Original Article
Curcumin prevents adriamycin-induced nephropathy MCP-1 expression through blocking histone acetylation

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Abstract: Objective: To investigate the anti-MCP-1 expression effect of curcumin in kidney disease model and the possible mechanisms associated with histone acetylation. Methods: ADR nephropathy was induced in male SD rats by twice intravenous injections of ADR at day 0 (5 mg·kg⁻¹) and 1 week later (2.5 mg·kg⁻¹). Curcumin treatment was started at week 6. EP300 and MCP-1 mRNA transcription were assessed by real-time PCR. The acetylation levels of the histones H3 and H4 in the promoters of MCP-1 were detected by ChIP-qPCR. And mouse podocytes were cultured to investigate the effect of curcumin against ADR-induced injury. Results: In animal model, curcumin intervention attenuated renal glomerulosclerosis and partially decreased transcription of EP300 and MCP-1 (P < 0.05) mRNA. Also, curcumin prevented an increase of acetylation of MCP-1 promoters H3 and H4 in ADR-nephropathy. In vitro cultured podocytes, compared with the untreated ADR group, pretreatment with low concentration of curcumin preserved podocyte cytoskeleton. Curcumin significantly reduced EP300 and MCP-1 mRNA (P < 0.01). Besides, curcumin prevented ADR-induced MCP-1 promoter hyper-acetylation in ADR injured podocytes by ChIP-qPCR. Conclusion: Curcumin prevents kidney fibrosis and decreases MCP-1 expression through modification of histone acetylation, and could be used as a therapy for patients with kidney disease.

Keywords: Curcumin, renal fibrosis, inflammation, podocyte, histone, acetylation

Introduction
Renal fibrosis is a complicated and important pathological change in the development of kidney disease. A variety of factors, including the transforming growth factor-β pathway, epithelial-mesenchymal transition, and inflammation are involved in this process [1]. Histone acetylation is a recognized histone modification that can regulate the production of certain proteins, and has spurred considerable interest [2, 3]. Studies have shown that histone activation participates in a transforming growth factor-β-induced kidney injury and streptozotocin (STZ)-induced diabetic nephropathy model [4].

Curcumin is a compound purified from the rhizomes of the plant Curcuma longa L [5]. Curcumin has been demonstrated to have anti-inflammatory, antioxidant, anti-proliferation, anti-bacterial and anti-cancer activities [6]. It could also protect against lipopolysaccharide-induced kidney injury by way of anti-inflammation [7]. In addition, curcumin is a histone acetylase inhibitor [8]. Studies have confirmed that inflammation take part in the progression of this kidney disease in a ADR-induced glomerulosclerosis model [9]. However, the role of histone modification of curcumin in glomerular disease is still unclear and its effect on MCP-1 expression also unknown. Thus we utilized the ADR-induced nephropathy and evaluated the effect of curcumin on renal monocyte chemotactic protein 1 (MCP-1) expression, and investigated the underlying mechanism of this effect.

Materials and methods
Reagents and chemicals
Fetal bovine serum, 1640 cell culture medium, trypsin, and Trizol RNA extraction reagent were purchased from Gibco Life Technologies (Grand Island, NY, USA). Reverse transcription-polymerase chain reaction (RT-PCR)-related reagents such as Moloney murine leukemia virus (M-MLV) reverse transcriptase, deoxyribonucleotide triphosphate (dNTP), random prim-
Histone deacetylation effect of curcumin in Kidney disease

ers and RNase inhibitor were obtained from Promega (Madison, Wisconsin, USA). Curcumin and valproic acid were from Sigma-Aldrich (St. Louis, MO, USA). Adriamycin was purchased from Sangon Biotech. (Shanghai, China). The SYBR Green Mix Kit was from TOYOBO (Osaka, Japan). PCR primers of glyceraldehyde-3-phosphate dehydrogenase (GAPDH), EP300 and MCP-1 were synthesized by Invitrogen (Shanghai, China). The chromatin immunoprecipitation (ChIP) and histone acetylation assay kits were purchased from Epigentek (NY, USA). Fluorescein phalloidin for labelling F-actin was from Invitrogen (Carlsbad, CA, USA).

Animals

Specific pathogen free Sprague Dawley rats (180 mg) were purchased from the Laboratory Animal Center of Ruijin Hospital, Shanghai Jiao Tong University School of Medicine (Shanghai, China). The animals were maintained under climate-controlled conditions with a 12-h light/dark cycle, and were fed standard rodent chow and water. All experiments were conducted in accordance with the National Institutes of Health Guide for the Care and with the approval of the Institutional Animal Care.

Animal experiments

Rats were divided into three groups: control group (animals that received an injection of saline by penis vein); ADR-induced kidney disease model group (animals that were given intravenous ADR 5 mg·kg⁻¹ and one week later 2.5 mg·kg⁻¹; the intervention group (animals that were modeled as model group, and 2 weeks later received curcumin 200 mg·kg⁻¹·d⁻¹).

Rats were sacrificed 8 weeks after the second time of ADR injection. Kidney tissues were fixed in 10% formalin to prepare paraffin-embedded tissue sections for immunohistochemistry, or used for MCP-1 mRNA detection and ChIP-qPCR. Serum and urine were collected for detecting Albumin-Creatinine Ratio (ACR) and serum creatinine (Scr).

Detection of mRNA expression in renal tissue by real-time PCR

mRNA expression in kidneys was measured via quantitative reverse transcriptase PCR. Total mRNA of the harvested kidneys was isolated using TRIzol and reverse-transcribed into cDNA as previously described. SYBR Green Real-Time PCR was performed in a 15-mL PCR mixture consisting of 7.5 mL of SYBR Green Real-Time PCR master mix (TOYOBO, Osaka, Japan) containing HotMaster Taq DNA polymerase and SYBR solution, 0.3 mL (10 mM) of each forward and reverse primer, 1 mL of cDNA, and 5.9 mL of nuclease-free water. PCR amplification reactions were performed in a Chromo4 Four-color Real-Time PCR Detection System (Bio-Rad, CA, USA) with the following thermal cycle conditions: initial denaturation 95°C for 3 min; 40 cycles of amplification at 95°C for 15 s; and annealing at 60°C for 45 s. Each sample was analyzed in triplicate. Data were normalized to GAPDH and calculated as the fold change in value of the treatment groups relative to the control groups in accordance with the 2⁻ΔΔCT method.

Primers were designed with Premier 5.0 software with the following sequences. For tissue: GAPDH, forward, 5'-AGTATGACTCCACCTCAGGG-CAA-3' and reverse, 5'-TCTCGCTCTGGAAAGAT-GGT-3'; MCP-1, forward, 5'-TAGCATTACGCTGCT-GTCT-3' and reverse, 5'-TGAGGTGTTGTGGGAAAAGA-3'; EP300, forward, 5'-GGAATACCA-ATGGTTGTG-3' and reverse, 5'-ATTGGGAGAATCTACATTG-3'. For podocytes: GAPDH, forward, 5'-TCATAGGCTCATTTTGTGTAT-3' and reverse, 5'-GCTCAAGCCGGTTTCTTACTCTC-3'; MCP-1, forward, 5'-TAGCATTACGCTGCTGCTCT-3' and reverse, 5'-TGAGGTGTTGTGGGAAAAGA-3'; EP300, forward, 5'-TGCACCCACAGCGCCCTAAT-3' and reverse, 5'-GTTCCAGGGTTTAACGTAAC-3'.
**Histone deacetylation effect of curcumin in Kidney disease**

**Figure 1.** Effects of curcumin on serum creatinine (A), urinary ACR (B) and Histologic changes (C, D). ADR Group denotes Adriamycin nephropathy model rat; ADR + curcumin group: ADR nephropathy model rat treated with curcumin 200 mg·kg⁻¹·d⁻¹ by intragastric administration for 6 weeks. (C) Representative micrographs of histologic changes in control group, ADR nephropathy rat model group and ADR + curcumin group by Masson staining (A: Magnification 200×, scale bar = 100 um; B: Magnification 400×, scale bar = 30 um). (D) Quantitative evaluation of fibrosis. The scope of collagen deposition was measured by collagen-rich area calculated as the percentage of the area. Means ± SD are given, *P < 0.05, **P < 0.01.

**In vitro experiments**

**Cell culture:** Immortalized mouse podocytes were cultured in accordance with the standards outlined by Professor Mundel of Mount Sinai School of Medicine, USA [10]. Briefly, the immortalized podocytes were cultured for 5 d at 33°C in 5% CO₂ under permissive conditions and were subsequently cultured for 2 wk at 37°C in 5% CO₂. By day 14, the podocytes had converted into spindle-shaped cells with small branches.

**Immunofluorescence assay:** Cells were washed once with PBS and fixed in 4% paraformaldehyde in PBS for 20 min at room temperature. After the cells had been washed three times with PBS, the nonspecific binding sites were blocked for 20 min with 3% bovine serum albumin at room temperature before being incubated with fluorescein-labelled phalloidin for F-actin detection for 20 min at room temperature. Subsequently, nuclei were stained with 4′,6′-diamidino-2-phenylindole dihydrochloride (DAPI) for 5 min at room temperature. Images were obtained with an LSM 510 laser scanning confocal microscope (Carl Zeiss, Thornwood, NY, USA). To visualize F-actin, Fluorescence microscope (Olympus) was used. The content of F-actin was determined by measuring the number of actin stress fibres. Ten fields per group were used for the measurement. The

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average content of F-actin in three independent experiments was determined.

**Statistical analyses**

Results are expressed as mean ± standard deviation (SD). Significant differences in the mean values were evaluated using Student’s t-test. Intergroup variation was measured by one way analysis of variance (ANOVA). Statistical significance was considered $P < 0.05$. Statistical analyses were performed with GraphPad Prism 5.

**Results**

**Curcumin treatment ameliorated biochemical parameters and renal pathology induced by ADR**

In ADR Group, urinary ACR were significantly higher than Control Group (43.79±6.875 vs. 5.263±1.116 mg·μmol$^{-1}$; $P < 0.01$). Urinary ACR in the Curcumin-treated group significantly decreased than that of the model group (22.27±2.214 vs. 43.79±6.875 mg·μmol$^{-1}$; $P < 0.05$) (Figure 1A). Compared with control group, Serum creatinine increased (52.66±15.68 vs. 19.25±1.367 μmol$^{-1}$; $P < 0.05$). Curcumin treatment did not change Serum creatinine compared with model group (30.12±4.498 vs. 52.66±15.68 μmol$^{-1}$, $P = 0.172$) (Figure 1B).

Renal pathology was evaluated after ADR injection. Extracellular matrix (ECM) deposition could be observed in the ADR-induced kidney disease model (both at 200× and 400× magnification), showing more fibrosis in the interstitial area and more sclerosis in the glomerular (Figure 1C). Quantitative data showed that the area of fibrosis was 3.43-fold that of the control group ($P < 0.01$; Figure 1D). The above distribution pattern of fibrosis in the ADR-induced kidney disease model was in accord with the literature, indicating the establishment of a renal fibrosis model.

Treatment with curcumin significantly reduced renal ECM accumulation, indicated by the smaller area of green color in tissue sections (Figure 1C). Quantitative data (Figure 1D) also showed that treatment with curcumin was associated with 1.9-fold less fibrosis ($P < 0.01$).

**Curcumin increased of EP300 mRNA transcription and decrease histone acetylation level**

Renal EP300 mRNA levels were 3.782-fold higher in the group treated with curcumin, compared with the control group ($P < 0.01$), while treatment with curcumin decreased renal EP300 mRNA expression to 1.737-fold of the control ($P < 0.01$; Figure 2A).

To further investigate the histone level of kidney cortex tissues treated with curcumin, we measured the histone levels. Both H3 (3.03-fold that of the control, $P < 0.01$) and H4 (1.6-fold that of the control, $P < 0.05$) acetylation..
levels were increased in the fibrosis model groups (Figure 3A, 3B). Curcumin treatment could decrease both H3 and H4 acetylation levels to 2.1-fold ($P < 0.01$) and 0.98-fold ($P < 0.01$) of the control (Figure 3A, 3B).

**Curcumin decreased MCP-1 transcription and histone acetylation of the MCP-1 promoter in ADR-induced kidney injury model**

Renal MCP-1 mRNA in the model group was significantly higher (86.86-fold) than that in the control ($P < 0.05$) and curcumin treatment could decrease ADR-induced MCP-1 expression to 4-fold ($P < 0.05$; Figure 2B). These results indicated that curcumin could decrease the inflammation of renal disease to some extent.

To understand the relationship between the changing histone acetylation function of curcumin and MCP-1 expression, we applied the ChIP-qPCR assay by anti-Acetyl-H3 and anti-Acetyl-H4 antibody to detect the histone acetylation of the MCP-1 promoter region. Values were presented as fold changes. Values are mean ± SD, *$P < 0.05$, **$P < 0.01$. 

**Figure 3. Effects of curcumin on ADR-induced renal histone acetylation levels. A, B: Kidney cortex tissue (40 mg per sample) was applied and the histone acetylation levels were detected at 450 nm wavelengths with a microplate reader (details in methods). C, D: Rat cortex kidney tissues by different treatment were applied. ChIP-qPCR assays were performed with anti-Acetyl-H3, anti-Acetyl-H4 and non-specific IgG antibodies and the binding was assessed on MCP-1 promoter region. Values were presented as fold changes. Values are mean ± SD, *$P < 0.05$, **$P < 0.01$.**
Histone deacetylation effect of curcumin in Kidney disease

Histone deacetylation effect of curcumin in Kidney disease

Figure 4. Curcumin protect ADR-caused podocyte injury. A: Podocytes were pretreated with curcumin (0.5 and 1 µg/mL) for 2 h and then stimulated with ADR (2.5 mg/mL) for 24 h. Podocytes were observed by inverted microscope; B: Podocytes were pretreated with curcumin (0.5 and 0.25 µg/mL) for 2 h and then stimulated with ADR (2.5 mg/mL) for 24 h. The nuclei were stained in blue and F-actin was stained in green (Original magnification ×400, scale bar = 50 µm). Data represent the mean ± SD, **P < 0.01.

Curcumin relieved podocyte injury induced by ADR

Stimulation of mouse podocytes with ADR could induce cell death significantly (Figure 4A). Pretreatment with curcumin ameliorated ADR-induced podocyte injury partly. To test whether curcumin has a role in the organization of the actin cytoskeleton, we used fluorescent phalloidin-labelled F-actin to observe actin rearrangement. After ADR stimulation for 24 h, the filament was dissolved, podocyte structure was disorganized, and compared to the ADR group, curcumin intervention could preserve some structure (Figure 4B). All of the above indicated that curcumin could ameliorate podocyte injury induced by ADR to a certain degree.

Curcumin changed EP300 and MCP-1 mRNA transcription and decreased MCP-1 promoter histone acetylation in podocyte

EP300 mRNA was 3.4-fold that of the control (P < 0.05) by ADR-treated for 24 hours and compared to ADR group (Figure 5A). Compared with the ADR group, curcumin could significantly reduce EP300 mRNA to 1.42-fold that of the control (P < 0.05).

Compared with the ADR group, curcumin (P < 0.01) treatment reduced the level of MCP-1 mRNA expression to 0.72-fold of control respectively (Figure 5B). Also, the MCP-1 promoter was hyperacetylated to 1.997-fold that of the control in the ADR group detected by ChIP-
Histone deacetylation effect of curcumin in Kidney disease

qPCR. Compared with the ADR group, curcumin intervention could decrease MCP-1 promoter acetylation levels significantly to 1.04-fold that of the control \((P < 0.05; \text{Figure 5C})\).

Discussion

Interstitial fibrosis and glomerular sclerosis are one of the characteristics in the development and progression of CKD, regardless of its etiology \([1]\). Therefore, understanding and halting the progression of fibrosis is an important clinical problem. It is well established that histone acetylation functions as a mediator of fibrosis in many diseases \([11-14]\). Emerging clinical and animal studies have revealed pivotal protective roles regarding histone acetylase in renal systems \([15, 16]\). However, to our knowledge, the histone acetylation effect of curcumin on renal injury has not been reported.

Our data obtained from this investigation are consistent with the suspect that curcumin has a renoprotective role against renal injury in ADR-induced kidney disease model by changing histone acetylation. In this study, 8 weeks after ADR injection we could see renal injury included glomerular barrier, inflammation, ECM deposition and sclerosis. And curcumin could protect the kidney from ADR-induced damage to some extent. Curcumin could affect histone acetylase and influence the histone acetylation levels of ADR-induced kidney model. These results indicate that curcumin could protect the kidney from injury by modifying the histone acetylation. In another word, changing the acetylation level of histone could relieve pathological change of disease models.

Curcumin has several important functions that may contribute to its renoprotective effect including inhibition of the production of reactive oxygen species and inflammation \([6]\). Our previous study showed that curcumin could decrease inflammation in lipopolysaccharide-induced kidney disease by inhibiting inflammation. Also, we demonstrated that this effect might be attributed to its inhibitory effects on MCP-1 mRNA expression and DNA-binding activity of NF-κB \([7]\). In addition, Yun et al. \([17]\) indicated that curcumin might influence MCP-1 expression via epigenetic changes involving NF-κB. In the present study, we found that curcumin might regulate the histone acetylation of the MCP-1 promoter by influencing EP300 expression, which agrees with another study \([8]\).

This result confirms the importance of histone acetylation. It is known that histone acetylase is associated with cell physiology and diseases \([18]\). It has been reported that the mutation of EP300 is related with the tumor incidence \([19]\). Furthermore, the application of a histone deacetylase (HDAC) inhibitor was associated with a decrease in the occurrence of heart failure in a heart failure model, and therefore a therapeutic effect \([20]\). In addition, the re-expression of HDAC2 could reinstitute an asthmatic response to glucocorticoid treatment \textit{in vitro} \([21]\). There are also studies that histone acetylation and kidney diseases are related; it has been shown that HDAC6 is associated with polycystic kidney \([22]\) and HDAC2 may affect Epithelial-mesenchymal transition \([4]\).

**Figure 5.** The mRNA transcription of EP300 and MCP-1 detected by real-time qPCR in different groups and the H3 and H4 acetylation of MCP-1 promoter by ChIP. Podocytes were pretreated with curcumin (0.5 µg/mL) for 2 h and then stimulated with ADR (2.5 mg/mL) for 24 h. The procedures were the same as previous described. Values are mean ± SD, \(*P < 0.05, **P < 0.01\).
Histone deacetylation effect of curcumin in Kidney disease

ADR-induced kidney injury is a model of human FSGS, characterized by glomerular sclerosis and tubulointerstitial fibrosis. In our study, we indicated that curcumin may protect podocytes in vitro by inhibiting MCP-1 expression and by restoring cytoskeleton, which may be due to changing histone acetylation levels. Studies has shown that podocyte injury was associated with proteinuria, and the impaired podocyte may undergo glomerular base membrane damage, cytoskeletal disorder, changes of foot process motility, podocyte apoptosis, inflammation, and Epithelial-mesenchymal transition [23, 24]. MCP-1, as an important inflammatory cytokine, can influence cell adhesion and induce inflammation and injury [25]. Therefore, MCP-1 could initiate and promote kidney damage and is associated with proteinuria [26, 27]. The most important cytoskeletal components of podocytes revealed recently include-actinin-4, podocin, nephrin, and integrin-linked kinase [28, 29]. Therefore, we predict that the cytoskeleton rescued by curcumin may be associated with recovery of the above proteins.

Although curcumin could protect podocytes from injury in the present study, we found that curcumin in high concentrations could lead to podocyte death. This is in accord with other literature. We presume that high doses of curcumin may cause cytoskeletal disorder and cell death. However, the mechanism of this opposite effect is unknown.

Conclusion

In summary, our study herein indicates that curcumin was able to attenuate ADR-induced kidney injury by changing histone acetylation levels and inhibiting MCP-1 expression. Mechanistic studies revealed that histone significantly changed histone acetylation of the MCP-1 promoter. Result of our in vitro study suggests that low doses of curcumin could protect podocytes from cytoskeletal disorder and MCP-1 expression caused by ADR, and these effects may be linked to changing histone acetylation. These findings provide a theoretical basis for further utilization of curcumin in renal disease.

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Disclosure of conflict of interest

None.

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Histone deacetylation effect of curcumin in Kidney disease


