RESEARCH ARTICLE

# Curcumin alleviates oxidative stress, inflammation, and renal fibrosis in remnant kidney through the Nrf2–keap1 pathway

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**Scope:** We hypothesized that curcumin, by increasing the expression of nuclear factor-erythroid-2-related factor 2 (Nrf2), could reduce oxidative stress, inflammation, and renal fibrosis in remnant kidney.

**Methods and results:** Sprague-Dawley rats were subjected to 5/6 nephrectomy and randomly assigned to untreated (Nx), curcumin-treated (75 mg/kg/day, orally), and telmisartan-treated groups (10 mg/kg/day, orally; as positive control). Sham-operated rats also served as controls. Five/sixth nephrectomy caused renal dysfunction, as evidenced by elevated proteinuria, blood urea nitrogen, and plasma creatinine, and decreased creatinine clearance that were ameliorated by curcumin or telmisartan treatment. The Nx rats demonstrated reduced Nrf2 protein expression, whereas the Kelch-like ECH-associated protein 1 was upregulated and heme oxygenase-1 level was significantly diminished. Consequently, Nx animals had significantly higher kidney malondialdehyde concentration and lower glutathione peroxidase activity, which was associated with the upregulation of nicotinamide adenine dinucleotide phosphatase oxidase subunit (p67$^{phox}$ and p22$^{phox}$), NF-kappaB p65, TNF-α, TGF-β1, cyclooxygenase-2, and fibronectin accumulation in remnant kidney. Interestingly, all of these changes were ameliorated by curcumin or telmisartan.

**Conclusion:** These findings demonstrate that, by modulating Nrf2-Keap1 pathway, the curcumin effectively attenuates oxidative stress, inflammation, and renal fibrosis, which suggest that curcumin hold promising potential for safe treatment of chronic kidney disease.

**Keywords:**
Chronic kidney disease / Curcumin / Fibrosis / Inflammation / Oxidative stress

## 1 Introduction

The pathogenesis of chronic kidney disease (CKD) involves a complex interaction of oxidative stress, inflammation, and fibrotic processes that lead to glomerular and tubulointerstitial scarring with subsequent progression toward end-stage renal disease [1, 2].

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**Abbreviations: ANG II**, angiotensin II; **ARB**, AT1 receptor blockers; **BP**, blood pressure; **BUN**, blood urea nitrogen; **CCr**, creatinine clearance; **CKD**, chronic kidney disease; **COX-2**, cyclooxygenase-2; **GPx**, glutathione peroxidase; **HO-1**, heme oxygenase-1; **Keap1**, Kelch-like erythroid cell-derived protein with cap-n-collar homology-associated protein 1; **MDA**, malondialdehyde; **NAD(P)H**, nicotinamide adenine dinucleotide phosphatase; **NF-κB**, nuclear factor-kappa-light-chain-enhancer of activated B cells; **Nrf2**, Nuclear factor-erythroid-2-related factor 2; **ROS**, reactive oxygen species; **SBP**, systolic blood pressure; **TBS**, tris-buffered saline; **TGF-β1**, transforming growth factor-β1; **TNF-α**, tumor necrosis factor-α

Oxidative stress may cause inflammation by direct toxic effects of reactive oxygen species (ROS) and by activation of redox-sensitive proinflammatory transcription factors and signal transduction pathways. Hence, oxidative stress and inflammation play a major role in the progression of renal injury [3]. The major ROS is superoxide, and in the kidney, this is mainly generated by NAD(P)H oxidase [4]. Redox systems including antioxidant and phase II detoxifying enzymes provide protection against ROS-induced tissue injury. Nuclear factor-erythroid-2-related factor 2 (Nrf2) is an important cytoprotective transcription factor that induces the expression of several antioxidant and phase II detoxifying enzymes such as catalase, superoxide dismutase, uridine 5′-diphospho (UDP)-glucuronosyltransferase, NAD(P)H:quinine oxidoreductase-1, heme oxygenase-1 (HO-1), glutamate cysteine ligase, glutathione *S*-transferase, glutathione peroxidase (GPx), and thioredoxin [5]. In resting cells, Nrf2 is sequestered in the cytoplasm as an inactive complex with the repressor Kelch-like ECH-associated protein 1 (Keap1). Under oxidative conditions, Nrf2 is released from Keap1 repression and translocates to the nucleus. After this translocation, Nrf2 forms heterodimers with other transcription factors, which in turn bind to the regulatory sequences encoding various antioxidants and phase II detoxifying enzymes [6]. Besides its role in regulating cellular detoxification and antioxidative defense, Nrf2 also has anti-inflammatory functions and thus represents a novel therapeutic approach for the treatment or prevention of inflammatory disorders [7].

Curcumin is a diferuloylmethane derived from the rhizomes of turmeric. Its chemopreventive effects have been extensively investigated and well defined [8]. Balogun et al. [9] reported that curcumin disrupted the Nrf2–Keap1 complex, leading to increases in the expression and activity of HO-1 in porcine renal epithelial proximal tubule cells. A very recent experimental study showed that short-term curcumin treatment in high-fat diet-fed mice ameliorated muscular oxidative stress by activating Nrf2 function [10]. Moreover, curcumin treatment has been shown to decrease macrophage infiltration in the kidneys of chronic renal failure rats and to block transactivation of nuclear factor-kappa-light-chain-enhancer of activated B cells (NF-κB), indicating that the anti-inflammatory property of curcumin may be responsible for alleviating disease in this animal model [11]. However, to the best of our knowledge, no attempt has been made to correlate the role of curcumin in Nrf2–Keap1 pathway and its downstream region in an experimental CKD model. The present study was designed to shed light on this issue.

## 2    Materials and methods

### 2.1    Study group

Male Sprague-Dawley rats with an average body weight of 150–200 g (age 6 wk of age) (Charles River, Japan Inc., Kanagawa, Japan) were used in this study. The experimental pro-

tocol was approved by the Institutional Animal Care and Use Committee of the University of Pharmacy and Applied Life Sciences. Animals were housed in a climate-controlled vivarium with a 12:12-h light–dark cycle and fed a standard laboratory diet and water ad libitum. The animals were randomly divided into four groups with six animals in each group. The control group underwent sham operation (sham), and the three other groups underwent 5/6 nephrectomy by surgical resection of the upper- and lower-thirds of the left kidney, followed by a right nephrectomy 7 days later. The procedures were carried out under general anesthesia (pentobarbital sodium, 50 mg/kg i.p.) using a sterile technique. In the second week after the second surgery, the animals were randomly divided into curcumin treatment (C75), telmisartan treatment (T10), and untreated (Nx) groups. The curcumin-treated group was subjected to gavage with 75 mg/kg curcumin daily [11]. The telmisartan group received 10 mg/kg oral telmisartan daily [12]. The untreated Nx animals received vehicle, which was 0.5% carboxymehtyl cellulose, daily. The animals were studied for 8 wk and in week 11 were killed, after which blood and kidney tissues were collected for further analysis. Twenty-four-hour urine samples were collected in metabolic cages at baseline, week 7, and week 10 for the determination of urinary levels of protein and creatinine.

### 2.2    Blood and urine chemistries

Blood samples were collected in EDTA vacutainer tubes by heart puncture. EDTA blood was centrifuge at $3000 \times g$ for 15 min at 4°C for separation of plasma. The collected plasma was utilized for the subsequent determination of creatinine, blood urea nitrogen (BUN), plasma total cholesterol, triglyceride, LDL cholesterol, and HDL cholesterol (SRL Laboratory, Japan). Urinary protein excretion was determined by the Bradford method [13]. Plasma and urinary creatinine levels were determined by Jaffe method [14]. BUN was determined by the diacetylmonoxamine method [15].

### 2.3    Measurement of malondialdehyde (MDA) content

Kidney tissues were rinsed, weighed, resuspended at 50 mg/mL in normal saline, and homogenized. After centrifugation at 5000 rpm for 10 min at 4°C, the supernatants were collected and analyzed with corresponding assay kits (OXItek, ZeptoMetrix Corporation, NY, USA) in accordance with the manufacturer's instruction.

### 2.4    Measurement of GPx activity

Kidney tissues were homogenized in six volumes (per weight of tissues) of cold GPx Assay Buffer and the mixture was centrifuged at 8000 rpm for 15 min at 4°C in accordance with the total GPx assay kit instructions (OXItek). GPx activity in kidney tissues was measured using a kinetic ultraviolet-visible

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spectrophotometer (Ultraspec 3100, Amersham Biosciences, Cambridge, UK). The oxidation of nicotinamide adenine dinucleotide phosphate reduced (NADPH) to nicotinamide adenine dinucleotide phosphate was measured by the decrease in absorbance at 340 nm.

## 2.5 Longitudinal measurement of blood pressure by tail plethysmography

Blood pressure (BP) was noninvasively measured by a volume pressure-recording sensor and an occlusion tail-cuff (CODA System, Kent Scientific, Torrington, CT, USA). In brief, before surgery, the conscious animal was placed in a restrainer and permitted to rest for 10 to 15 min. The cuff was then placed on the tail and was inflated and released several times to condition the animal to the procedure. After stabilization, BP was measured three times, and the average of the recorded values was used. In this study, we report the mean systolic BP (SBP) of each group. On the third day before surgery, BP was recorded (week 0). Two weeks after the second surgery, the animals were retrained and BP was recorded (week 3). Similar BP recording was carried out in weeks 6 and 10 postsurgery.

## 2.6 Kidney homogenates and nuclear extracts preparation

Briefly, 100 mg of kidney cortex was homogenized in 0.5 mL of buffer A containing 10 mM HEPES, pH 7.8, 10 mM KCl, 2 mM MgCl$_2$, 1 mM DTT, 0.1 mM EDTA, 0.1 mM PMSF, 1 $\mu$M pepstatin, and 1 mM *p*-aminobenzamidine using a tissue homogenizer for 20 s. Homogenates were kept on ice for 15 min, and then 125 $\mu$L of a 10% Nonidet p40 solution were added and mixed for 15 s and the mixture was centrifuged for 2 min at 12 000 rpm. The supernatant containing cytosolic proteins was collected. The pelleted nuclei were washed once with 200 $\mu$L of buffer A plus 25 $\mu$L of 10% Nonidet p40, centrifuged, then suspended in 50 $\mu$L of buffer B containing 50 mM HEPES, pH 7.8, 50 mM KCl, 300 mM NaCl, 0.1 mM EDTA, 1 mM DTT, 0.1 mM PMSF, and 10% v/v glycerol, mixed for 20 min, and centrifuged for 5 min at 12 000 rpm. The supernatant containing nuclear proteins was stored at –80°C [16]. The protein concentrations in kidney homogenates and nuclear extracts were determined by the bicinchoninic acid method.

## 2.7 Western blot analysis

Protein abundance of cycloxygenase-2 (COX-2), NADPH oxidase subunits (p22$^{phox}$ and p67$^{phox}$), transforming growth factor (TGF)-$\beta$1, tumor necrosis factor (TNF)-$\alpha$, HO-1, Keap1, Nrf2 nuclear translocation, and p65 NF-$\kappa$B nuclear translocation were measured by the Western blot technique. Antibodies against COX-2, p22$^{phox}$, p67$^{phox}$, TNF-$\alpha$, HO-1, Keap1, and Nrf2 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibodies against p65 NF-$\kappa$B were pur-

chased from Cell Signaling Technology (Denver, CO, USA). Antibody against TGF-$\beta$1 was purchased from Promega (Madison, WI, USA). Antibodies to $\beta$-actin (Cell Signaling Technology) and Lamin A (Santa Cruz, CA, USA) were used for measurements of the housekeeping proteins for cytosolic and nuclear target proteins, respectively. Aliquots containing 70 $\mu$g of total protein (cytosol) and 50 $\mu$g of total protein (nuclear extracts) were separated on a 7.5–12.5% SDS PAGE gel, and proteins were transferred to a nitrocellulose membrane. After being briefly washed in Tris-buffered saline (TBS; 10 mM Tris HCl, 0.85% NaCl, pH 7.2) containing 0.05% Tween-20 (TBS-T) and blocked for 1 h in 5% nonfat dry milk, membranes were incubated with appropriate antibodies in 5% nonfat dry milk overnight at 4°C. After being washed three times for 5 min in TBS-T, membranes were subsequently incubated with secondary antibody appropriately diluted in TBS-T for 1 h at room temperature. The membrane was washed four times and detection was performed using the enhanced chemiluminescence system (Amersham Biosciences, Buckinghamshire, UK) and exposed to X-ray film.

## 2.8 Immunohistochemistry of fibronectin

Formalin-fixed, paraffin-embedded kidney tissue sections were used for immunohistochemical staining. After deparaffinization and hydration, the slides were washed in TBS. Endogenous peroxidase activity was quenched by incubating the slides in methanol and 0.3% H$_2$O$_2$ in methanol. After overnight incubation with the primary antibody, that is, fibronectin (diluted 1:50) (Santa Cruz, CA), at 4°C, the slides were washed in TBS and then horseradish peroxidase-conjugated secondary antibody was added and the slides were further incubated at room temperature for 1 h. The slides were washed in TBS and incubated with diaminobenzidine tetrahydrochloride as the substrate and counterstained with hematoxylin. A negative control without primary antibody was included in the experiment to verify the antibody specificity. For morphometry analysis, the percentage of positive cells (stained brown) was determined with a computer-assisted color image analyzer (CIA-102; Olympus, Tokyo, Japan). Ten random fields per kidney were studied at ×100 magnification for comparison of the different groups [17].

## 2.9 Histopathological analysis

Light microscopy was performed in the formalin-fixed sections (4 $\mu$m) stained with Azan Mallory to stain collagen as an indicator of fibrosis and periodic acid-Schiff to analyze glomerulosclerosis and tubulointerstitial injury. Glomerulosclerosis was graded from 0 to 4 (grade 0; normal, grade 1; <25% involvement of the glomerular tuft, grade 2; 25–50% involvement of the glomerular tuft, grade 3; 50–75% involvement of the glomerular tuft, and grade 4; sclerosis in more than 75% of the glomerular tuft) and the score was calculated with the following formula: [(1 × *n* glomeruli with

1+) + (2 × *n* glomeruli with 2+) + (3 × *n* glomeruli with 3+) + (4 × *n* glomeruli with 4+)] × 100/total number of glomeruli examined. Tubulointerstitial damage was graded according to the extension (%) of tubular damage (infiltration, fibrosis, and tubular dilatation/atrophy). Interstitial fibrosis was determined in ten random fields of the cortical area at ×100 magnification with use of a computer-assisted color image analyzer (CIA-102; Olympus), with the observer blind to the study group. The results are presented as the ratio of the fibrotic area to the whole area of the kidney. The same instrument was used to determine glomerulosclerosis and tubulointerstitial injury [17].

### 2.10 Determination of TNF-α expression by quantitative real-time RT-PCR

Kidney tissues were preserved by immersion in RNAlater (Ambion Inc., Austin, TX, USA) immediately after sampling. Total RNA was extracted after homogenization using Ultra Turrax T8 (IKA Labortechinik, Staufen, Germany) in TRIzol reagent (Invitrogen Corp., Carlsbad, CA, USA) according to the standard protocol. cDNA was synthesized by reverse transcription using total RNA (2 µg) as a template (Super Script II; Invitrogen Corp). Gene expression analysis was performed by real-time RT-PCR (Smart cycler; Cepheid, Sunnyvale, CA, USA) using cDNA synthesized from the samples specimen. Primer sequences were as follow: TNF-α (forward), CCCCAAAGGGATGAGAAGTT, (reverse), CACTTGGTGGTTTGCTACGA; glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (forward) GCTCATTTCCTGGTATGACAACG, (reverse), AGGGGTCTACATGGCAACTG. Real-time RT-PCR, monitoring with a TaqMan probe (TaqMan Gene expression assays; Applied Biosystems, Foster City, CA, USA) was performed according to the following protocol: 600 s at 95°C, followed by thermal cycles of 15 s at 95°C, and 60 s at 60°C for extension. Rel-

ative standard curves representing several tenfold dilutions of cDNA from kidney tissue samples were used for linear regression analysis of other samples. Results were normalized to GAPDH mRNA as an internal control and shown as relative mRNA levels.

### 2.11 Statistical analysis

Data are expressed as mean ± SEM and were analyzed using one-way analysis of variance (ANOVA) followed by Tukey's methods for post hoc analysis and two-tailed t-test when appropriate. A value of $p < 0.05$ was considered statistically significant. For statistical analysis, GraphPad Prism 5 software (San Diego, CA, USA) was used.

## 3 Results

### 3.1 Effect of curcumin and telmisartan on general data

The final total number of animals used in this study was 24 (sham (6), Nx (6), T10 (6), C75 (6)). The survival rate after Nx surgery was 64.29% (18 of 28), this was not different between the three Nx groups (60% for Nx versus 66.67% for T10 versus 66.67% for C75, $p > 0.05$) (data not shown). Results obtained at 10 wk after 5/6 nephrectomy are summarized in Table 1. Compared with the sham group, the 5/6 nephrectomy groups had significantly decreased body weight, creatinine clearance (CCr), and HDL cholesterol, and increased left kidney weight, plasma creatinine, BUN, total cholesterol, LDL cholesterol, and triglyceride. Both curcumin and telmisartan treatments resulted in significant reductions of plasma creatinine and left kidney weight and an increase of CCr. The curcumin-treated and telmisartan-treated rats did not show any significant changes in body weight, total cholesterol, triglyceride, and HDL cholesterol in comparison

**Table 1.** General data obtained at 10 wk following surgery[a)]

|  | Sham ($n = 6$) | Nx ($n = 6$) | T10 ($n = 6$) | C75 ($n = 6$) |
|---|---|---|---|---|
| Body weight (g) | 517.5 ± 8.5 | 468.3 ± 5.5[b)] | 507.5 ± 23.1 | 494.5 ± 13.3 |
| Left kidney weight (g) | 1.45 ± 0.06 | 1.96 ± 0.07[b)] | 1.52 ± 0.05[d)] | 1.54 ± 0.04[d)] |
| Plasma creatinine (mg/dL) | 0.65 ± 0.3 | 2.27 ± 0.18[b)] | 0.82 ± 0.34[e)] | 1.04 ± 0.29[e)] |
| BUN (mg/dL) | 14 ± 0.9 | 32.5 ± 0.8[b)] | 31.8 ± 0.6[b)] | 28.2 ± 1.6[b)] |
| CCr (mL/min/kg) | 5.3 ± 1.4 | 1.3 ± 0.08[c)] | 2.8 ± 0.4[e)] | 3.2 ± 0.6[e)] |
| Total cholesterol (mg/dL) | 49.8 ± 3.1 | 73.8 ± 7.6[c)] | 69.8 ± 8.6 | 59 ± 7.8 |
| Triglyceride (mg/dL) | 25 ± 3.1 | 55.8 ± 11.5[c)] | 33.5 ± 5.4 | 29.8 ± 2.4 |
| LDL cholesterol (mg/dL) | 18 ± 0.7 | 58.5 ± 11.8[c)] | 27.3 ± 5[e)] | 22.8 ± 2.5[e)] |
| HDL cholesterol (mg/dL) | 26.7 ± 1.7 | 18 ± 2.5[c)] | 25.7 ± 3.3 | 24.7 ± 2.6 |

a) Values are given as mean ± SEM.
b) $p < 0.01$ versus sham based on two-tailed t-test.
c) $p < 0.05$ versus sham based on two-tailed t-test.
d) $p < 0.01$ versus Nx based on two-tailed t-test.
e) $p < 0.05$ versus Nx based on two-tailed t-test.
BUN, blood urea nitrogen; CCr, creatinine clearance; Nx, untreated 5/6 nephrectomy; T10, 5/6 nephrectomy + telmisartan 10 mg/kg/day; C75, 5/6 nephrectomy + curcumin 75 mg/kg/day.
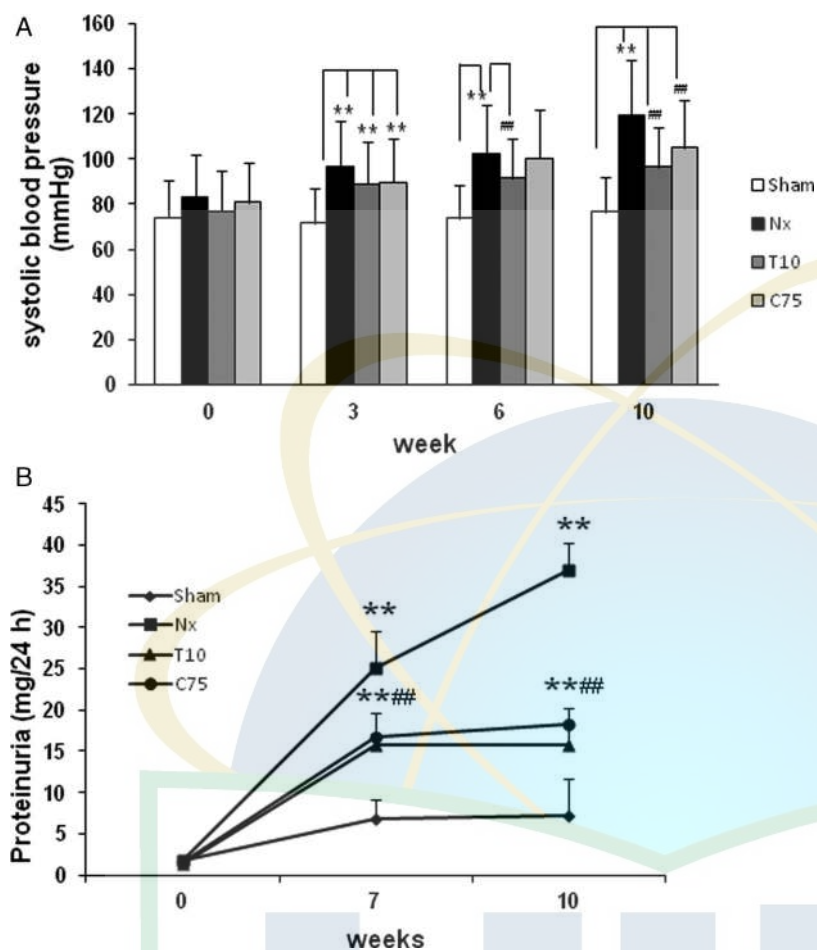
**Figure 1.** Time-course study of (A) systolic blood pressure (SBP) and (B) proteinuria in sham-operated (sham), untreated 5/6 nephrectomized (Nx), telmisartan-treated 5/6 nephrectomized (T10), and curcumin-treated 5/6 nephrectomized (C75) rats. Values are means ± SEM; $n = 6$ animals per group. [**]$p < 0.05$ versus sham, [##]$p < 0.05$ versus Nx based on two-tailed *t*-test.

to those of the Nx rats. As shown in Fig. 1, at week 3 after the operation, the SBP was markedly elevated in the 5/6 nephrectomy groups compared with that in the sham group ($p < 0.01$). Telmisartan treatment significantly curtailed the increase in SBP throughout the entire experimental period ($p < 0.01$). In the curcumin group, the SBP was parallel to that in the Nx group until week 10, but was subsequently reduced, although not to the extent in the telmisartan-treated animals ($p < 0.01$). The Nx group exhibited a significant gradual increase in proteinuria. Curcumin was as effective as telmisartan in reducing proteinuria ($p < 0.01$).

### 3.2 Effect of curcumin and telmisartan on Nrf2–Keap1 pathway

We next examined whether 8-wk treatment with curcumin or telmisartan affected the renal expression of Nrf2, Keap1, and HO-1. As shown in Fig. 2, 5/6 nephrectomy markedly reduced the nuclear expression of Nrf2 and increased the cytoplasmic Keap1 abundance. This was associated with significant reduction of renal tissue HO-1 protein abundance in the remnant kidney. Administration of curcumin or telmisartan increased the nuclear expression of Nrf by 1.6%, and

reduced the cytoplasmic Keap1 abundance to 1.8% and 2%, respectively, and increased the HO-1 protein abundance in the remnant kidney by 1.9% and 1.8%, respectively. The effects of curcumin and telmisartan treatments on Nrf2, Keap1, and HO-1 protein expression were not significantly different.

### 3.3 Effect of curcumin and telmisartan on oxidative stress and inflammatory parameter

Compared with the Nx group, curcumin- and telmisartan-treated groups exhibited significantly attenuated p67[phox] protein expression, by 2.1-fold and twofold, respectively (Fig. 3A). Western blot analysis also demonstrated a significant increase of p22[phox] protein expression by twofold in the Nx group compared with that in the sham group, which was significantly attenuated by treatment with curcumin by 1.2-fold. Telmisartan treatment also reduced the expression of p22[phox], but this did not reach significance (Fig. 3B). Furthermore, the Nx group exhibited a significant increase in the level of MDA (1.9-fold elevation) compared with the sham group, and chronic treatment with curcumin as well as telmisartan significantly reduced the level of MDA in the remnant rat kidney by
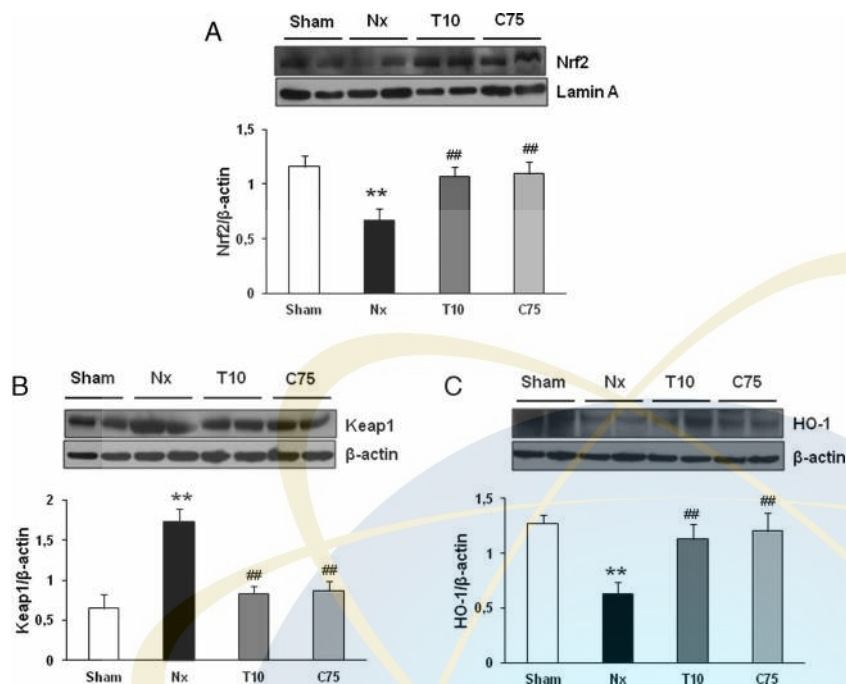
**Figure 2.** Representative Western blots and group data depicting (A) Nrf2, (B) Keap1, and (C) HO-1 abundance in the remnant kidney tissues of sham-operated (sham), untreated 5/6 nephrectomized (Nx), telmisartan-treated 5/6 nephrectomized (T10), and curcumin-treated 5/6 nephrectomized (C75) rats. Equal amounts of protein sample (50 μg) obtained from the nuclear fraction were applied to each lane for Nrf2 and Lamin A and equal amounts of protein sample (70 μg) obtained from cytosolic fraction were applied to each lane for Keap1, HO-1, and β-actin. Each bar represents mean ± SEM. $n = 5$–6 experiments. $^{**}p < 0.05$ versus sham, $^{##}p < 0.05$ versus Nx based on one-way ANOVA followed by Tukey's test.

1.7-fold and 1.5-fold, respectively (Fig. 3C). GPx activity in the kidney was also significantly lower in the Nx group than in the sham group. Chronic treatment with curcumin or telmisartan prevented this decrease in GPx activity (Fig. 3D). The increase of oxidative stress parameter was associated with a significant increase in NF-κB activity, as indicated by the increased phosphorylation of NF-κB in the nuclear fraction of the Nx group. Both curcumin and telmisartan treatments resulted in decreased activity of NF-κB (Fig. 4A) and, as a re-

sult, decreases in the protein and mRNA expression of TNF-α (Fig. 4C and D). Similarly, COX-2 abundance was significantly increased in the remnant kidney of the Nx group compared with that of the sham group, which was significantly attenuated by treatment with curcumin as well as telmisartan ($p < 0.05$) (Fig. 4B). The differences of NAD(P)H oxidase subunit p67$^{phox}$, MDA level, GPx activity, NF-κB activity, and COX-2 between curcumin- and telmisartan-treated groups were not significant.
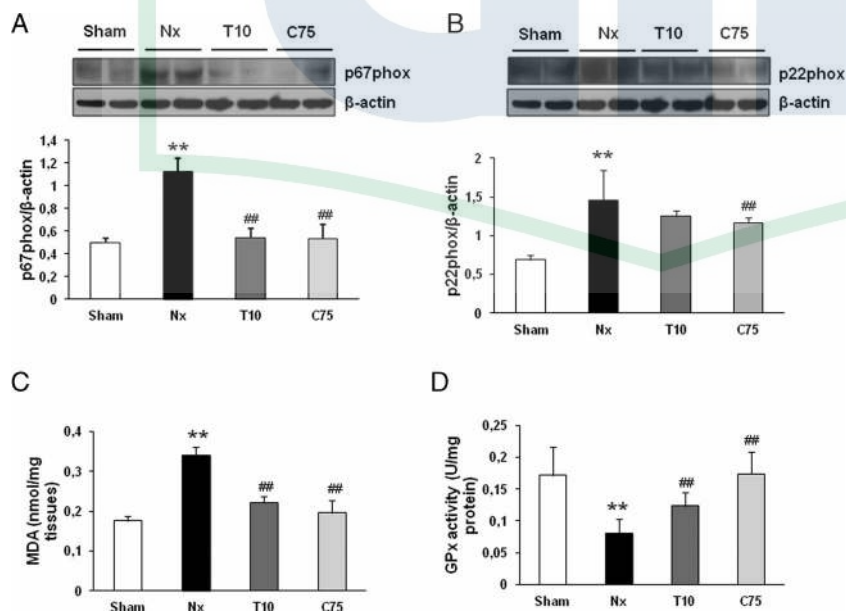


**Figure 3.** Representative Western blots and group data depicting (A) p67$^{phox}$ and (B) p22$^{phox}$ abundance, (C) malondialdehyde (MDA) concentration, and (D) GPx activity in the remnant kidney tissues of sham-operated (sham), untreated 5/6 nephrectomized (Nx), telmisartan-treated 5/6 nephrectomized (T10), and curcumin-treated 5/6 nephrectomized (C75) rats. Equal amounts of protein sample (70 μg) were applied to each lane. Each bar represents mean ± SEM. $n = 5$–6 experiments. $^{**}p < 0.05$ versus sham, $^{##}p < 0.05$ versus Nx based on one-way ANOVA followed by Tukey's test.

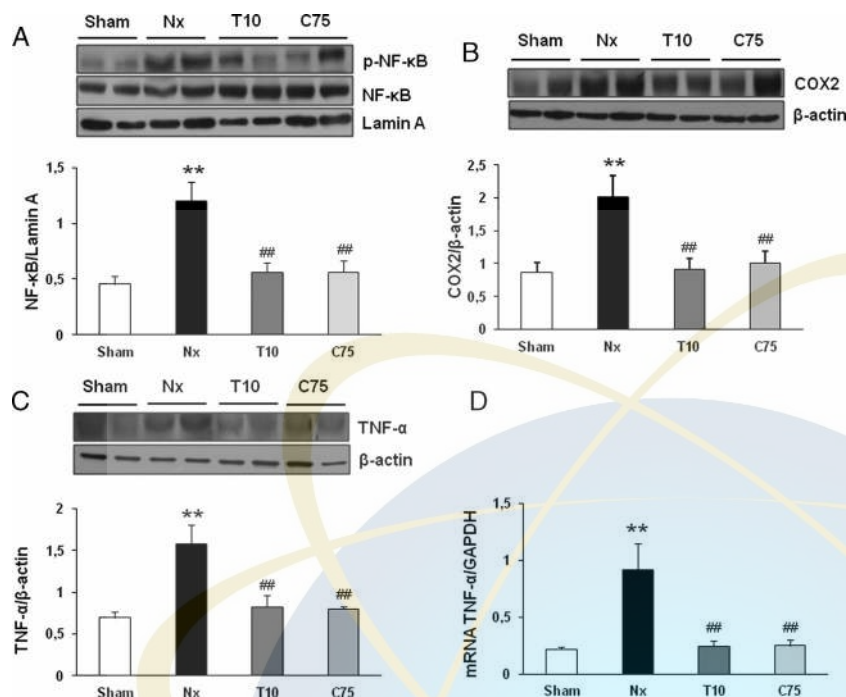*Mol. Nutr. Food Res.* 2013, 57, 1649–1659

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**Figure 4.** Representative Western blots and group data depicting (A) p-NF-κB, (B) COX-2, (C) TNF-α protein, and TNF-α mRNA in the remnant kidney tissues of sham-operated (sham), untreated 5/6 nephrectomized (Nx), telmisartan-treated 5/6 nephrectomized (T10), and curcumin-treated 5/6 nephrectomized (C75) rats. Equal amounts of protein sample (50 μg) obtained from nuclear fraction were applied to each lane for p-NF-κB, NF-κB, and Lamin A and equal amounts of protein sample (70 μg) obtained from cytosolic fraction were applied to each lane for COX-2 and TNF-α. Each bar represents mean ± SEM. $n = 5$–6 experiments. $^{**}p < 0.05$ versus sham, $^{\#\#}p < 0.05$ versus Nx based on one-way ANOVA followed by Tukey's test.

### 3.4 Effect of curcumin and telmisartan on TGF-β1 protein expression and fibronectin accumulation

Besides NF-κB, TGF-β1 also plays an important role in modulating inflammatory responses and fibrosis. We thus further examined the effect of curcumin or telmisartan on TGF-β1 protein expression in the remnant kidney. As shown in Figs. 5 and 6E–H, increased TGF-β1 protein expression and fibronectin accumulation were detected in the remnant kidney of Nx group and curcumin as well as telmisartan treatments could eliminate this response.

### 3.5 Effect of curcumin and telmisartan on histological findings

Data on the effect of curcumin and telmisartan on the histology are shown in Fig. 6. As expected, kidneys from the Nx group showed extensive fibrosis (Fig. 6B), significant glomerulosclerosis, tubular atrophy, and tubulointersitital injury (Fig. 6J). Treatment with telmisartan or with curcumin reduced fibrosis, glomerulosclerosis index, as well as tubulointerstitial injury in 5/6 nephrectomy rats ($p < 0.05$).

## 4 Discussion

The removal of kidney, representing two-thirds of the total functioning renal mass, by surgical procedures in this study was sufficient to trigger the progression of CKD, as evidenced by proteinuria, decreased CCr, increased BUN, and structural damage to the remnant kidney. This was as-
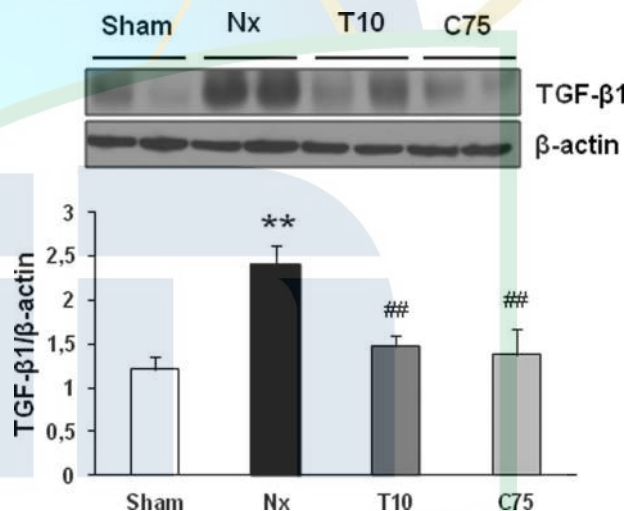


**Figure 5.** Representative Western blot and group data depicting TGF-β1 in the remnant kidney tissues of sham operated (sham), untreated 5/6 nephrectomized (Nx), telmisartan-treated 5/6 nephrectomized (T10), and curcumin-treated 5/6 nephrectomized (C75) rats. Equal amounts of protein sample (70 μg) obtained from cytosolic fraction were applied to each lane. Each bar represents mean ± SEM. $n = 5$–6 experiments. $^{**}p < 0.05$ versus sham, $^{\#\#}p < 0.05$ versus Nx based on one-way ANOVA followed by Tukey's test.

sociated with the upregulation of oxidative, inflammatory, and fibrotic pathway. Excess angiotensin II (ANG II) production by the renin–ANG system is responsible for the gradual decline of kidney function in CKD. These effects are triggered by signaling via ANG II type 1 receptor [18]. Numerous
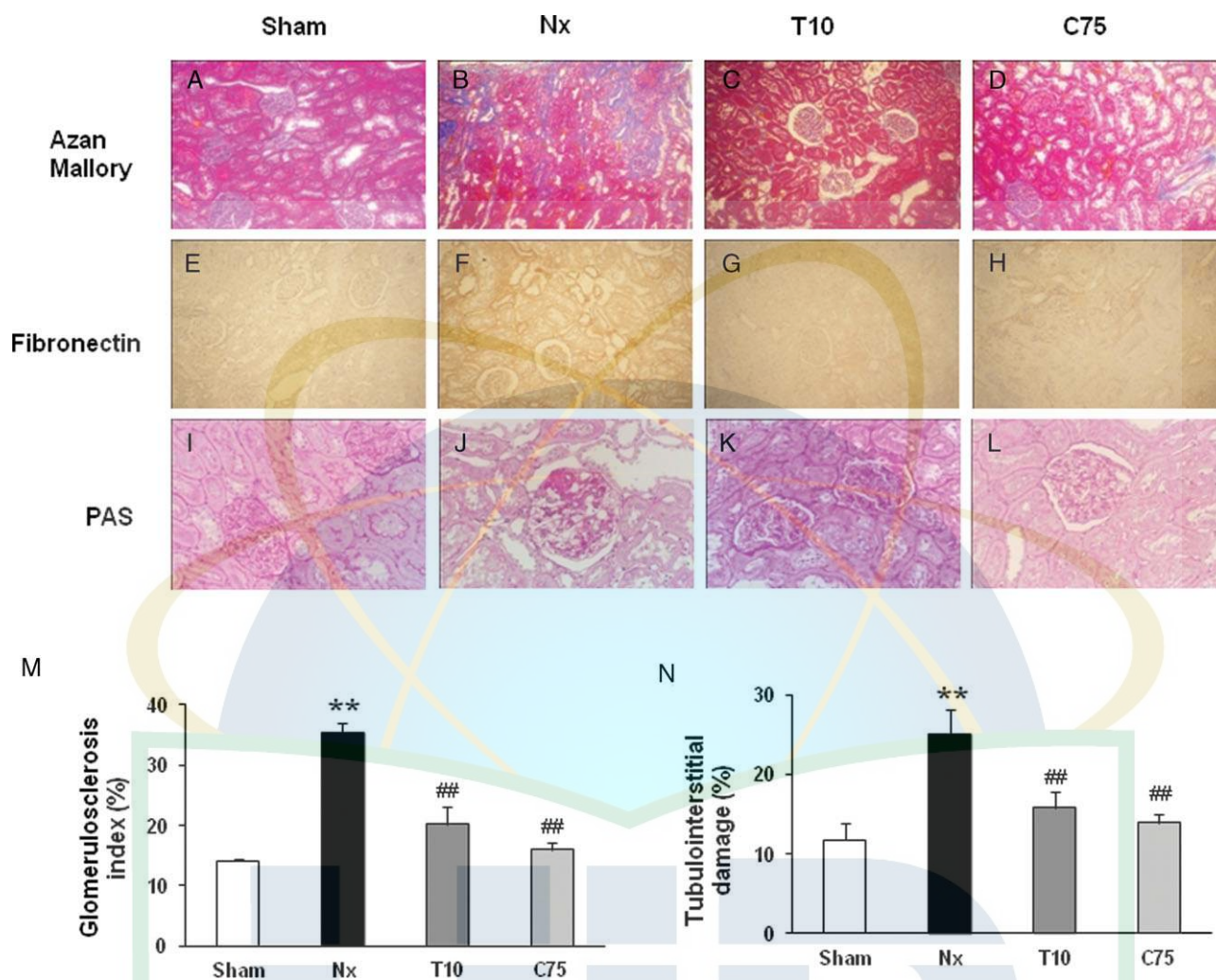
**Figure 6.** Representative photomicrograph Azan Mallory staining (A–D) for fibrosis of the cross-sectional tissue slices of kidney. Fibrosis is indicated by the blue area (magnification ×100), immunohistochemical staining of fibronectin is strong in epithelial tubular cells in the Nx group (E–H) (magnification ×100), and PAS staining (I–L) (magnification ×200) of the remnant kidney is shown in sham operated (sham), untreated 5/6 nephrectomized (Nx), telmisartan-treated 5/6 nephrectomized (T10), and curcumin-treated 5/6 nephrectomized (C75). Bar graphs depict the corresponding glomerulosclerosis index (M) and tubulointerstitial injury index (N). ** $p < 0.05$ versus sham, ## $p < 0.05$ versus Nx based on one-way ANOVA followed by Tukey's test.

clinical and experimental studies have revealed that activation of ANG II type 1 receptor by ANG II can account for upregulation/activation of NAD(P)H oxidase and oxidative stress and that intervention by AT1 receptor blockers (ARBs) shows efficacy in slowing the onset of kidney disease and/or slowing progression of CKD [19, 20]. In fact, an experimental study of spontaneous focal glomerulosclerosis rats has indicated that ARB treatment prevented nephropathy, suppressed oxidative stress, inflammatory process, and endoplasmic reticulum stress-induced apoptosis as well as restored Nrf2 activation and expression of the antioxidant enzymes [21, 22]. A very recent study has revealed that telmisartan, an ARB, could suppress NAD(P)H oxidase and subsequently upregulated Nrf2, leading to the amelioration of renal oxidative

stress and diabetic renal changes [23]. Moreover, it has been demonstrated that telmisartan attenuated the progression of CKD by blocking vascular dysfunction and inflammation in subtotal nephrectomy rat model [24]. To verify whether curcumin might have a beneficial role in CKD, we compared it with telmisartan. We showed here that curcumin gavage was as effective as telmisartan in alleviating the deterioration of renal function, as shown by biochemical and histological parameters, even though curcumin treatment could not significantly ameliorate the increased of SBP compared with telmisartan treatment.

During the pathogenesis of CKD, perturbations in cellular oxidant handling influence downstream cell signaling and, in the kidney, promote renal cell fibrosis and decrease

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regenerative ability of cells [25]. Pivotal to the antioxidant response is the transcription factor Nrf2. Under basal conditions, Nrf2 is mainly found sequestered in the cytoplasm as an inactive complex bound to a repressor molecule known as Keap1, which facilitates its ubiquitination. When challenged by oxidative stress derived from accumulation of ROS, Nrf2 can quickly translocate into the nucleus and induce genes encoding antioxidant and phase II detoxifying enzymes, such as HO-1 [26]. Actually, disruption of Nrf2 in mice diminishes or abrogates the induction of these genes [27]. Moreover, Nrf2 gene ablation has been shown to cause a lupus-like autoimmune nephritis and exacerbate diabetes-induced inflammation, oxidative stress, and renal injury in experimental animals [28, 29]. It has been demonstrated that impaired Nrf2 activation contributes to the severity of oxidative stress, inflammation, and the progression of tissue damage in the remnant kidney model [16]. Interestingly, curcumin has antioxidant and anti-inflammatory effects and in vitro studies have indicated its action in triggering Nrf2 signaling [9, 30]. Another study also reported that dimethoxycurcumin and curcumin induced HO-1 expression and Nrf2 nuclear translocation in RAW264.7 macrophages [31]. We therefore proposed the working hypothesis of this study that curcumin could alleviate oxidative stress and inflammation in the CKD model by its action via Nrf2 activation. We found that the remnant kidney in the CKD animals showed marked upregulation of NAD(P)H oxidase isoforms (p67$^{phox}$ and p22$^{phox}$), significant elevation of lipid peroxidation product MDA, as well as depression of antioxidant activity GPx, denoting the presence of oxidation, while curcumin and telmisartan administration significantly reverses this deleterious effect. To further investigate the underlying mechanism of curcumin and telmisartan on regulating redox balance, we examined Nrf2 signaling in remnant kidney tissues. We found that CKD led to a reduced nuclear expression of Nrf2 and increased cytoplasmic expression of Keap1, along with decreased levels of Nrf2 target gene products, HO-1 in remnant kidney. Kim and Vaziri [16] have recently reported that Nrf2 activity (nuclear translocation) was mildly reduced at 6 wk and markedly reduced at 12 wk after 5/6 nephrectomy. This was accompanied by reduced or unchanged of the Nrf2 target genes, HO-1, at 6 wk; and significantly diminished at 12 wk. In the present study, although curcumin and telmisartan administration slightly abrogated these changes; the differences reach a statistical significant. This perhaps related with the limited follow-up time in our model. Nevertheless, as far as we know, our study is the first evidence that treatment with curcumin and telmisartan for 8 wk starting 2 wk after 5/6 nephrectomy significantly reduced the degree of kidney structural damage and reversed oxidative stress in remnant kidney, and that these improvements were associated with increased nuclear expression of Nrf2. Importantly, this was achieved without increased mortality. We hence suggest that the activation of the major oxidative defense machinery Nrf2 in kidney is a novel mechanism behind curcumin's alleviation of CKD induced-oxidative stress.

Furthermore, we here show that CKD animals exhibited significant activation of NF-κB in the remnant kidney as evidenced by increased translocation of p65, the active subunit of this transcription factor. NF-κB is the general transcription factor that plays a crucial role in mediating inflammation in the kidney because it regulates the expression of numerous genes involved in inflammation, including cytokines and adhesion molecules [32, 33]. In the CKD model, increased activity of the NF-κB system was reported, and its decreased activity was associated with amelioration of proteinuria and renal structural damage [34, 35]. In fact, besides Nrf2's role in regulating cellular antioxidative defense, it also has anti-inflammatory functions [7]. For instance, the increased susceptibility of Nrf2-deficient mice to dextran sulfate sodium-induced colitis and colorectal cancer was associated with decreased expression of antioxidant/phase II detoxifying enzymes in parallel with upregulation of proinflammatory cytokines, such as COX-2 and TNF-α [36]. It has also been reported that lipopolysachharide-induced NF-κB activation could be attenuated by diverse Nrf2 activators, such as phenethyl isothiocyanate and curcumin [37]. Furthermore, it was shown that Nrf2$^{-/-}$ mice exhibit prolonged inflammation during cutaneous wound healing and show enhanced bronchial inflammation [38, 39]. These findings suggest that Nrf2 deficiency and the resulting impaired antioxidant activity is important for the susceptibility to inflammatory disease. Our CKD rats showed NF-κB activation, upregulation of COX-2 and TNF-α protein, as well as mRNA expression of TNF-α. In fact, our findings are in agreement with the findings of previous study suggesting that inflammation plays a key role in renal failure [11]. Curcumin and telmisartan downregulated the protein expression of COX-2, and the protein and mRNA expression of TNF-α, and prevented NF-κB activation. This suggests that curcumin and telmisartan treatments prevented inflammatory response and this effect might be mediated by their ability to activate Nrf2 signaling.

Oxidative stress and inflammation drive heavy lipid accumulation in the remnant kidney by promoting LDL proinflammatory and limiting HDL anti-inflammatory activities, leading to progressive glomerulosclerosis and tubulointerstitial injury in this model [40]. TGF-β1 overexpression, extracellular matrix deposition, and loss of glomerular architecture define glomerulosclerosis [41, 42]. TGF-β1 induces fibrosis during the process of tissue repair, and contributes to the pathogenesis of a variety of renal diseases [43]. Previous study indicated the interaction between the TGF-β1 and Nrf2 pathways, in which the activation of the TGF-β1 pathway was reported to inhibit Nrf2-dependent expression of the γ-glutamylcysteine synthetase catalytic subunit, resulting in elevated ROS levels. This in turn further activated the TGF-β1 pathway, leading to excessive production of extracellular matrix [44]. The CKD rats employed in the present study exhibited marked glomerulosclerosis, tubulointerstitial fibrosis, fibronectin accumulation, and increased TGF-β1 expression. This was accompanied by increased total cholesterol, triglyceride, and LDL cholesterol, and decreased plasma HDL

cholesterol. Curcumin and telmisartan treatment clearly ameliorated the structural damage to remnant kidney and resulted in mild reduction of plasma triglyceride, total cholesterol, and LDL cholesterol, and an increase of HDL cholesterol; these improvements were most probably achieved via the activation of Nrf2 signaling.

In conclusion, long-term curcumin treatment attenuates oxidative stress, inflammation, and renal fibrosis in animals with CKD. All the above results suggest that the beneficial effect of curcumin occurs at least in part through modulation of Nrf2–Keap1 signaling pathway. Based on the above results and due to the safety profile as well as low cost of curcumin, we believe that these studies might facilitate future clinical trials with curcumin in the treatment of CKD.

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