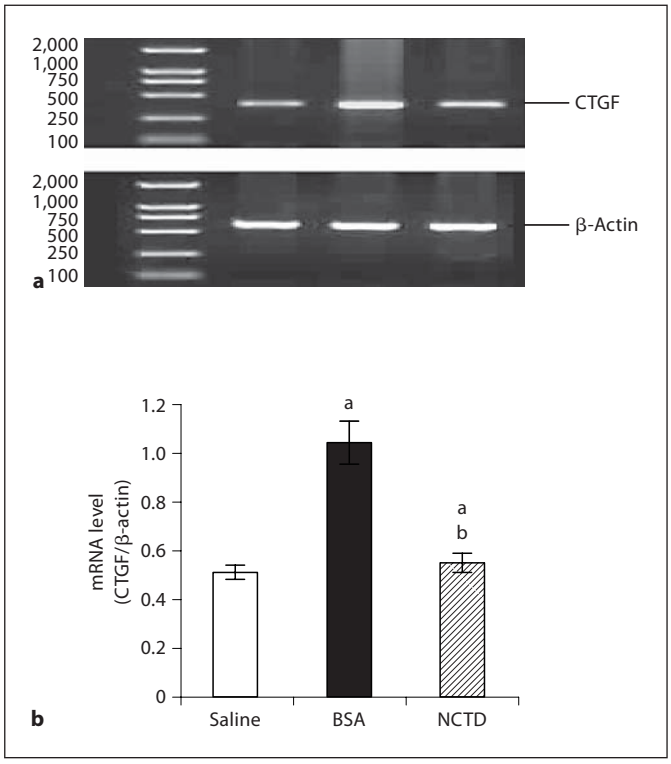

Fig. 9. NCTD inhibits the expression of CTGF mRNA in protein overload nephropathy (n = 9 in each group). A representative RT-PCR experiment in different animals of 3 groups (**a**) and data of densitometric analysis expressed as arbitrary units of mean SEM from each group (**b**). [a] $p < 0.01$ versus control; [b] $p < 0.01$ versus BSA group.

nephropathy model was produced in rats, resulting in interstitial inflammation and fibrosis development in the 9th week. A relatively low dose of NCTD resulted in a substantial reduction in proteinuria in this model, and markedly ameliorates the development of the intrarenal inflammation and interstitial fibrosis characteristic of protein overload nephropathy. However, the effects of NCTD on tubulointerstitial disease cannot be completely explained by the reduction in proteinuria, which occurred relatively late and well after the interstitial inflammation and fibrosis had already occurred. The ability of NCTD to reduce proteinuria is obviously of potential clinical importance and may represent a viable treatment option for proteinuric renal diseases in humans. Moreover, our study revealed that NCTD had no influence on routine blood, serum blood urea nitrogen, creatinine, and the level of albumin, globin and alanine transaminase, indicating that it is well tolerated and would be a relatively safe drug.

Persistent proteinuria triggers tubulointerstitial damage by inducing interstitial inflammation and fibrosis. The other goal of this study was to elucidate for the first time the mechanisms of the beneficial effects of NCTD on the development of tubulointerstitial damage caused by proteinuria.

Several publications indicate that the interaction of proximal tubular cells with proteinuric tubular fluid results in their production of proinflammatory mediators by activation of NF-κB [26–29]. The incubation of tubular epithelial cells with albumin, in concentrations similar to those found in the urine from patients with nephrotic syndrome, induced an increase in the NF-κB activity and an upregulation of proinflammatory molecules [30, 31]. These data suggest that NF-κB is activated in the presence of increased protein traffic. The predominant form of NF-κB is a heterodimer of p65 and p50 proteins. The presence of p65 in the cytoplasm of renal cells suggests that the NF-κB heterotrimeric complex was still in its inactive form. In contrast, the localization of p65 in the nucleus indicated that the NF-κB translocated into the nucleus and, therefore, was able to activate the transcription of NF-κB-dependent genes. The present study demonstrated that proteinuria induced by BSA administration led to activation of NF-κB, which confirms previous reports. Our results showed that renal cortical NF-κB mRNA and protein expression were increased in rats with severe proteinuria and renal damage. The score of tubulointerstitial lesions was related to NF-κB p65 mRNA and the positive expression of NF-κB p65. NCTD significantly prevented this increase, although interstitial monocyte/macrophage infiltration and tubular injury were only partially reduced. NCTD markedly attenuates the interstitial inflammatory responses associated with proteinuria-induced renal disease.

In the kidney, enhanced expression of CTGF had been found in proliferative and fibrotic lesions of various human and experimental renal diseases, including glomerulonephritis and diabetic nephropathy. CTGF, as a downstream mediator of transforming growth factor-β1, is a novel profibrotic factor that contributes to renal fibrosis and tubuloepithelial transdifferentiation [32–34]. In this study, CTGF expression was upregulated in rats with protein overload nephropathy and related to tubulointerstitial fibrosis. The proteinuria-associated interstitial fibrosis might be induced by protein filtered from glomeruli triggering CTGF production. NCTD significantly inhibited renal fibrosis with concomitant inhibition of CTGF gene and protein expression. These results suggest that the antifibrotic effects of NCTD may be mediated by

CTGF in this animal model of protein overload nephropathy. This is the first demonstration that the blockade of CTGF by NCTD treatment in vivo significantly ameliorates the development of renal interstitial fibrosis.

Cantharidin is well known as a potent serine/threonine PP1 and PP2A inhibitor, with less potent inhibitory activity for PP2B. However, NCTD was found to be a highly selective PP2B inhibitor without inhibiting PP1 or PP2A. The immunosuppressant drugs FK506 and cyclosporin A bind to immunophilins, and these complexes selectively inhibit PP2B (calcineurin), leading to the suppression of T cell proliferation [35]. A previous study demonstrated that NCTD could markedly inhibit lymphocyte proliferation stimulated by the mitogens concanavalin A or lipopolysaccharide in vitro [36]. Further studies are needed to determine whether the protective effect of NCTD is similar to FK506 and cyclosporin A by inhibiting infiltration of a T-cell-mediated renal injury in the proteinuric kidney.

In this study, we provide novel evidence that NCTD, in relatively low doses compared with the dosages used for antitumor therapy (usually, the dose of NCTD used in humans is 20–30 mg/day and in rats is 0.5–1 mg/kg/day), substantially ameliorates the progression of proteinuria-induced renal disease by at least three mechanisms: attenuation of proteinuria, inhibition of interstitial inflammation and reduction of intrarenal fibrosis. The beneficial effects of NCTD appear to be related to the inhibition of NF-κB and CTGF expression. These suggest the potential usefulness for the therapeutic application of NCTD in renal diseases with severe proteinuria and tubulointerstitial damage, other than immunosuppression, in particular the prevention of inflammation and fibrosis. Additional studies will be necessary to thoroughly elucidate the mechanisms that are responsible for the protective effects of NCTD on proteinuria-associated progressive renal disease and to determine whether NCTD has similar effects in humans with proteinuric states.

References

1 Liu FY, Li Y, Peng YM, Yang L, Duan SB, Li J, Chen X, Xia YC, Guo N, Xu XQ: Relationship between clinical predictors and tubulointerstitial damage in adult-onset primary nephrotic syndrome. Arch Med Res 2006;37:981–986.

2 Hirschberg R, Wang S: Proteinuria and growth factors in the development of tubulointerstitial injury and scarring in kidney disease. Curr Opin Nephrol Hypertens 2005;14:43–52.

3 Palmer BF: Proteinuria as a therapeutic target in patients with chronic kidney disease. Am J Nephrol 2007;27:287–293.

4 McCluskey A, Bowyer MC, Collins E, Sim ATR, Sakoff JA, Baldwin ML: Anhydride modified cantharidin analogues: synthesis, inhibition of protein phosphatases 1 and 2A and anticancer activity. Bioorg Med Chem Lett 2000;10:1687–1690.

5 Sakoff JA, Ackland SP, Baldwin ML, Keane MA, McCluskey A: Anticancer activity and protein phosphatase 1 and 2A inhibition of a new generation of cantharidin analogues. Invest New Drugs 2002;20:1–11.

6 Wei CM, Wang BJ, Ma Y, Sun ZP, Li XL, Guo RC: Pharmacokinetics and biodistribution of ^{3}H-norcantharidin in mice. Yao Xue Xue Bao 2007;42:516–519.

7 Chen YN, Chen JC, Yin SC, Wang GS, Tsauer W, Hsu SF, Hsu SL: Effector mechanisms of norcantharidin-induced mitotic arrest and apoptosis in human hepatoma cells. Int J Cancer 2002;100:158–165.

8 Liu XH, Blazsek I, Comisso M, Legras S, Marion S, Quittet P, Anjo A, Wang GS, Misset JL: Effects of norcantharidin, a protein phosphatase type-2A inhibitor, on the growth of normal and malignant haemopoietic cells. Eur J Cancer 1995;31A:953–963.

9 Baba Y, Hirukawa N, Tanohira N, Sodeoka M: Structure-based design of a highly selective catalytic site-directed inhibitor of Ser/Thr protein phosphatase 2B (calcineurin). J Am Chem Soc 2003;125:9740–9749.

10 Eddy AA, Kim H, López-Guisa J, Oda T, Soloway PD: Interstitial fibrosis in mice with overload proteinuria: deficiency of TIMP-1 is not protective. Kidney Int 2000;58:618–628.

11 Bonegio RG, Fuhro R, Wang Z, Valeri CR, Andry C, Salant DJ, Lieberthal W: Rapamycin ameliorates proteinuria-associated tubulointerstitial inflammation and fibrosis in experimental membranous nephropathy. J Am Soc Nephrol 2005;16:2063–2072.

12 Nakayama H, Ikebe T, Beppu M, Shirasuna K: High expression levels of nuclear factor kappaB, IkappaB kinase alpha and Akt kinase in squamous cell carcinoma of the oral cavity. Cancer 2001;92:3037–3044.

13 Wilmer WA, Rovin BH, Hebert CJ, Rao SV, Kumor K, Hebert LA: Management of glomerular proteinuria: a commentary. J Am Soc Nephrol 2003;14:3217–3232.

14 MacKinnon M, Shurraw S, Akbari A, Knoll GA, Jaffey J, Clark HD: Combination therapy with an angiotensin receptor blocker and an ACE inhibitor in proteinuric renal disease: a systematic review of the efficacy and safety data. Am J Kidney Dis 2006;48:8–20.

15 Doggrell SA: ACE inhibitors and AT-1-receptor antagonists COOPERATE in non-diabetic renal disease. Expert Opin Pharmacother 2003;4:1185–1188.

16 Nakao N, Yoshimura A, Morita H, Takada M, Kayano T, Ideura T: Combination treatment of angiotensin-II receptor blocker and angiotensin-converting-enzyme inhibitor in non-diabetic renal disease (COOPERATE): a randomised controlled trial. Lancet 2003;361:117–124.

17 Taal MW, Brenner BM: Combination ACEI and ARB therapy: additional benefit in renoprotection? Curr Opin Nephrol Hypertens 2002;11:377–381.

18 Ha IS, Um EY, Jung HR, Park HW, Cheong HI, Choi Y: Glucocorticoid diminishes vascular endothelial growth factor and exacerbates proteinuria in rats with mesangial proliferative glomerulonephritis. Am J Kidney Dis 2002;39:1001–1010.

19 Peng A, Gu Y, Lin SY: Herbal treatment for renal diseases. Ann Acad Med Singapore 2005;34:44–51.

20 Williams LA, Moller W, Merisor E, Kraus W, Rosner H: In vitro anti-proliferation/cytotoxic activity of cantharidin (Spanish fly) and related derivatives. West Indian Med J 2003;52:10–13.

21 Ho YP, To KK, Au-Yeung SC, Wang X, Lin G, Han X: Potential new antitumor agents from an innovative combination of demethylcantharidin, a modified traditional Chinese medicine, with a platinum moiety. J Med Chem 2001;44:2065–2068.
22 McCluskey A, Ackland SP, Bowyer MC, Baldwin ML, Garner J, Walkom CC, Sakoff JA: Cantharidin analogues: synthesis and evaluation of growth inhibition in a panel of selected tumour cell lines. Bioorg Chem 2003;31:68–79.
23 Yang EB, Tang WY, Zhang K, Cheng LY, Mack PO: Norcantharidin inhibits growth of human Hep G2 cell-transplanted tumor in nude mice and prolongs host survival. Cancer Lett 1997;117:93–98.
24 Yi SN, Wass J, Vincent P, Iland H: Inhibitory effect of norcantharidin on K562 human myeloid leukemia cells in vitro. Leuk Res 1991;15:883–886.
25 Weening JJ, Van Guldener C, Daha MR, Klar N, van der Wal A, Prins FA: The pathophysiology of protein-overload proteinuria. Am J Pathol 1987;129:64–73.
26 Schieppati A, Remuzzi G: Proteinuria and its consequences in renal disease. Acta Paediatr Suppl 2003;92:9–13.
27 Morigi M, Macconi D, Zoja C, Donadelli R, Buelli S, Zanchi C, Ghilardi M, Remuzzi G: Protein overload–induced NF-κB activation in proximal tubular cells requires H_2O_2 through a PKC dependent pathway. J Am Soc Nephrol 2002;13:1179–1189.
28 Zoja C, Donadelli R, Colleoni S, Figliuzzi M, Bonazzola S, Morigi M, Remuzzi G: Protein overload stimulates RANTES production by proximal tubular cells depending on NF-κB activation. Kidney Int 1998;53:1608–1615.
29 Wang Y, Rangan GK, Tay Y-C, Wang Y, Harris DC: Induction of monocyte chemoattractant protein-1 by albumin is mediated by nuclear factor κB in proximal tubule cells. J Am Soc Nephrol 1999;10:1204–1213.
30 Mezzano SA, Barría M, Droguett MA, Burgos ME, Ardiles LG, Flores C, Egido J: Tubular NF-κB and AP-1 activation in human proteinuric renal disease. Kidney Int 2001; 60:1366–1377.
31 Gomez-Garre D, Largo R, Tejera N, Fortes J, Manzarbeitia F, Eqido J: Activation of NF-κB in tubular epithelial cells of rats with intense proteinuria. Role of angiotensin II and endothelin-1. Hypertension 2001;37:1171–1178.
32 Perbal B: CCN proteins: multifunctional signalling regulators. Lancet 2004;363:62–64.
33 Rupérez M, Ruiz-Ortega M, Esteban V, Lorenzo O, Mezzano S, Plaza JJ, Eqido J: Angiotensin II increases connective tissue growth factor in the kidney. Am J Pathol 2003;163:1937–1947.
34 Okada H, Kikuta T, Kobayashi T, Inoue T, Kanno Y, Takigawa M, Sugaya T, Kopp JB, Suzuki H: Connective tissue growth factor expressed in tubular epithelium plays a pivotal role in renal fibrogenesis. J Am Soc Nephrol 2005;16:133–143.
35 Miller JL, Ericson SG: Cyclosporin A and tacrolimus (FK506) differentially alter T-cell receptor expression in vivo. Immunopharmacol Immunotoxicol 2007;29:105–118.
36 Yan MS, Xiue S, Wei LX, Piao XH: The preliminary observation on immuno-suppressive effect of norcantharidin in mice. Immunopharmacol Immunotoxicol 1993;15:79–85.