

# Attenuation of Inflammation by Emodin in Lipopolysaccharide-induced Acute Kidney Injury via Inhibition of Toll-like Receptor 2 Signal Pathway

Ying Li, Weijian Xiong, Jing Yang, Jin Zhong, Ling Zhang, Jing Zheng, Hong Liu, Qi Zhang, Xiaoqin Ouyang, Lei Lei, Xiao Yu

Department of Nephrology,  
Chongqing Hospital of  
Traditional Chinese Medicine,  
Chongqing, China

**Keywords.** emodin, toll-like  
receptor 2, lipopolysaccharides,  
inflammation, acute kidney  
injury

**Introduction.** Emodin, an anthraquinone derivative from the Chinese herb *Radix et Rhizoma Rhe*, has been reported to possess anti-inflammatory property in vivo and in vitro. However, the effect of emodin on inflammation in lipopolysaccharide (LPS)-induced acute kidney injury as an immunomodulator has yet to be determined. This study aimed to investigate whether emodin had protective effects against LPS-induced acute kidney injury by inhibiting toll-like receptor 2 (TLR2) signal pathway in normal rat kidney epithelial cells (NRK-52E).

**Materials and Methods.** The NRK-52E cells were incubated with LPS with and without the indicated concentrations of emodin for 24 hours. The TLR2 and NF- $\kappa$ B protein level was detected by Western blot method. Tumor necrosis factor (TNF)- $\alpha$ , interleukin (IL)-1 $\beta$ , and IL-6 protein levels were measured using an enzyme-linked immunosorbent assay. The mRNA expression of TLR2, NF- $\kappa$ B, TNF- $\alpha$ , IL-1 $\beta$ , and IL-6 was detected using a real-time polymerase chain reaction.

**Results.** A concentration of 10<sup>2</sup> ng/mL of LPS significantly upregulated mRNA and protein levels of TLR2 and NF- $\kappa$ B and increased TNF- $\alpha$ , IL-1 $\beta$ , and IL-6 mRNA and protein levels. At doses of 20  $\mu$ M and 40  $\mu$ M, emodin was able to inhibit LPS-induced TLR2, NF- $\kappa$ B, TNF- $\alpha$ , IL-1 $\beta$  and IL-6 mRNA and protein expressions in cultured NRK-52E cells.

**Conclusions.** These results demonstrate that an elevated expression of inflammatory cytokines and TLR2 in cells stimulated by LPS were simultaneously inhibited by emodin. Therefore, emodin attenuates the inflammation by inhibiting TLR2-mediated NF- $\kappa$ B signal pathway, which may contribute to the immune inflammation regulation of emodin in LPS-induced acute kidney injury.

IJKD 2015;9:202-8  
www.ijkd.org

## INTRODUCTION

Acute kidney injury (AKI) is a common severe emergency disease, which contributes significantly to the morbidity and mortality of hospitalized patients. Therefore, how to stop its progress has become the focus of many studies.<sup>1-3</sup> Recent studies have

highlighted the role of the innate immune system in initiating the inflammatory cascade which leads to detrimental changes in AKI. It has been clear that toll-like receptors (TLRs) play a key role in the recognition of exogenous pathogen-associated molecular patterns during the pathogenesis of sepsis-

induced AKI.<sup>4,6</sup> Of the known mammalian TLRs, TLR2 has received particular attention. Toll-like receptor 2 is involved in the signaling pathway of the lipopolysaccharide (LPS) receptor complex.<sup>7</sup> Virulent uropathogenic strains (*Escherichia coli*) express P fimbriae, which bind to the glycolipid receptors of uroepithelial and kidney tubular cells, triggering TLR2 activation with subsequent recruitment of leukocytes and release of proinflammatory cytokines.<sup>8</sup> Cytokines are expressed in response to stimulation by LPS and a synthetic TLR2 agonist in renal tubular epithelial cells.<sup>9</sup>

Emodin (1,3,8-trihydroxy-6-methyl-anthraquinone) is a biologically active natural anthraquinone extracted from the roots and rhizomes of *Rheum palmatum* (Chinese name *DaHuang*), which is one of the most effective traditional Chinese medicines for infection and has now been officially listed in the Chinese Pharmacopoeia.<sup>10,11</sup> Emodin has been confirmed to possess antimicrobial, anti-inflammatory, anti-ulcerogenic, anti-cancer, immunosuppressive, and chemopreventive effects.<sup>12,13</sup> Furthermore, the anti-inflammatory properties of emodin have been well-established in animal experiments and clinical studies.<sup>14-17</sup> It was recently reported that amelioration of tubular epithelial cells injury by emodin may partly contribute to the suppression of TLR4 expression<sup>18</sup>; however, their relevance to TLR2 in tubular epithelial cells has not been investigated.

In this study, we utilized a normal rat kidney epithelial cells (NRK-52E) LPS-induced injury model to assess the effect of emodin on progressive renal damage, and to associate emodin immune regulatory mechanism with TLR2-nuclear factor kappa-light-chain-enhancer of activated B cells (NF- $\kappa$ B) signaling pathway.

## MATERIALS AND METHODS

### Antibodies and Reagents

Emodin (molecular weight, 270.24; purity, 95%) was purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Dulbecco's modified Eagle's medium (DMEM)-F12 culture medium and fetal calf serum were purchased from Gibco (Carlsbad, CA, USA). All other cell culture reagents were obtained from Sigma (St Louis, MO, USA). The LPS from *E coli* (serotype, 0111:B4; cat no,

L-2630) was obtained from Sigma. The CytoTox 96 nonradioactive cytotoxicity assay kit was obtained from Promega Corporation (Madison, WI, USA). TRIzol reagent was purchased from Invitrogen (Thermo Fisher Scientific, Waltham, Massachusetts, USA). Reagents for real-time polymerase chain reaction were purchased from Takara Biotechnology (Dalian, China). The anti-TLR2 and anti-p65 antibodies were from Cell Signaling Technology Co (Boston, MA, USA). Concentrations of tumor necrosis factor (TNF)- $\alpha$ , interleukin (IL)-1 $\beta$ , and IL-6 were determined in culture supernatants using commercially available enzyme-linked immunosorbent assay kits (R&D Systems, Minneapolis, MN, USA).

### Lipopolysaccharide Stimulation and Treatment With Emodin

The NRK-52E cells were grown until confluent. Culture medium was changed to serum-free conditions 24 hours prior to stimulation with LPS (10<sup>2</sup> ng/mL). Experiments were performed in triplicate using 3 consecutive wells of 6-well plates. Each experiment was repeated at least 3 times. The cells were incubated with LPS and/or the indicated concentrations of emodin (20  $\mu$ M and 40  $\mu$ M) for 24 hours.

### Real-time Polymerase Chain Reaction Analysis

Total RNA was extracted from NRK-52E cells using TRIzol. Complementary DNA was synthesized using oligo (dT)16 (Applied Biosystems) and the SuperScript III reverse transcriptase kit. Complementary DNA was amplified in 1 $\times$ Universal Master Mix (Applied Biosystems) with gene-specific primers and probe on Rotor-Gene 6000. Specific TaqMan primers and probes for TLR2, P65, TNF- $\alpha$ , IL-1 $\beta$ , IL-6, and glyceraldehyde 3-phosphate dehydrogenase were previously described. All of the results are expressed as ratios to glyceraldehyde 3-phosphate dehydrogenase.

### Western Blot Analysis

The cells were washed with phosphate-buffered saline and lysed in cells lysis buffer containing 20 mM of Tris-hydrogen chloride, 150 mM of sodium chloride, 1 mM of disodium ethylenediaminetetraacetate, 1 mM of ethylene glycol tetraacetic acid, 1% triton, 2.5 mM sodium pyrophosphate, 1 mM of  $\beta$ -glycerophosphate, 1

mM of sodium orthovanadate, and 1 µg/mL of leupeptin. Cell lysates were sonicated briefly, centrifuged at 12 000 g (15 minutes, 4°C) and supernatants 20 µg of supernatant protein were electrophoresis on a 4% to 15% polyacrylamide gel. Proteins were then transferred to a polyvinylidene difluoride membrane. After electrotransfer, the blots were blocked for 1 hour at room temperature in blocking buffer containing 20 mM of Tris, 137 mM of sodium chloride, 0.1% of tween 20, and 5% of milk (pH, 7.6). The blots were then incubated with primary antibodies (anti-TLR2 and anti-p65) with 1:1000-dilutions in blocking buffer overnight. Following several washes in buffer containing 20 mM of Tris- hydrogen chloride, 137 mM of sodium chloride, and 0.1% of tween 20 (pH, 7.6), the blots were incubated in 1:2000-dilution of antirabbit immunoglobulin G horseradish peroxidase-linked as secondary antibody diluted in blocking buffer 1 hour at room temperature. Following several washes in buffer, the immunoreactive proteins were visualized and quantified by densitometric analysis using Image J software (Wayne Rasband, National Institutes of Health, Stapleton, NY, USA). For all the Western blots, glyceraldehyde 3-phosphate dehydrogenase was used as internal control.

### Enzyme-linked Immunosorbent Assay

To quantify the level of TNF-α, IL-1β, and IL-6 protein expression under different experimental conditions, the total TNF-α, IL-1β, and IL-6 protein in the culture supernatant was measured using a commercial sandwich enzyme-linked immunosorbent assay kit for TNF-α, IL-1β, and IL-6 according to the manufacturer’s instructions. Samples were assayed in duplicate.

### Statistical Analysis

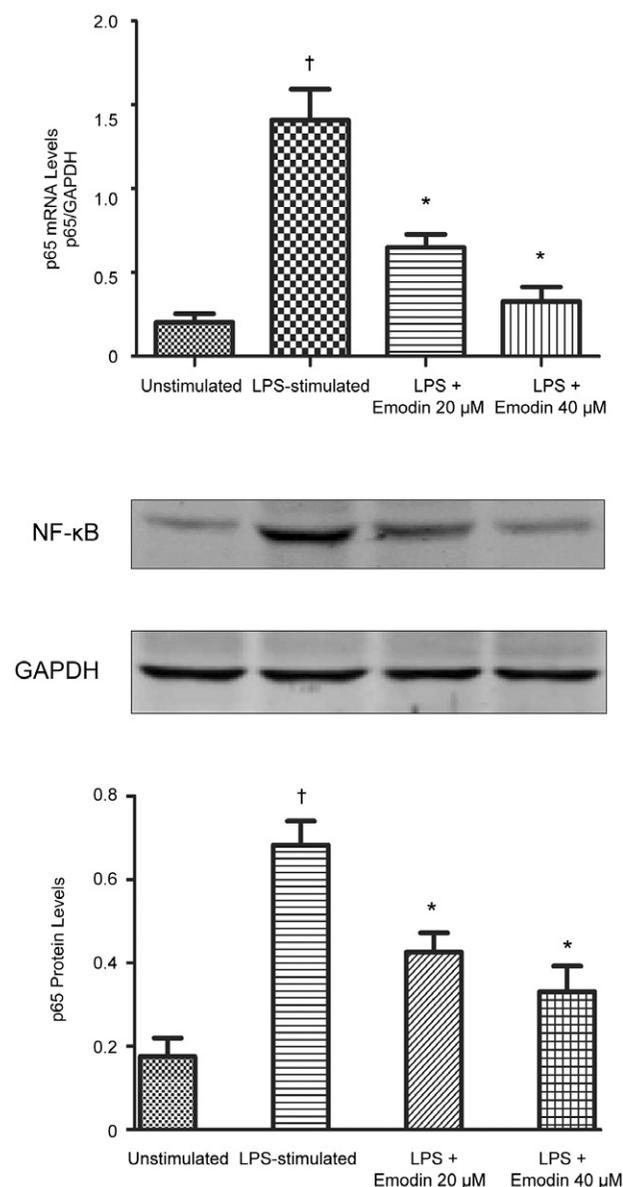
All data were expressed as mean ± standard deviation. Statistical analysis was carried out using the SPSS software (Statistical Package for the Social Sciences, version 13.0, SPSS Inc, Chicago, Ill, USA). Statistical significance was assessed by the 1-way analysis of variance test and the *t* test. *P* values less than .05 were considered significant.

## RESULTS

### Toll-like Receptor2 mRNA and Protein Expression

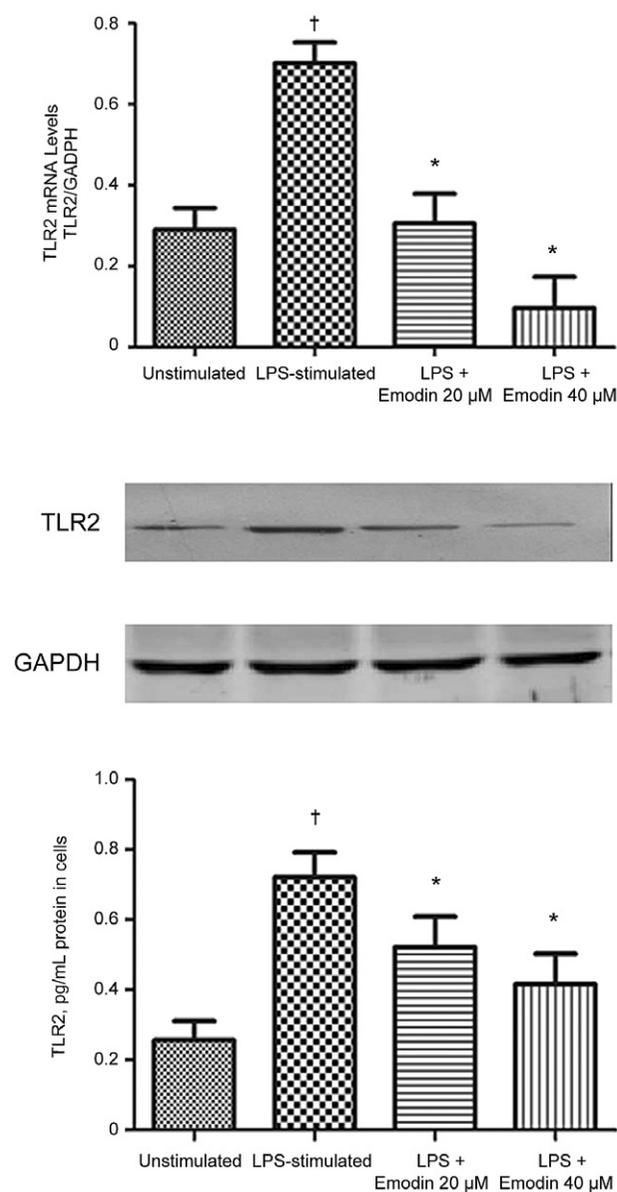
Emodin was able to inhibit LPS-stimulated

*TLR2* mRNA expression in cultured cells, but with distinct differences in action intensity (*P* < .05). At a dose of 40 µM, emodin demonstrated maximum suppression of *TLR2* mRNA expression in LPS-stimulated cells (*P* < .05, Figure 1). Similar to



**Figure 1.** Effects of emodin on LPS-stimulated NF-κB mRNA and protein secretion in NRK-52E cells. The NRK-52E cells were incubated with LPS 10<sup>2</sup> ng/mL in the presence of emodin for 24 hours and NF-κB mRNA and protein were evaluated by real-time polymerase chain reaction and Western blot, respectively. Top, Emodin inhibited the expression of NF-κB mRNA at doses of 20 µM and 40 µM, respectively. Bottom, Effect of emodin on NF-κB protein expression in NRK-52E cells. Western blot of the NF-κB protein expression was performed in NRK-52E cells from the control and treated cells with emodin at doses of 20 µM and 40 µM for 24 hours, respectively. \**P* < .05 versus unstimulated NRK-52E cells †*P* < .01 versus unstimulated NRK-52E cells

its effect on TLR2 protein expression, emodin inhibited the LPS-upregulated synthesis of the TLR2 surface protein in a dose-dependent manner ( $P < .05$ , Figure 2).



**Figure 2.** Effects of emodin on LPS-stimulated TLR2 mRNA and protein production in NRK-52E cells. The NRK-52E cells were incubated with LPS  $10^2$  ng/mL in the presence of emodin for 24 hours and TLR2 mRNA and protein were evaluated by real-time polymerase chain reaction and Western blot, respectively. Top, Emodin inhibited the expression of TLR2 mRNA at doses of 20  $\mu$ M and 40  $\mu$ M, respectively. The inhibitory effect of emodin demonstrates a dose-dependent manner. Bottom, Effect of emodin on TLR2 protein expression in NRK-52E cells. Western blot of the TLR2 protein expression was performed in NRK-52E cells from the control and treated cells with emodin at doses of 20  $\mu$ M and 40  $\mu$ M for 24 hours, respectively. \* $P < .05$  versus unstimulated NRK-52E cells <sup>†</sup> $P < .01$  versus unstimulated NRK-52E cells

### Nuclear Factor- $\kappa$ B mRNA and Protein Expression

The levels of NF- $\kappa$ B mRNA and protein significantly increased in LPS-stimulated cells, which indicated that NF- $\kappa$ B took an active role in inflammatory response to the injury. Compared with those in LPS-induced cells, NF- $\kappa$ B mRNA and protein were both significantly downregulated in emodin-treated cells at both doses ( $P < .05$ , Figure 1).

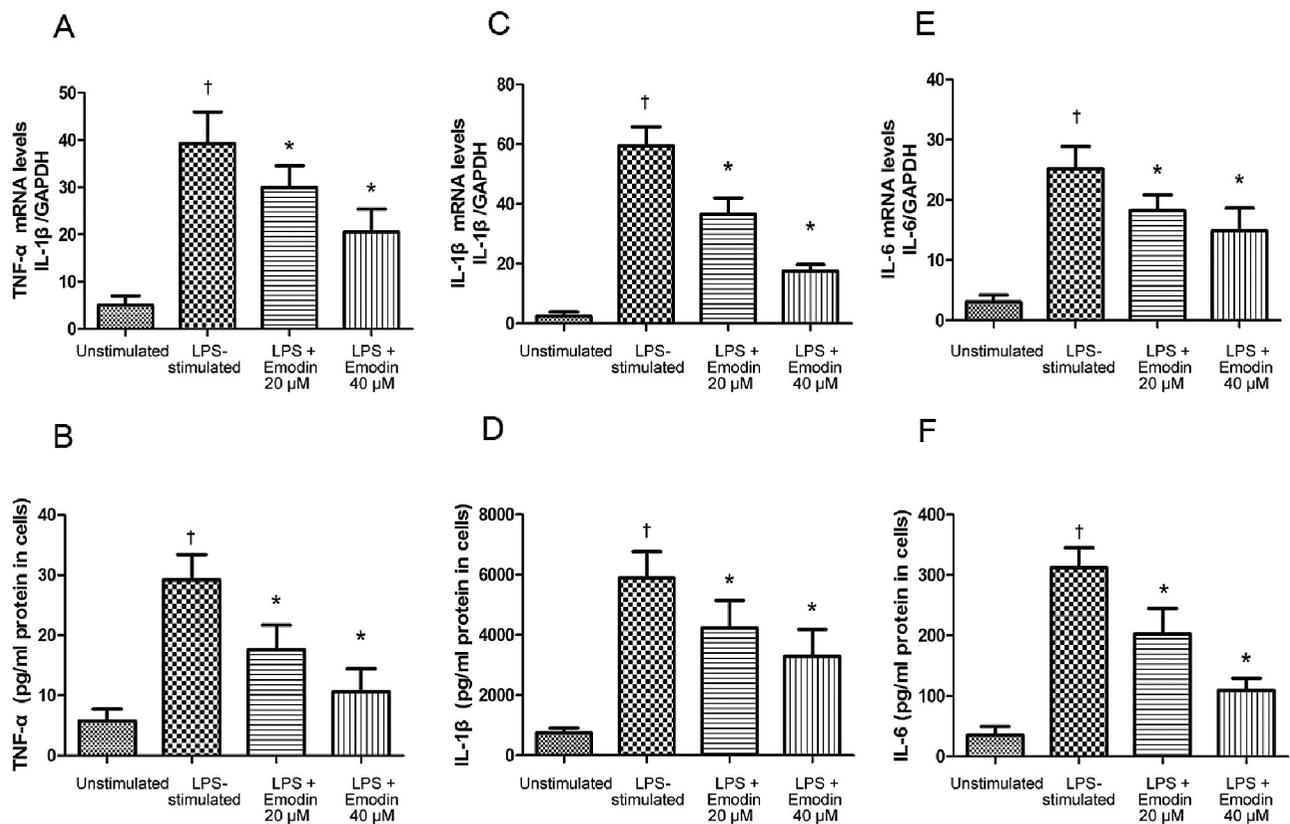
### Inflammatory Cytokines mRNA and Protein Expression

Expression of *TNF- $\alpha$* , *IL-1 $\beta$* , and *IL-6* mRNA and protein was measured by real-time polymerase chain reaction and Western blot. Emodin decreased LPS-induced *TNF- $\alpha$* , *IL-1 $\beta$* , and *IL-6* mRNA expression ( $P < .05$ , Figures 3A to 3C). At a dose of 20  $\mu$ M, the inhibitory effects of emodin on *TNF- $\alpha$* , *IL-1 $\beta$* , and *IL-6* mRNA secretion were significant, but were lower than the concentration of 40  $\mu$ M compared with LPS-stimulated cells ( $P < .05$ ). Emodin at both concentrations had an effect on *TNF- $\alpha$* , *IL-1 $\beta$* , and *IL-6* protein secretion ( $P < .05$ , Figures 3D to 3F).

### DISCUSSION

Our results demonstrated for the first time, to the best of our knowledge, that emodin inhibited LPS-induced TLR2 and NF- $\kappa$ B expressions in cultured NRK-52E cells, as well as partly blocked LPS-stimulated *TNF- $\alpha$* , *IL-1 $\beta$* , and *IL-6* upregulation. Certain data from the previous studies demonstrated that emodin attenuated LPS-mediated inflammatory response in mouse mammary epithelial cells and liver.<sup>13,14</sup> In the present study, the effect of emodin in the LPS-induced NRK-52E cells is likely to be interrelated to downregulate TLR2, NF- $\kappa$ B, and inflammatory cytokines. It is considered that an increased understanding of this process may lead to therapies that are able to effectively prevent or reverse inflammation in AKI.

Lipopolysaccharide is one of the most studied immunostimulatory components of bacteria that can induce systemic inflammation when excessive signaling occurs.<sup>19</sup> Thus, it has been widely used as a stimulating factor in research on TLR2 in infective diseases. We investigated the effects of emodin on cultured NRK-52E cells. The results are consistent with previous findings that TLR2 mRNA and protein expression were upregulated by LPS,<sup>7</sup> which also paralleled the severity of increased *TNF- $\alpha$* ,



**Figure 3.** Effects of emodin on LPS-stimulated inflammatory cytokines mRNA and protein secretion in NRK-52E cells. The NRK-52E cells were incubated with LPS  $10^2$  ng/mL in the presence of emodin for 24 hours and inflammatory cytokines mRNA and protein were evaluated by real-time polymerase chain reaction and enzyme-linked immunosorbent assay, respectively. A, Emodin decreased the expression of *TNF-α* mRNA at doses of 20  $\mu$ M and 40  $\mu$ M, respectively. B, Emodin inhibited the expression of *IL-1β* mRNA at both doses. C, Effect of emodin on the expression of *IL-6* mRNA with dose-dependent manner. D, The expression of *TNF-α* protein was analyzed by enzyme-linked immunosorbent assay. E, Emodin decreased the expression of *IL-1β* protein at both doses. F, The inhibitory effect of emodin on *IL-6* protein expression demonstrates a dose-dependent manner.

\* $P < .05$  versus unstimulated NRK-52E cells  
 $\dagger P < .01$  versus unstimulated NRK-52E cells

IL-1 $\beta$ , and IL-6 mRNA and protein expression in cultured mouse tubular epithelial cells, supporting the hypothesis that LPS is the key molecule in the overexpression of TLR2 and cytokines.

Previous reports investigating significant differences between wild-type and TLR2 knockout mice have demonstrated that TLR2's role is significant in renal pathophysiological conditions.<sup>20</sup> Leemans and colleagues revealed that the kidneys of TLR2 knockout mice in wild-type hosts remained susceptible to ischemic AKI.<sup>21</sup> Tubular epithelial cells from TLR2-knockout mice failed to respond to LPS, and cytokines released by them were decreased.<sup>1</sup> These data suggest that the TLR2 expresses on renal parenchyma plays a crucial role in the induction of inflammation and injury. In the present study, we demonstrated that the expression of TLR2 was significantly upregulated 24

hours after stimulation with  $10^2$  ng/mL of LPS. The detection of TLR2 on NRK-52E cells also revealed a potential site for inflammation initiation based on TLR2. Leemans and colleagues also provided compelling evidence that the activation of TLR2 present on renal parenchymal cells triggers an innate immune response that mediates progressive renal failure.<sup>22</sup> Thus, TLR2 is critical in the progression of immunoreactivity. Therefore, the inhibition of TLR2 overexpression has become particularly significant for the management of AKI.

Emodin is the main effective composition in certain Chinese herbs. Previous studies have revealed that the laboratory signs of rat renal lesions were significantly ameliorated by emodin, as demonstrated by decreased blood creatinine and urea and 24-hour urine protein in the kidney failure models after administration with emodin.<sup>12,23</sup>

A previous study demonstrated that emodin significantly suppressed the expression of ocular surface TLR4, at the level of mRNA transcription and protein synthesis.<sup>17</sup> Li and colleagues revealed that emodin might ameliorate LPS-induced acute pancreatitis by inhibiting activation of NF- $\kappa$ B signaling.<sup>18</sup> However, no studies have yet described emodin's ability to suppress LPS-induced TLR2 expression in the kidney. In the present study, it was observed that emodin effectively reversed LPS-upregulated TLR2 mRNA and protein expression at concentrations of 20  $\mu$ M and 40  $\mu$ M in cultured NRK-52E cells. When compared with LPS-stimulated group, co-culture with emodin significantly reversed LPS-induced expression of TLR2 in cells.

While we have demonstrated that the LPS-induced activation of TLR2 was suppressed by emodin in the present study, the ligation of TLR2-NF- $\kappa$ B induced production of proinflammatory cytokines cannot be ignored. Consequently, we examined the inhibitory effects of emodin on the overexpression of TNF- $\alpha$ , IL-1 $\beta$ , and IL-6 in NRK-52E cells. Tubular epithelial cells respond to local infection, with the release of multiple cytokines, chemokines, and other factors that are considered to orchestrate the cellular constituents of the innate immune response.<sup>24</sup> Moreover, the expression of TLR2 may be involved in regulating immune cells to synthesize cytokines via activating NF- $\kappa$ B.<sup>25</sup> The results from the present study demonstrate a compelling contribution of emodin in renal inflammatory disease.

## CONCLUSIONS

The present study shows the role of emodin as an immunoregulator in LPS-induced AKI, most likely acting through the suppression of TLR2-triggered innate immune responses. These encouraging results put emodin to one of the candidates for therapy in LPS-induced AKI treatment.

## ACKNOWLEDGEMENTS

This work was supported by grants from the Applied Basic Research Program of the Public Health Bureau Foundation of Chongqing Province (zy201403005 and zy201403004).

## CONFLICT OF INTEREST

None declared.

## REFERENCES

1. Kellum JA. Acute kidney injury. *Crit Care Med*. 2008;36:S141-S145.
2. Theilig F, Kriz W, Jerichow T, et al. Abrogation of protein uptake through megalin-deficient proximal tubules does not safeguard against tubulointerstitial injury. *J Am Soc Nephrol*. 2007;18:1824-34.
3. Ostermann M, Chang RW. Acute kidney injury in the intensive care unit according to RIFLE. *Crit Care Med*. 2007;35:1837-43.
4. Akira S, Uematsu S, Takeuchi O. Pathogen recognition and innate immunity. *Cell*. 2006;124:783-801.
5. Lee HK, Iwasaki A. Innate control of adaptive immunity: dendritic cells and beyond. *Semin Immunol*. 2007;19:48-55.
6. Zager RA, Johnson AC, Lund S, Randolph-Habecker J. Toll-like receptor (TLR4) shedding and depletion: acute proximal tubular cell responses to hypoxic and toxic injury. *Am J Physiol Renal Physiol*. 2007;292:F304-F312.
7. Werts C, Tapping RI, Mathison JC, et al. Leptospiral lipopolysaccharide activates cells through a TLR2-dependent mechanism. *Nat Immunol*. 2001;2:346-52.
8. Scherberich JE, Hartinger A. Impact of Toll-like receptor signalling on urinary tract infection. *Int J Antimicrob Agents*. 2008;31 Suppl 1:S9-14.
9. Chowdhury P, Sacks SH, Sheerin NS. Toll-like receptors TLR2 and TLR4 initiate the innate immune response of the renal tubular epithelium to bacterial products. *Clin Exp Immunol*. 2006;145:346-56.
10. China Pharmacopoeia Committee. *Pharmacopoeia of the People's Republic of China (First Division of 2000 ed)*. Beijing: China Chemical Industry Press; p. 181999.
11. Li S, Dong X, Wu H, Zhang L, Zhang D. Progress in studies on pharmacological effect of rhubarb and its active components. *Med Recapitulate*. 2005;1:76-8.
12. Kwak HJ, Park MJ, Park CM, et al. Emodin inhibits vascular endothelial growth factor-A-induced angiogenesis by blocking receptor-2 (KDR/Flk-1) phosphorylation. *Int J Cancer*. 2006;118:2711-20.
13. Jia X, Iwanowycz S, Wang J, et al. Emodin attenuates systemic and liver inflammation in hyperlipidemic mice administrated with lipopolysaccharides. *Exp Biol Med (Maywood)*. 2014;239:1025-35.
14. Yang Z, Zhou E, Wei D, et al. Emodin inhibits LPS-induced inflammatory response by activating PPAR-gamma in mouse mammary epithelial cells. *Int Immunopharmacol*. 2014;21:354-60.
15. Lei T, Zhou XC, Cai SQ. The clinical observation of different administration route and doses of rhubarb in acute pancreatitis. *Guide China Med*. 2011;28:1671-8194.
16. Chen RF, Shen YC, Huang HS, et al. Evaluation of the anti-inflammatory and cytotoxic effects of anthraquinones and anthracenes derivatives in human leucocytes. *J Pharm Pharmacol*. 2004;56:915-9.
17. Kitano A, Saika S, Yamanaka O, et al. Emodin suppression of ocular surface inflammatory reaction. *Invest Ophthalmol Vis Sci*. 2007;48:5013-22.
18. Li Z, Xia X, Zhang S, Zhang A, Bo W, Zhou R. Up-

- regulation of Toll-like receptor 4 was suppressed by emodin and baicalin in the setting of acute pancreatitis. *Biomed Pharmacother.* 2009;63:120-8.
19. Beutler B, Rietschel ET. Innate immune sensing and its roots: the story of endotoxin. *Nat Rev Immunol.* 2003;3:169-76.
  20. El-Achkar TM, Dagher PC. Renal Toll-like receptors: recent advances and implications for disease. *Nat Clin Pract Nephrol.* 2006;2:568-81.
  21. Leemans JC, Stokman G, Claessen N, et al. Renal-associated TLR2 mediates ischemia/reperfusion injury in the kidney. *J Clin Invest.* 2005;115:2894-903.
  22. Leemans JC, Butter LM, Pulskens WP, et al. The role of Toll-like receptor 2 in inflammation and fibrosis during progressive renal injury. *PLoS One.* 2009;4:e5704.
  23. Wang J, Huang H, Liu P, et al. Inhibition of phosphorylation of p38 MAPK involved in the protection of nephropathy by emodin in diabetic rats. *Eur J Pharmacol.* 2006;553:297-303.
  24. Yeboah MM, Xue X, Duan B, et al. Cholinergic agonists attenuate renal ischemia-reperfusion injury in rats. *Kidney Int.* 2008;74:62-9.
  25. Ando M, Shibuya A, Tsuchiya K, Akiba T, Nitta K. Reduced expression of Toll-like receptor 4 contributes to impaired cytokine response of monocytes in uremic patients. *Kidney Int.* 2006;70:358-62.

Correspondence to:

Weijian Xiong, MSc  
Department of Nephrology, Chongqing Hospital of Traditional Chinese Medicine, Chongqing 400021, China  
Fax: +86 23 6706 3859  
E-mail: weijianxiong\_zyy@163.com

Received April 2014  
Revised September 2014  
Accepted October 2014