Diosgenin attenuates hepatic stellate cell activation through transforming growth factor-β/Smad signaling pathway

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Abstract: Activation of hepatic stellate cells (HSC) plays a pivotal role in the development of hepatic fibrosis. Transforming growth factor-β1 (TGF-β1) is considered to be the main stimuli factor responsible for the activation of HSC. Diosgenin is a steroidal saponin found in several plants including Solanum and Dioscorea species, and it inhibited high glucose-induced renal tubular fibrosis. However, the effects of diosgenin against hepatic fibrosis remain elusive. Therefore, in this study, we investigated the effects of diosgenin on TGF-β1-induced HSCs and elucidate the possible mechanism of its anti-fibrotic effect. Our results demonstrated that diosgenin inhibited TGF-β1-induced HSC proliferation, reduced the expression of collagen I and α-smooth muscle actin (α-SMA), as well as the expression of TGF-β receptor I (TGF-β RI) and II. Moreover, diosgenin suppressed TGF-β1-induced phosphorylation of Smad3 in HSCs. In conclusion, our data demonstrate that diosgenin inhibited HSC-T6 cell proliferation and activation, at least in part, via the TGF-β1/Smad signaling pathway. These results provide that diosgenin may have potential to treat liver fibrosis.

Keywords: Diosgenin, hepatic stellate cells (HSC), transforming growth factor-β1 (TGF-β1)

Introduction

Liver fibrosis is a major cause of morbidity and mortality from hepatic diseases. It is the result of wound-healing after repeat injury by alcohol, cholestasis, chronic hepatitis, or drugs, and is characterized by the excessive accumulation of extracellular matrix (ECM), which is mainly composed of collagens [1]. Hepatic stellate cells (HSCs) are the most relevant cell type for the development of liver fibrosis, and the activation of HSCs plays an important role during the initiation and development of liver fibrosis [2]. In the healthy liver, HSCs have a quiescent phenotype, but, after liver injury, these quiescent HSCs are exposed to profibrogenic factors, and undergo a process of activation to a myofibroblastic phenotype, finally resulting in the excess production and deposition of ECM components [3]. Therefore, the inactivation of HSCs is the main approach for preventing the progression of liver fibrosis [4]. However, the therapeutic drugs of liver fibrosis are still insufficient. Thus, there is a need to find new agents which can reverse HSC activation.

Diosgenin is a steroidal saponin found in several plants including Solanum and Dioscorea species. A growing body of evidence indicates that diosgenin possesses important pharmacological properties, such as anti-inflammatory [5], anti-atherosclerosis [6], anti-tumor [7] and antioxidant activities [8]. Most recently, one study showed that diosgenin inhibited high glucose-induced renal tubular fibrosis [9]. However, there are no studies focusing on the effects of diosgenin against hepatic fibrosis remain elusive. Therefore, in this study, we investigated the effects of diosgenin on TGF-β1-induced HSCs and elucidate the possible mechanism of its anti-fibrotic effect.
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Materials and methods

Cell culture

The human HSC cell line, HSC-T6, was purchased from the American Type Culture Collection (Manassas, VA, USA). HSC-T6 cells were grown in DMEM supplemented with 10% heat-inactivated fetal bovine serum (FBS) at 37°C in a humidified 5% CO₂ atmosphere.

MTT assay

The cell proliferation assay was carried out using the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT; Sigma-Aldrich, St. Louis, MO, USA) method. The cells were seeded in a 96-well (1×10⁴ cells/well) and treated with TGF-β1 or TGF-β1 + diosgenin (25, 50 and 100 μM) for 24 h. Then MTT was added to the cells at a final concentration of 0.5 mg/ml before the end of the experiment and incubated at 37°C for 4 h. The supernatant was removed, and the crystals were dissolved in 150 μl dimethyl sulfoxide. The absorbance was measured at 570 nm in a microplate reader (Thermo Lab systems, Waltham, MA, USA).

Caspase 3 activity assay

HSC-T6 cells cultured in 100-mm-diameter dishes were harvested and pelleted by centrifugation. The medium supernatant was discarded, and the cell pellet was washed in 1 mL of ice-cooled PBS. Caspase 3 (DEVDase) activity was determined by a colorimetric caspase 3 activity kit (Abcam) following the manufacturer’s instructions.

Quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA was extracted using Trizol Reagent according to the manufacturer’s instructions (Invitrogen, Carlsbad, CA, USA). Then, 2 μg of total RNA was transcribed to first-strand cDNA using TaqMan reverse transcription reagents (Applied Biosystems, Foster City, CA). The following primers were used: collagen I, 5’-TGACTGGAAGAGCGGAGTAGCTACT-3’ (sense), 5’-GCTGTGGGCTATTGCACACAA-3’ (antisense); α-SMA, CCGAGATCTCACCAGACTACC (sense), 5’-TCCAGACGCAACACGAC-3’ (antisense); and β-actin 5’-CCAGCCTGGATGGCTACGT-3’ (antisense). These primers were all synthesized by Sangon Biotech Co., Ltd. (Shanghai, China). The PCR procedure was as followed: 95°C for 3 min, followed by 40 cycles of 94°C for 30 s, 59°C for 20 s, and 72°C for 40 s, and finally a single cycle at 72°C for 5 min. For relative quantification, the levels of individual gene mRNA transcripts were firstly normalized to the control β-actin. Subsequently, the differential expression of these genes was analyzed according to the 2⁻ΔΔCt method.

Western blot

HTC-6 cells were washed with ice-cold PBS and lysed with RIPA lysis buffer (Beyotime, China). The lysates were sonicated for 5 s on ice and centrifuged at 10,000× g for 5 min. The supernatants were collected and the protein concentration was determined using the Bio-Rad Protein Assay (Bio-Rad Laboratories, Hercules, CA).
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CA). Protein (30 μg) was separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidene fluoride (PVDF) membranes (Millipore, Bedford, MA, USA). The membrane was incubated with the primary antibody for collagen α type I, α-smooth muscle actin (α-SMA), TGF-β RI, TGF-β RII, phospho-Smad 2, Smad 2, phospho-Smad3, Smad3, GAPDH (Santa Cruz Biotechnology, CA, USA) at 4°C overnight. After washing with TBST, blots were then incubated with horseradish peroxidase-linked secondary antibodies (Invitrogen, Carlsbad, CA, USA) at room temperature for 1 h. The specific protein bands were developed using a chemiluminescent substrate and imaged using a gel scanner. β-actin was used as the internal control.

Statistical analysis

Results were presented as mean ± S.D. Statistical analysis was performed using One-way ANOVA analysis. Statistical significance was determined at the level of *P*<0.05.

Results

**Diosgenin treatment suppressed TGF-β1-induced production of type I collagen and α-SMA**

We examined whether diosgenin is able to inhibit the fibrotic effects of TGF-β1 on ECM expression in HSCs. As shown in Figure 3, TGF-β1 significantly increased the protein expression of induced a considerable increase in collagen I and α-SMA. However, diosgenin reserved TGF-β1-induced collagen I and α-SMA protein level in a dose dependent manner.

**Diosgenin inhibited TGF-β RI and II expression in hepatic stellate cells**

Because TGF-β/Smad signaling pathways perform a significant role in liver fibrosis and TGF-β receptor binding initiates the signaling cascade, therefore, we examined the effect of diosgenin on TGF-β RI and II expression levels in TGF-β1-stimulated HSC-T6 cells. As shown in Figure 4, TGF-β1 significantly increased TGF-β RI and TGF-β RII expression in HSC-T6 cells. However, diosgenin treatments dramatically suppressed the TGF-β1-enhanced TGF-β RI and TGF-β RII expression in HSC-T6 cells.

**Diosgenin inhibited TGF-β1-induced phosphorylation of Smad3 in hepatic stellate cells**

The TGF-β1-mediated signaling pathway depends on the phosphorylation of Smad 2/3. To further evaluate understand the molecular mechanisms responsible for the inhibition of

![Figure 2. Effect of diosgenin on TGF-β1-induced apoptosis in HSC-T6 cells. HSC-T6 cells were stimulated with TGF-β1 after treatment with various concentrations of diosgenin for 24 h. Caspase 3 activity was measured using Caspase 3 colorimetric assay kits. Values were expressed as mean ± SD of three independent experiments.](image-url)
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HSCs activation, the protein levels of Smad 2/3 were analyzed. As shown in Figure 5, TGF-β1 treatment stimulated phosphorylation of Smad3, however, diosgenin prevented TGF-β1-induced phosphorylation of Smad3 in HSC-T6 cells.
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**Discussion**

Our results demonstrated that diosgenin inhibited TGF-β1-induced HSC proliferation, reduced the expression of collagen I and α-SMA, as well as the expression of TGF-β RI and TGF-β RII. Moreover, diosgenin suppressed TGF-β1-induced phosphorylation of Smad3 in HSCs.

The induction of proliferation is an early step after HSC activation and is stimulated by a variety of cytokines. Among many cytokines, platelet-derived growth factor (PDGF) and TGF-β1 are considered the most prominent mediators of this process. PDGF is one of the most potent mitogen for activated HSC [10], while TGF-β1 is widely accepted as the strongest stimulus for the transdifferentiation of HSCs [11]. In this study, we used TGFβ1 to induce HSC activation. Our data showed that TGF-β1 significantly induced HSC proliferation. These findings are in agreement with earlier reports that TGF-β1 promoted HSC proliferation. In addition, we found that diosgenin inhibited TGF-β1-induced HSC proliferation, and diosgenin had no significant effect on cell apoptosis. These data suggested that diosgenin inhibited TGF-β1-induced HSC proliferation without affecting cell apoptosis.

Activated HSCs are the principal cell type promoting synthesis and deposition of ECM proteins. There is extensive evidence demonstrating that TGF-β1 plays an essential role in modulating ECM expression [12-14]. Previous studies showed that TGF-β1 upregulated expression of α-SMA and collagen I in human HSCs [15-17]. In line with these reports, in this study, we found that TGF-β1 increased the expression of collagen I and α-SMA, while, diosgenin inhibited TGF-β1-induced the expression of collagen I and α-SMA in HSC-T6 cells. These data suggested that diosgenin inhibited TGF-β1-induced HSC activation by reducing the expression of collagen I and α-SMA.

A growing body of evidence demonstrated that the TGF-β1/Smad signaling pathway is a key mediator of progressive liver fibrosis [18-20]. TGF-β signals through a heteromeric receptor complex of type II and type I receptor serine-threonine kinases, which activates the downstream Smad signal pathway. After TGFβ binding to the receptor complex, Smad 2/3 is phosphorylated and binds with SMAD 4 to form multimers, then activated R-Smads translocate to the nucleus and induce transcriptional modulation of target genes, including ECM proteins [21]. So, inhibition of TGF-β1/Smad signaling may be an important therapeutic approach for liver fibrosis. Liu et al. reported that Smad3 specific inhibitor, Naringenin, decreases the expression of ECM induced by TGF-β1 in cul-
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Yang et al. reported that astaxanthin decreased TGFβ1-induced α-SMA and procollagen type 1, alpha 1 (Col1A1) mRNA as well as α-SMA protein levels. It also attenuated TGFβ1-induced Smad3 phosphorylation and nuclear translocation with a concomitant inhibition of Smad3, Smad 7, Tβ RI and Tβ RII expression [23]. In agreement with previous data, in the present study, we found that TGF-β1 significantly increased Tβ RI and Tβ RII expression, as well as the phosphorylation level of Smad3 in HSC-T6 cells; whereas co-treatment of diosgenin eliminated these changes. These results suggest that diosgenin attenuated liver fibrosis by inhibiting the TGF-β1/Smad signaling pathway.

In conclusion, our data demonstrate that diosgenin inhibited HSC-T6 cell proliferation and activation, at least in part, via the TGF-β1/Smad signaling pathway. These results provide that diosgenin may have potential to treat liver fibrosis.

Disclosure of conflict of interest

None.

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