Int J Clin Exp Pathol 2019;12(3):867-875
www.ijcep.com /ISSN:1936-2625/IJCEP0088263

Original Article

Dioscin ameliorates peritoneal fibrosis by inhibiting epithelial-to-mesenchymal transition of human peritoneal mesothelial cells via the TLR4/MyD88/NF-κB signaling pathway

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Received November 12, 2018; Accepted January 9, 2019; Epub March 1, 2019; Published March 15, 2019

Abstract: Objective: To investigate the effect of dioscin on lipopolysaccharide (LPS)-induced peritoneal fibrosis and its underlying mechanism. Methods: The human peritoneal mesothelial cell line (HMrSV5) was treated with LPS, followed by treatment with different concentrations of dioscin (0.25, 0.5 or 1.0 μg/ml). Toll-like receptor (TLR) 4 gene transfection was performed and dioscin (0.5 μg/ml) was used in mechanism research. Then morphological observation was carried out, and LPS-related markers of epithelial mesenchymal transition (EMT) as well as fibrosis markers were detected by western blotting. qRT-PCR and ELISA assay were applied to measure inflammatory factors. Furthermore, TLR4/MyD88/NF-κB pathway related proteins were assessed. Results: Dioscin inhibited LPS-induced morphologic changes, significantly reduced the levels of markers of EMT including N-cadherin, matrix metalloproteinase-2 (MMP-2), MMP-9 and vimentin, and elevated the levels of E-cadherin and zonula occludens protein 1 (ZO-1). Decreased levels of fibrosis markers α-smooth muscle actin (α-SMA), collagen I and fibronectin were found in dioscin groups. Additionally, dioscin downregulated interleukin-6 (IL-6), IL-1β and tumor necrosis factor alpha (TNF-α). Dioscin inhibited EMT and fibrosis through triggering the TLR4/MyD88/NF-κB signaling pathway by decreasing expressions of TLR4, myeloid differentiation factor 88 (MyD88), nuclear factor κB (NF-κB), transforming growth factor-β1 (TGF-β1), phosphorylated Smad2 (p-Smad2), α-SMA, collagen I and fibronectin. Conclusion: This study provides a novel and efficient remedy to alleviate PD-associated fibrosis for patients undergoing long-term peritoneal dialysis.

Keywords: Dioscin, epithelial mesenchymal transition, peritoneal fibrosis, TLR4/MyD88/NF-κB

Introduction

Currently, peritoneal dialysis (PD) has received increasing attention as a common treatment remedy for patients with end-stage renal disease (ESRD), rather than hemodialysis and kidney transplantation. Unfortunately, long-term preservation of the peritoneal membrane integrity, peritoneal dialysis solution, and peritonitis are considered as important challenges in PD maintenance [1], which often lead to peritoneal fibrosis and an ending of dialysis. Previous studies demonstrated that inhibition of EGF receptor, periostin-binding DNA aptamer treatment, and the dipeptide alanyl-glutamine hindered the development and progression of peritoneal fibrosis [2-4]. Although the modern research has reached certain progress on peritoneal fibrosis therapy, there is no ideal therapeutic drug for peritoneal fibrosis.

It was reported that chronic inflammation, oxidative stress, and epithelial-mesenchymal transition (EMT) of peritoneal mesenchymal cells (MCs) contributed to PD-induced fibrosis [5, 6], and a previous study revealed that EMT of MCs was the protagonist and initiator of peritoneal fibrosis [7]. EMT, which had previously been described in chronic inflammatory and fibrogenic diseases, was a mature dynamic process of epithelial degeneration and mesenchymal phenotype. With the development of EMT, the polarized, inactive epithelial cells lose tight junctions and related adherence, and then rear-

range the cytoskeleton, which promote the loss of peritoneal function [8]. Fresh evidence unveiled that chronic inflammation-caused EMT of MCs had an important role in peritoneal fibrosis [9]. Moreover, several signaling pathways have been shown to participate in the EMT process of peritoneal fibrosis [10]. Although the role of EMT in peritoneal fibrosis has been explored extensively, there are no ideal drugs available for patients with peritoneal fibrosis. Therefore, it is urgent to develop a novel and effective remedy to treat peritoneal fibrosis of patients undergoing long-term PD.

As a natural steroid saponin, dioscin is isolated from traditional herb, such as *Dioscorea nipponica* Makino [11]. Previous studies demonstrated that dioscin had potent effects on antitumor, ameliorating cerebral ischemia/reperfusion injury, attenuating liver injury and suppressing liver fibrosis [12-14]. Additionally, dioscin was proved to suppress alcohol-induced fibrosis by attenuating hepatic stellate cell activation by triggering TLR4/MyD88/NF-κB pathway [15]. Dioscin also alleviated LPS-caused kidney injury through the microRNA let-7i/TLR4/MyD88 signaling pathway [16]. However, the effect of diosicin on peritoneal fibrosis and its underlying molecular mechanism are still poorly elucidated.

In the present study, we therefore attempted to explore the effects of dioscin on EMT, peritoneal fibrosis, and inflammation induced by LPS in HMrSV5 cells. Further, the molecular mechanism of dioscin alleviating LPS-caused peritoneal fibrosis was investigated. The present study provides a novel strategy for patients with long-term PD to alleviate PD-associated fibrosis.

Methods

Drug

Dioscin was purified from *Dioscorea nipponica* Makino, and the purity of Dioscin was greater than 98%. Then dioscin was dissolved in 0.1% dimethylsulfoxide (DMSO, Sinopharm Chemical Reagent Co., Ltd., Shanghai, China) for all *in vitro* studies.

Cell culture

HMrSV5 cell line (Shanghai Cell Bank, Chinese Academy of Sciences) was maintained in Dulbecco's Modified Eagle Medium (DMEM, Gibco, USA), supplemented with 10% fetal bovine serum (Thermo Fisher Scientific, USA) at 37°C in humidified 5% CO_2, and cells were subcultured every 2 days. HMrSV5 cells with a density of 3×10^4 cells/cm^2 were grown to 80% confluence in a 6 cm culture dish. Then the cells were exposed to 100 ng/ml lipopolysaccharide (LPS, Sigma, St Louis, MO, USA) in experimental groups, while cells in negative control group was treated with DMEM only. After LPS stimulation, cells were treated with different concentrations of dioscin (0.25, 0.5 or 1.0 μg/ml).

Reverse transcriptase-polymerase chain reaction (RT-PCR)

Trizol reagent (Invitrogen, Carlsbad, CA) was utilized to extract total cellular RNA of HMrSV5 cells. First-strand cDNA was synthesized using SuperScript® III cDNA Synthesis Kit (Invitrogen). RT-PCR was performed using the QuantiTect SYBR Green PCR kit (QiaGen). Primer pairs used for amplification as follows: TNF-α, 5'-TGTAGCCCATGTTGTAGCAAACC-3' (forward) and 5'-GAGGACCTGGGAGTAGATGAGGTA-3' (reverse); IL-1, 5'-CTGAGCACCTTCTTTCCCTTCA-3' (forward) and 5'-TGGACCAGACATCACCAAGCT-3' (reverse); IL-6, 5'-TGGCTGAAAAAGATGGATGCT-3' (forward) and 5'-TCTGCACAGCTCTGGCTTGT-3' (reverse); GAPDH, 5'-GCACCGTCAAGGCTGAGAAC-3' (forward) and 5'-TGGTGAAGACGCCAGTGGA-3' (reverse).

Enzyme-linked immunosorbent assay (ELISA)

TNF-α, IL-1 and IL-6 were detected in culture medium using sandwich ELISA kits according to the manufacturer's instructions.

TLR4 gene transfection

HMrSV5 cells were transfected with pPICZA plus TLR4 plasmid DNA for 24 h using Lipofectamine Plus Reagent (Invitrogen, CA, USA) following manufacturer's instructions. Once completed, cells were treated by serum deprivation for 24 h, and then exposed to LPS (100 ng/ml) for another 24 h in the presence or absence of dioscin (0.5 μg/ml).

Western blotting

Cells were harvested by whole protein lysate (Beyotime, Shanghai, China), and centrifuged at 12000 g for 20 min at 4°C. Proteins from

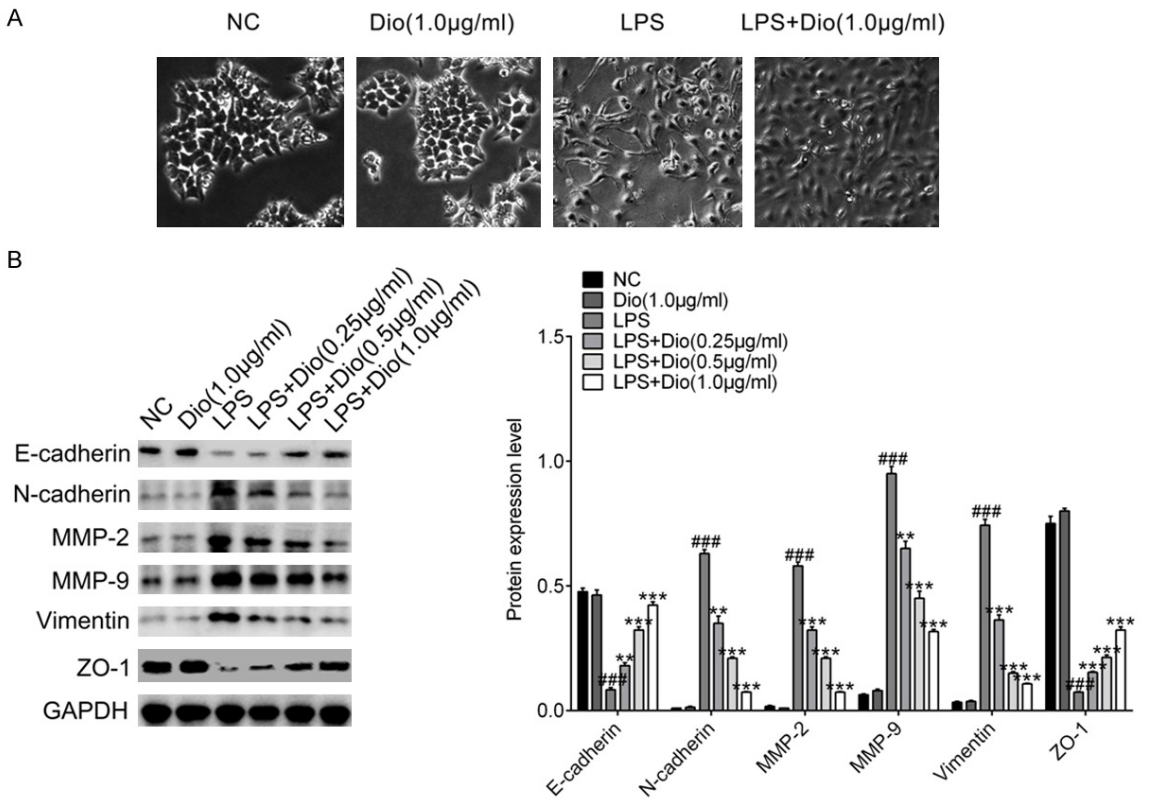


Figure 1. Dioscin suppresses EMT caused by LPS in HMrSV5 cells. A. Morphological observation of HMrSV5 cells in NC, Dio, LPS and LPS+ Dio groups. B. Western blotting for detecting protein levels of E-cadherin, N-cadherin, MMP-2, MMP-9, vimentin and ZO-1. $^{###}P < 0.001$ compared with negative control group or dioscin (1.0 μg/ml) group. $^{**}P < 0.01$ and $^{***}P < 0.001$ compared with LPS group. NC, negative control; LPS, lipopolysaccharide; Dio, dioscin; MMP, matrix metalloproteinase; ZO-1, zonula occludens protein 1.

total cell lysates were then electrophoresed onto 6% SDS-polyacrylamide gels (Invitrogen) and transferred from gels to nitrocellulose membranes (Bio-Rad). Blots were blocked in Tris-buffered saline (TBS) buffer (blocking buffer, 50 mM Tris-HCL and 150 mM NaCL) harboring 5% non-fat milk for 2 h at room temperature. Then the membranes were incubated with following primary antibodies: α-SMA (1: 100, Proteintech Group), collagen I (1:100, Proteintech Group), fibronectin (1:250, Abcam), TLR4 (1:1000, Proteintech Group), MyD88 (1: 1000, Proteintech Group), NF-κB (1:1000, Proteintech Group), TGF-β1 (1:1000, Proteintech Group), p-Smad2/Smad2 (1:1000, Proteintech Group), respectively. GAPDH (1:1000, Cell Biolabs) on the same membrane was used as a loading control. The membranes were subsequently incubated with horseradish peroxidase-conjugated anti-rabbit or anti-mouse IgG (Promega). Finally, blots were visualized by chemiluminescence reagents (Pierce) and radiographed on Kodak X-ray films.

Immunofluorescence assay of α-SMA, collagen I, fibronectin

HMrSV5 cells were seeded in six-well plates for 24 h. After being treated with different doses of dioscin (0.25, 0.5 or 1.0 μg/ml), cells were fixed in 4% formaldehyde in PBS for 5 min, permeabilized with 0.1% Triton-X 100 for 5 min, and blocked with 2% bovine serum albumin at room temperature. Then, fixed cells were incubated with anti-α-SMA antibody (1:100, Proteintech Group), anti-fibronectin antibody (1: 250, Abcam), and anti-collagen I antibody (1: 100, Proteintech Group) overnight at 4°C. Subsequently, the cells were incubated with a fluorescein-labelled secondary antibody for 1 h. After being washed with PBS for 3 times, cell nuclei were stained with DAPI (5 μg/ml). Finally, all the samples were examined by a laser scan-

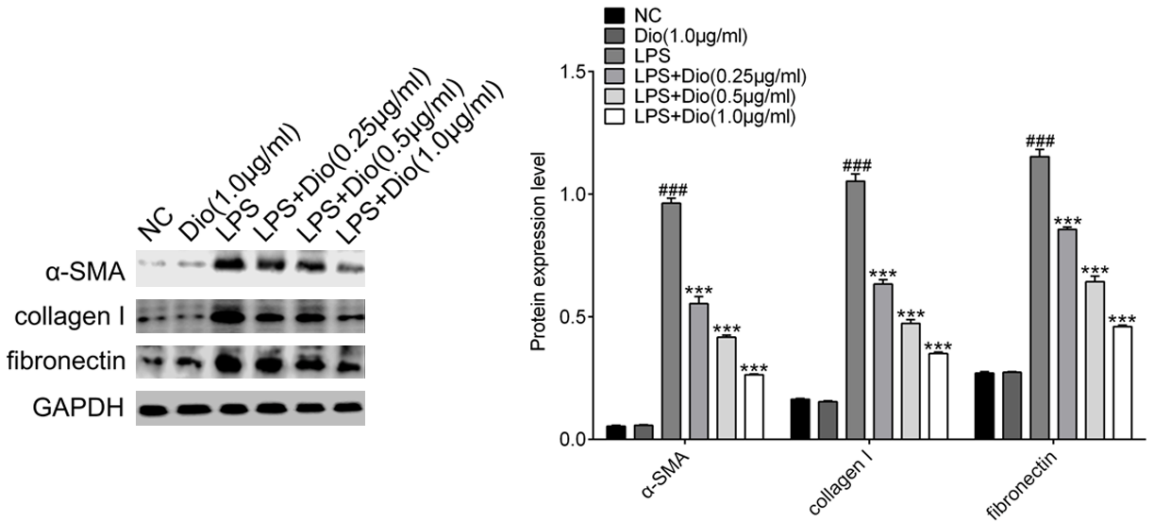


Figure 2. Dioscin inhibits LPS-induced fibrosis in HMrSV5 cells. Western blotting for detecting protein levels of α-SMA, collagen I and fibronectin. ###$P < 0.001$ compared with negative control group or dioscin (1.0 μg/ml) group. ***$P < 0.001$ compared with LPS group. NC, negative control; LPS, lipopolysaccharide; Dio, Dioscin; α-SMA, α-smooth muscle actin.

ning confocal microscope (Olympus, Tokyo, Japan).

Statistical analysis

SPSS16.0 statistical software (SPSS Inc, Chicago, IL) was utilized for all statistical analysis. All data were exhibited as the mean and standard deviation (SD). Statistical analysis of the quantitative data for multiple group comparisons was utilized by ANOVA, following Tukey's multiple comparison tests. Paired comparisons were performed using the *t* test. The level of statistical significance was 0.05.

Results

Dioscin inhibits EMT of HMrSV5 cells treated by LPS

Morphological changes of HMrSV5 cells were observed in negative control, dioscin, LPS and LPS+ dioscin groups. As shown in **Figure 1A**, cells in negative control group and dioscin group exhibited normal morphology, whereas HMrSV5 cells were loosely arranged, and apparent fusiform shape were found in LPS group. However, EMT could be attenuated after being treated by dioscin.

Then, the EMT-related markers were detected by western blotting. Protein expression levels of N-cadherin, MMP-2, MMP-9 and Vimentin were significantly increased, while the levels of E-cadherin and ZO-1 were visibly reduced after LPS challenge. Contrary to LPS-treated group, downregulated levels of N-cadherin, MMP-2, MMP-9 and vimentin, as well as elevated levels of E-cadherin and ZO-1 were remarkably observed after being treated with different concentrations of dioscin (0.25, 0.5 and 1.0 μg/ml). Importantly, dioscin effectively inhibited LPS-induced EMT of HMrSV5 with dose-dependent manner (**Figure 1B**).

Dioscin rehabilitates LPS-induced fibrosis in HMrSV5 cells

Furthermore, fibrosis markers including α-SMA, collagen I and fibronectin were examined. **Figure 2** revealed higher protein levels of α-SMA, collagen I, and fibronectin in LPS-treated group compared with NC group or dioscin group. On the contrary, the levels of α-SMA, collagen I and fibronectin were clearly downregulated in dioscin-treated groups in a dose-dependent manner.

Dioscin alleviates LPS-associated inflammation in HMrSV5 cells

To investigate the effect of dioscin on inflammation caused by LPS in HMrSV5 cells, levels of IL-6, IL-1β and TNF-α were further detected by qRT-PCR and ELISA assay. **Figure 3** showed

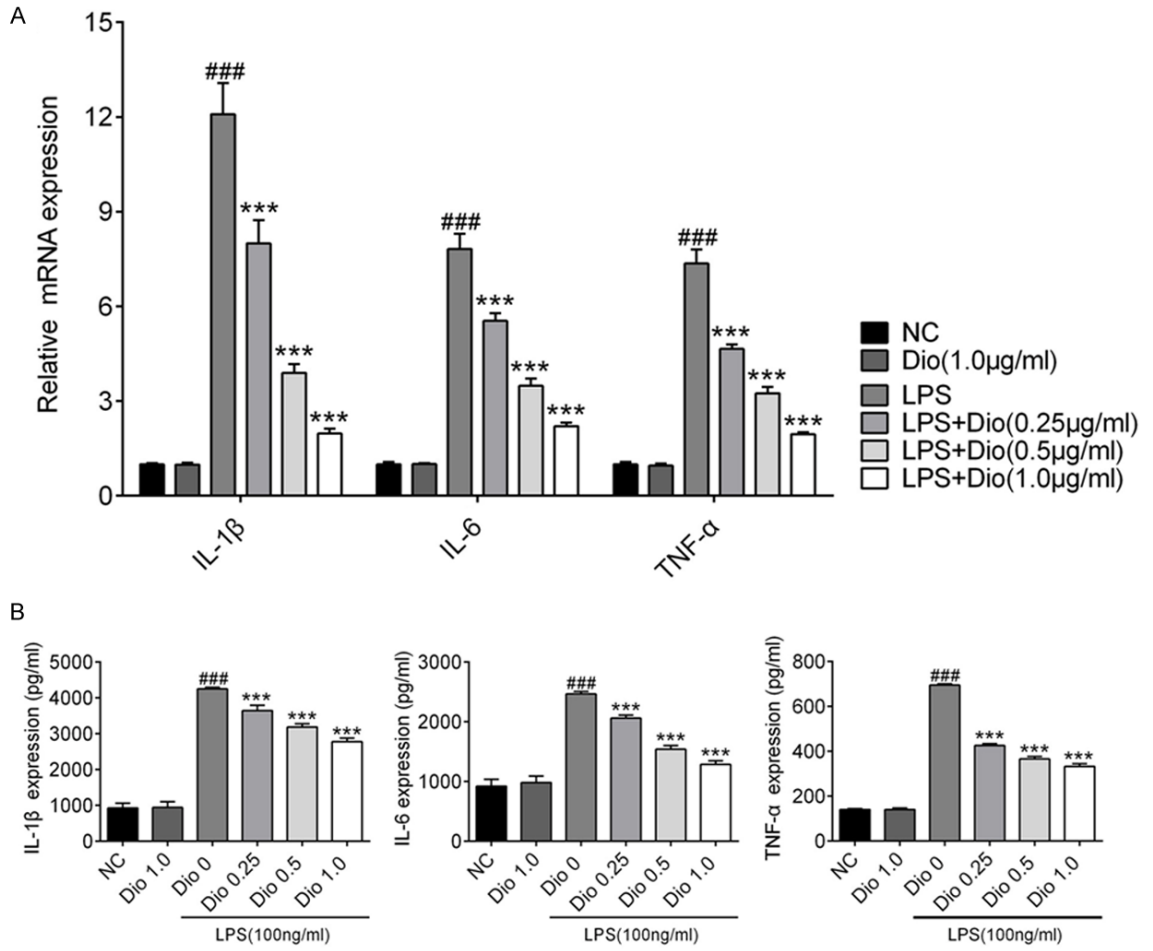


Figure 3. Dioscin alleviates inflammation caused by LPS in HMrSV5 cells. A. Expression of IL-6, IL-1β and TNF-α were measured by qRT-PCR. B. ELISA assay for detecting expressions of IL-6, IL-1β and TNF-α. $^{###}P < 0.001$ compared with negative control group or dioscin (1.0 μg/ml) group. $^{***}P < 0.001$ compared with LPS group. NC, negative control; LPS, lipopolysaccharide; Dio, Dioscin; IL-6, interleukin-6; IL-1β, interleukin-1β; TNF-α, tumor necrosis factor alpha.

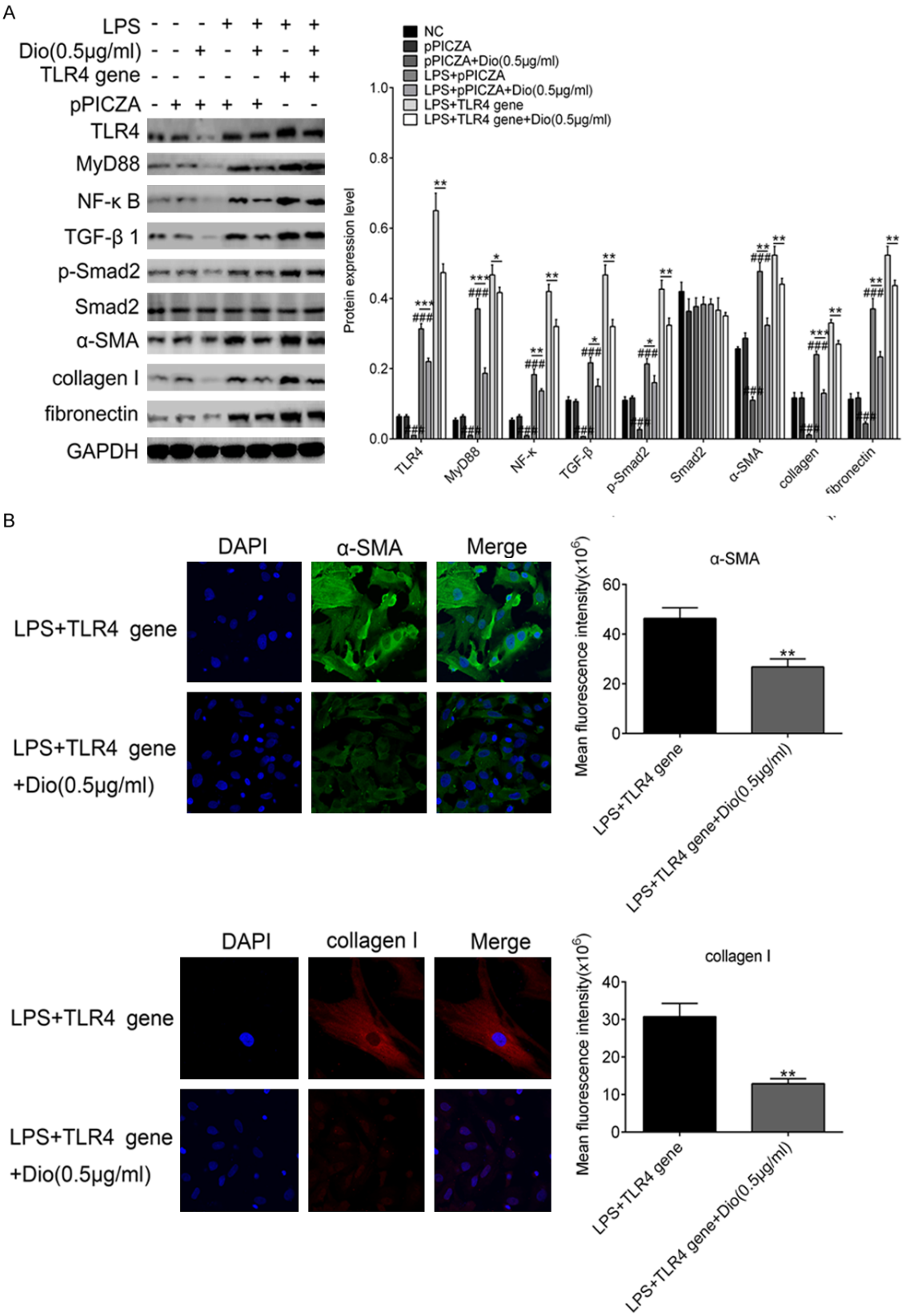
that LPS led to a severe inflammation response in HMrSV5 cells, for which IL-6, IL-1β and TNF-α were markedly elevated. The increased levels of IL-6, IL-1β and TNF-α were significantly decreased after dioscin treatment.

Dioscin inhibits EMT and fibrosis in HMrSV5 cells via the TLR4/MyD88/NF-κB pathway

As shown in **Figure 4A**, protein levels of TLR4, MyD88, NF-κB, TGF-β1, p-Smad, α-SMA, collagen I and fibronectin were both markedly elevated in LPS+pPICZA group and LPS+TLR4 group, and decreased expression levels were reversely found in dioscin (0.5 μg/ml) treated groups. Moreover, immunofluorescence assay unveiled that dioscin (0.5 μg/ml) significantly downregulated the levels of α-SMA, collagen I and fibronectin after transfection (**Figure 4B**).

Discussion

Peritoneal fibrosis, a normal response to peritoneal member injury, can be caused by bioincompatible dialysate solutions, peritonitis, uremia, or chronic inflammation. Peritoneal dialysis is still limited by dialysis failure due to peritoneal membrane fibrosis induced by inflammation. Therefore, it is necessary to seek ideal prevention and treatment for peritoneal fibrosis. Dioscin, a natural steroid saponin, has effects of being anti-inflammatory, anti-tumor, and anti-fibrosis [17-20]. However, few papers discussed the treatment of peritoneal fibrosis with dioscin. Thus, in the present study, we attempted to investigate the effect of dioscin against LPS-induced peritoneal fibrosis and its underlying mechanism.


A
LPS
Dio(0.5μg/ml)
TLR4 gene
pPICZA
TLR4
MyD88
NF-κ B
TGF-β 1
p-Smad2
Smad2
α-SMA
collagen I
fibronectin
GAPDH
NC
pPICZA
pPICZA+Dio(0.5μg/ml)
LPS+pPICZA
LPS+pPICZA+Dio(0.5μg/ml)
LPS+TLR4 gene
LPS+TLR4 gene+Dio(0.5μg/ml)
Protein expression level
TLR4
MyD88
NF-κ
TGF-β
p-Smad2
Smad2
α-SMA
collagen
fibronectin
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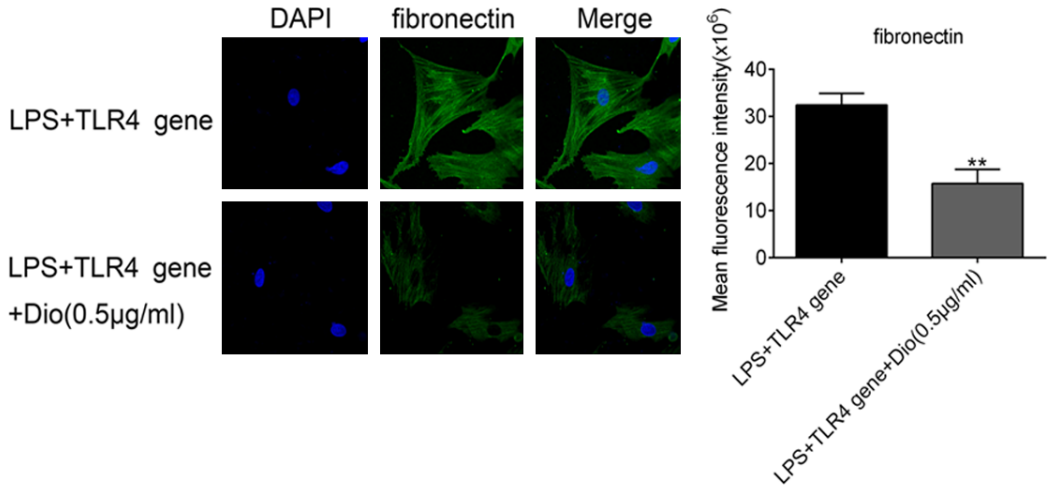

Figure 4. Dioscin inhibits EMT and fibrosis through TLR4/MyD88/NF-κB pathway in HMrSV5 cells. A. Western blotting for assessing protein levels of TLR4, MyD88, NF-κB, TGF-β1, p-Smad2, Smad2, α-SMA, collagen I and fibronectin. B. Immunofluorescence assay for detecting expressions of α-SMA, collagen I and fibronectin in LPS+TLR4 group and LPS+TLR4+dioscin (0.5 μg/ml) group. $^{###}P < 0.001$ compared with negative control group or Ppicza group. $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ compared with LPS+ pPICZA group or LPS+TLR4 group. NC, negative control; LPS, lipopolysaccharide; Dio, Dioscin; α-SMA, α-smooth muscle actin; MyD88, myeloid differentiation factor 88; NF-κB, nuclear factor κB; TGF-β1, transforming growth factor-β1; TLR4, Toll-like receptor (TLR) 4.

In this study, we demonstrated that dioscin significantly reduced the levels of LPS-related markers of EMT, including N-cadherin, MMP-2, MMP-9 and vimentin, and upregulated the levels of E-cadherin and ZO-1. Substantial decreased levels of fibrosis markers, such as α-SMA, collagen I and fibronectin, were further observed in the dioscin-treated group. Additionally, dioscin suppressed inflammation by reducing the expression levels of IL-6, IL-1β and TNF-α. Furthermore, dioscin was proved to alleviate peritoneal fibrosis through inhibiting LPS-induced activation of TLR4/MyD88/NF-κB signaling pathway by decreasing expression levels of TLR4, MyD88, NF-κB, TGF-β1, p-Smad2, α-SMA, collagen I and fibronectin.

It was well known that EMT of MCs played a central role in the onset of peritoneal fibrosis, and EMT might characterized by loss of epithelial cell markers and/or overexpression of mesenchymal cell markers in an inflammatory microenvironment [21]. Literature reported by Tanjore unveiled that during endoplasmic reticulum stress, decreased levels of E-cadherin and ZO-1 and increased α-smooth muscle actin (SMA) were observed [22]. Shi found downregulated E-cadherin and elevated α-SMA by directly co-culturing HMrSV5 cells and M1 macrophages, implying the occurrence of EMT in MCs [23]. In the present study, epithelial markers E-cadherin and ZO-1, and mesenchymal marker N-cadherin, vimentin and α-SMA were determined following LPS stimulation and dioscin treatment. We revealed that dioscin significantly reduced the levels of N-cadherin and vimentin, and upregulated the levels of E-cadherin and ZO-1.

Repeated inflammation was proved to be closely linked to peritoneal fibrosis [20]. Pro-inflammatory cytokines including IL-1β, IL-6 and TNF-α stimulated by LPS participated in the balance between the pro-inflammatory and anti-inflammatory responses, in which NF-κB signaling modulated inflammatory actions [24]. Kim et al [25] showed high molecular weight peptide fraction of *Mytilus edulis* hydrolysates exhibited a better anti-inflammatory effect by inhibiting NF-κB and MAPK signaling pathways. Park [26] suggested that Mito-TEMPO alleviated LPS-associated pro-inflammatory response by preventing MAPK and NF-κB pathways. In addition, Sunny et al [27] revealed that kolaviron suppressed LPS-associated inflammation through decreasing the expression of several inflammatory transcription factors including IL-6, IL-1α, IL-33, IL-1β, and IFNβ1-1. Importantly, kol-v exerted inhibitory effects on IL-6 by inhibiting ERK1/2, NF-κB, Akt, p-c-JUN and JNK

pathways. In the present study, dioscin suppressed inflammation by reducing the expression levels of IL-6, IL-1β and TNF-α. To further clearly investigate the mechanism action of dioscin, we examined the protein levels of TLR4, MyD88, NF-κB in HMrSV5 cells after TLR4 overexpression. Our findings indicated that the inhibition of LPS-induced inflammation by dioscin might be mediated by TLR4/MyD88/NF-κB pathway.

Choi and his colleagues unveiled that peritoneal inflammation and fibrosis were significantly observed after 50 mM glucose stimulation in human peritoneal MCs partly by activating TLR4/MyD88/NF-κB signaling pathway, suggesting that TLR4 might be a therapeutic target for alleviating inflammation and fibrosis in PD [28]. Our findings also implied that dioscin markedly attenuated peritoneal fibrosis accompanied with the reduction of TGF-β1, p-Smad2, α-SMA, collagen I, and fibronectin. To further explore whether overexpression of TLR4 gene could mediate peritoneal fibrosis, immunofluorescence assay was performed to examine the expressions of fibrosis markers α-SMA, collagen I, and fibronectin in HMrSV5 cells after TLR4 transfection. Our data indicated that TLR4 regulated the changes in α-SMA, collagen I and fibronectin intervened by dioscin, suggesting that TLR4 might be a therapeutic target in PD-associated fibrosis. Raby et al [29] indicated the influence of peritoneal TLR4 on PD-associated fibrosis and provided a therapeutic remedy for peritoneal fibrosis treatment, which is consistent with our findings.

In the present study, we first studied the role of dioscin in peritoneal fibrosis and elucidated an anti-fibrotic role by inhibiting TLR4/MyD88/NF-κB signaling pathway. There is a limitation in this study. We explored the role of dioscin on LPS-induced peritoneal fibrosis and its action of mechanism *in vitro*, however, the inhibitory effect on peritoneal fibrosis of dioscin *in vivo* is remain elusive. Therefore, further *in vivo* study still need to be performed.

In conclusion, we confirmed that dioscin attenuated peritoneal fibrosis through inhibiting the TLR4/MyD88/NF-κB signaling pathway, which suggested that dioscin represented a novel and efficient therapeutic opportunity for patients undergoing long-term PD.

Disclosure of conflict of interest

None.

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