Total flavones of *Desmodium styracifolium* antagonize calcium oxalate monohydrate-triggered IL-2Rβ expression in renal epithelial cells

Haijie Xie, Rui Yang, Yong Xu, Chunyu Liu*
Urolithiasis Treatment Center of Tianjin, Tianjin Institute of Urology, The Second Hospital of Tianjin Medical University, 23 Pingjiang Road, Hexi District, Tianjin 300211, China

*For correspondence: Email: chunyuliu2017@126.com; Tel: +86-022-88328091

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**Abstract**

**Purpose:** To explore the effect of total flavone of Desmodium styracifolium (TFDS) on calcium oxalate monohydrate (COM)-triggered IL-2Rβ expression in human kidney proximal tubular epithelial cells.

**Methods:** Human kidney proximal tubular epithelial cell line HK-2 was treated with COM, TFDS or both. The expression of IL-2Rβ was evaluated by quantitative polymerase chain reaction (qPCR) or flow cytometry. The responsiveness of HK-2 cells to IL-2 was determined by enzyme-linked immunosorbent assay (ELISA), qPCR and western blot. The signaling mechanism underlying the effect of TFDS was studied using western blot and qPCR. The clinical relevance of IL-2Rβ to renal inflammation was investigated by re-analyzing a Gene Expression Omnibus (GEO) dataset.

**Results:** Total flavones of Desmodium styracifolium (TFDS) antagonize COM-triggered IL-2Rβ expression in HK-2 cells, thus reducing the responsiveness of HK-2 cells to IL-2 stimulation. Mechanistically, TFDS dampens IL-2Rβ expression by preventing the activation of STAT3. The level of IL-2Rβ is positively correlated with the inflammatory status of the kidney.

**Conclusions:** The total flavones of Desmodium styracifolium (TFDS) prevent the upregulation of IL-2Rβ in renal epithelial cells upon COM stimulation in a STAT3-dependent manner.

**Keywords:** Total flavones, Desmodium styracifolium, Calcium oxalate, Urinary calculi, IL-2Rβ, STAT3 signaling

INTRODUCTION

Urinary calculi is a common disease with an approximate prevalence of 5 - 45 % [1]. Oxalate calculi represents nearly 70 - 80 % cases of urinary calculi [2]. In terms of etiology, calcium oxalate monohydrate (COM) leads to the damage of renal tubular epithelial cells. The adherence of calcium oxalate monohydrate crystal to the lesion site facilitates renal crystallization and the formation of urinary calculi [3,4]. Although various have approaches have been used in the treatment of urinary calculi such as extracorporeal shock wave lithotripsy and percutaneous nephroscopie, the cellular and molecular basis of urinary calculi are still far from clear. This lack of understanding hinders further development of therapeutic strategies.
Desmodium styracifolium, a traditional Chinese medicinal herb, is used in the treatment of various renal diseases such as kidney stone or kidney damage. Nevertheless, the detailed mechanisms of Desmodium styracifolium as a kidney stone inhibitor remain elusive. Previous studies have reported that total flavone of Desmodium styracifolium (TFDS) inhibited the formation of calcium oxalate monohydrate crystal in the kidneys of hyperoxaluria rats. This work also indicated that TFDS alleviated COM-induced apoptosis and autophagy of kidney proximal tubular epithelial cells [5]. The exact effect of TFDS on the function of renal tubular epithelial cells remains largely unknown. Nevertheless, the detailed therapeutic mechanisms of TFDS remain to be further explored. Previous reports have indicated that renal tubular epithelial cells express functional IL-2 receptor which may contribute to renal disorders [6,7].

EXPERIMENTAL

Reagents

Desmodium styracifolium was obtained from Xi’an Tianrui Biological Technology Company (Xi’an, China), COM crystals were prepared as previously described [5]. STAT3 inhibitor S3I-201 was purchased from Selleck (Houston, USA), p38 inhibitor SB203580 was purchased from Beyotime (Shanghai, China). Human IL-2 was purchased from Peprotech.

Cell culture

HK-2 human kidney proximal tubular epithelial cell line was obtained from Shanghai Cell Bank of Chinese Academy of Science and was maintained in DMEM medium containing 10 % fetal bovine serum and 1X Penicillin-Streptomycin. Cells were cultured in 5 % CO₂ incubator at 37 °C.

Quantitative PCR (qPCR)

Total RNA was extracted using Trizol method and then reverse transcribed into cDNA using reverse transcription kit from QIAGEN (Duesseldorf, Germany). QPCR was conducted using the following parameters: pre-denaturing at 95 °C for 10 min; denaturing at 95 °C for 20 s; annealing/extending at 61 °C for 50 s.

Enzyme-linked Immunosorbert assay (ELISA)

The level of complement C3 was evaluated using ELISA kit from Abcam (Cambridge, USA).

Flow cytometry

HK-2 cells were digested with trypsin for 2-3 min and centrifuged at 300 g for 5 min. Cell pellets were resuspended in PBS containing PE-anti-human IL-2Rβ (Biolegend, San Diego, USA) or isotype control (Biolegend). Staining was performed at 4 °C in dark for 20 min. Flow cytometry was conducted on a FACS Calibur.

Western blot

Total cell lysates were subjected to sodium dodecyl sulphate-polyacrylamide gel electrophoresis then transferred to PVDF membranes which were then blocked with 5 % bovine serum albumin. The membranes were probed with appropriate primary antibodies and secondary antibodies. All antibodies were purchased from Cell Signaling Technology (USA).

Statistical analysis

Unpaired Student’s t test was performed using SPSS 17.0 software. P < 0.05 was considered statistically significant.

RESULTS

COM upregulated expression of IL-2Rβ in HK-2 cells

First, HK-2 cells were treated with COM to examine its effect on human proximal tubular epithelial cells. As shown in Figure 1, COM stimulation led to an increase in IL-2Rβ mRNA level, peaking at 3 h and then progressively declined. Therefore, COM functions as an inducer of IL-2Rβ expression in human proximal tubular epithelial cells.

Figure 1: COM upregulates IL-2β expression in HK-2 cells. (a) HK-2 cells were treated with 2 mM COM for indicated time, the expression of IL-2β was evaluated by QPCR. (b) HK-2 cells were treated with different concentrations of COM for 2 h, the expression of IL-2β was evaluated by QPCR. Data are mean ± SD; *p < 0.05; **p < 0.01; ***p < 0.001.
TFDS antagonizes COM-induced expressions of IL-2Rβ in HK-2 cells

The effect of TFDS on COM-induced IL-2Rβ expression was investigated. Although COM markedly upregulated mRNA level of IL-2Rβ in HK-2 cells, concomitant treatment with TFDS significantly compromised the effect of COM, as evidenced by the results that the mRNA level of IL-2Rβ in COM+TFDS group was reduced by 60% compared to COM alone group (Figure 2 a). Next, the expression of IL-2Rβ on cell surface was evaluated by flow cytometry. Similar to QPCR results, the IL-2Rβ-inducing role of COM was largely antagonized by TFDS. On the other hand, TFDS treatment did not affect the basal expression of IL-2Rβ in the absence of COM (Figure 2 b and c). Thus, TFDS functions as an antagonist of COM in terms of IL-2Rβ expression.

TFDS decreases IL-2 responsiveness in COM-treated HK-2 cells

To investigate if TFDS affects IL-2-induced gene expression, HK-2 cells were treated with COM in the absence or presence of TFDS, followed by IL-2 stimulation. As shown in Figure 3 a, COM pretreatment significantly increased the production of C3 as well as the production of CD25 in IL-2 stimulated HK-2 cells. However, in COM+TFDS group, IL-2 triggered C3 production and CD25 expression were significantly inhibited compared with COM group. In terms of IL-2 signaling, COM pretreatment led to enhanced STAT5 activation in IL-2 treated HK-2 cells, which was markedly suppressed by TFDS (Figure 3 b). Thus, through preventing COM-induced IL-2β expression, TFDS reduced the responsiveness of HK-2 cells to IL-2 stimulation.

Figure 3: TFDS reduces the activation of IL-2 signaling in HK-2 cells HK-2 cells were pretreated with 2 mM COM in the absence or presence of TFDS for 3 h, followed by stimulation with 20 ng/mL IL-2, the level of complement C3 in culture supernatant was evaluated by ELISA (a), the level of CD25 was detected by QPCR (b), the phosphorylation of STAT5 was examined by western blot (c). Data are mean ± SD; *p < 0.05, **p < 0.01

STAT3 signaling, but not p38 signaling is responsible for the TFDS-mediated downregulation of IL-2β

Since it is reported that COM induced strong activation of p38 signaling in human renal tubular epithelial cells, TFDS might downregulate IL-2β by suppressing p38 activation. Surprisingly, although pretreatment of HK-2 cells with SB203580 (p38 inhibitor) partially prevented COM-induced upregulation of IL-2Rβ, TFDS showed a synergistic effect with SB203580 to cause a further reduction in IL-2Rβ level. Besides p38, COM did not prominently increase the phosphorylation of ERK and JNK – the other two MAPKs (Figure 4 a).

Figure 4: TFDS compromises the function of COM in a STAT3-dependent manner. (a) HK-2 cells were treated with 2 mM COM, the phosphorylation of indicated proteins was evaluated by western blot. (b) HK-2 cells were pretreated with SB203580 or S3I-201 or DMSO for 2 h, followed by stimulation with COM or COM plus TFDS, (b) the level of IL-2Rβ was evaluated by QPCR, (c) the level of complement C3 was detected by ELISA (c). Data are mean ± SD; *p < 0.05, **p < 0.01

Interestingly, the phosphorylation of STAT3 markedly enhanced by COM (Figure 4 a). Importantly, TFDS failed to downregulate IL-2Rβ expression when HK-2 cells were pretreated with S3I-201, a specific STAT3 inhibitor (Figure 4 b).
suggested that there was no synergistic effect between TFDS and STAT3 inhibition in preventing C3 production in HK-2 cells. Furthermore, STAT3 inhibition, but not p38 inhibition abrogated the ability of TFDS to reduce IL-2β expression in COM-stimulated HK-2 cells. Therefore, TFDS antagonize COM-induced IL-2β expression primarily by inhibiting the activation of STAT3 signaling.

**IL-2Rβ level correlates with the inflammatory status in kidney**

Finally, the possible clinical relevance of IL-2Rβ to renal inflammation was investigated. By re-analyzing a dataset in GEO database, significant positive correlations between the level of IL-2Rβ and the levels of complement C3, TNF-α, and IL-6 were observed in a renal cancer patient cohort (Figure 5). Thus, increased expression of IL-2Rβ may be associated with enhanced renal inflammation.

**DISCUSSION**

As a traditional Chinese herb, the therapeutic effect of Desmodium styrecolium in alleviating renal diseases has been increasingly recognized, especially in the treatment of urinary calculi. It is reported that Desmodium styrecolium inhibited calcium oxalate stone formation through increasing urinary citrate excretion and reducing urinary calcium as an antioxidant [8]. Rodgers found that Desmodium styrecolium treatment resulted in the decreased particle size of precipitated crystals and the supersaturation of calcium oxalate in synthetic urine [9].

Although it has been reported that COM can induce p38 signaling in HK-2 cells [5,10], this study found that STAT3 signaling, but not p38 signaling was responsible for the effect of TFDS in antagonizing COM-induced IL-2b expression in human renal tubular epithelial cells. Previous studies have linked renal inflammation with IL-2 signaling [6,7]. In this work, TFDS was identified as an antagonist against COM-triggered IL-2β expression and complement C3 production, which was thought to be involved in the pathogenesis of renal inflammation.

Interleukin-2 receptor is composed of IL-2Rα, IL-2Rβ and IL-2Rγ chain. Although IL-2 receptor is predominantly expressed on immune cells, especially T cells, Gerritsma JS have indicated that human proximal tubular epithelial cells can also express IL-2 receptor [6,7]. From functional perspective, IL-2 was reported to induce human proximal tubular epithelial cells to produce complement C3, which can lead to immune complex-associated renal inflammation [6]. Taken together, TFDS antagonizes multiple COM-induced detrimental effects on renal epithelial cells, while through distinct signaling mechanism.

**CONCLUSION**

This study identifies a novel function of total flavones of Desmodium styrecolium (TFDS) in suppressing IL-2 signaling. Due to its synergistic effect with p38 inhibition, TFDS may achieve higher efficacy when used in combination with p38 inhibitors.

**DECLARATIONS**

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**Conflict of interest**

No conflict of interest is associated with this work.

**Contribution of authors**

We declare that this work was done by the researchers listed in this article. All liabilities related with the content of this article will be borne by the authors. Haijie Xie and Rui Yang designed all the experiments and revised the manuscript. Yong Xu and Chunyu Liu performed the experiments, wrote the paper. Haijie Xie and Rui Yang contributed equally to this work; Yong Xu and Chunyu Liu contributed equally to this work.

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