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Article

Hesperidin inhibits the epithelial to mesenchymal transition induced by transforming growth factor-β1 in A549 cells through Smad signaling in the cytoplasm

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Hesperidin, a natural compound, suppresses the epithelial-to-mesenchymal transition through the TGF-β1/Smad signaling pathway. However, studies on the detailed effects and mechanisms of hesperidin are rare. The present study showed that, for A549 alveolar epithelial cells, the anti-proliferative effects of hesperidin occurred in a dose-dependent manner, with an IC50= 216.8 μM at 48 h. TGF-β1 was used to activate the Smad signaling pathway and induce the epithelial to mesenchymal transition in cells. Treatment with hesperidin or SB431542 was used for antagonism of Smad pathway activation. Hesperidin inhibited the increase in α-SMA and Col1α-1 and the decrease in E-cadherin in a dose-dependent manner from concentration of 20 μM to 60 μM, as assessed by both ELISA and Western blotting assays; however, there was no significant effect on cellular morphological alterations. Moreover, the Western blotting assay showed that, in the cytoplasm, hesperidin and SB431542 had no significant effect on the protein expression of Smad 2, 3, 4, or 7 as well as 2/3. However, 60 μM hesperidin and SB431542 significantly decreased p-Smad2/3 protein expression. From the above results, it is concluded that hesperidin can partly inhibit the epithelial to mesenchymal transition in human alveolar epithelial cells; the effect accounts for the blockage of the phosphorylation of Smad2/3 in the cytoplasm rather than a change in Smad protein production in the cytoplasm.

Keywords: Hesperidin. Epithelial-to-mesenchymal transition (EMT). Smad signaling pathway. Inhibition.

INTRODUCTION

Idiopathic pulmonary fibrosis (IPF) is a chronic and progressive parenchymal lung disease with an incidence of 6.8-8.8 cases per 100,000 and a poor prognosis with a median survival of only 2-3 years (King, Pardo, Selman, 2011; Nalysnyk *et al*., 2012). The pathogenesis of IPF is characterized by the formation of fibrotic foci through the excessive accumulation of activated fibroblasts and myofibroblasts (Selman, Pardo, 2003). Furthermore, there is a close link between pulmonary fibrosis and epithelial injury. In particular, the injured cells show an E-cadherin decrease, an α-SMA increase, and morphological changes, appearing to undergo a mesenchymal transition. Currently, many reports support that transitional alveolar epithelial cells are one of the main sources of fibroblasts and myofibroblasts (Kim *et al*., 2006; Willis *et al.*, 2005).

TGF-β1 is a cytokine that is present at an abnormally high level in patients with lung fibrosis, with an obvious mesenchymal transition of alveolar epithelial cells, indicating TGF-β1 as a possible inducer of epithelial-to-mesenchymal transition (EMT). *In vitro*, TGF-β1 induces EMT in some alveolar epithelial cells, including A549 cells, RLE-6TN cells (Gao *et al*., 2017) and primary alveolar epithelial cells (Kim *et al*., 2006). Therefore, the TGF-β1-induced EMT model has been widely applied to understand the efficacy and mechanism of drugs against pulmonary fibrosis (Baek *et al*., 2016; Coward, Saini, Jenkins, 2010; Yang, Chen, Sun, 2013; Yu *et al*., 2016a).

Hesperidin is a flavonoid glycoside that is abundant in oranges and lemons and has a wide range of

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pharmacological activities, including anti-inflammatory, anti-viral, and anticancer activities (Iranshahi *et al*., 2015). Recently, some studies reported that hesperidin inhibits liver fibrosis through the Wnt/β-catenin pathway (Lin *et al*., 2015) and the TGF-β1/Smad signaling pathway (Wu *et al*., 2015). Furthermore, in a bleomycin-induced pulmonary fibrosis rat model, hesperidin shows a beneficial effect (Görmeli Cemile Ayse *et al*., 2016). In our study, hesperidin showed an inhibitory effect on EMT induced by TGF-β1 in A549 cells (Yu *et al*., 2016a). However, the detailed mechanism of hesperidin in blocking the TGF-β1 signaling pathway is still unclear.

Smad proteins are the intracellular kinase-substrate of TGF-β1-receptor complexes, with molecular weights ranging from 42-60 kD, and are mainly located in the cytoplasm, transmitting signals from the membrane to the nucleus of the TGF-β1 signaling pathway. The levels of Smad protein synthesis and Smad phosphorylation have pivotal effects on signal transduction. Therefore, in this study, Smad protein expression, including the expression of Smad 2, Smad 3, Smad 4, Smad 2/3, Smad7, and p-Smad 2/3 in the cytoplasm, was assayed to explore the mechanism of hesperidin in A549 cells undergoing EMT.

MATERIAL AND METHODS

Hesperidin

Hesperidin was purchased from the Chengdu Must Bio-Technology Co., Ltd. (Chengdu, Sichuan province, China), dissolved in DMSO as a 200 mM stock solution and stored at -20 °C.

Reagents and assay kits

Recombinant human TGF-β1 was purchased from Pepro Tech Ltd. (Rocky Hill, NJ, USA). SB431542 was purchased from Sigma-Aldrich (St. Louis, MO, USA). The Cell Counting Kit-8 was purchased from Beijing Solarbio Science & Technology Co., Ltd (Beijing, China). The human soluble ECAD ELISA Kit was purchased from Boster Biological Technology Co., Ltd. (Wuhan, Hubei province, China). The human Col-1α and α-SMA ELISA Kits were purchased from Cloud-Clone Corp (Wuhan, Hubei province, China).

Cell culture

A549 cells were purchased from the Institute of Biochemistry and Cell Biology, Shanghai Institutes for Biological Sciences of Chinese Academy of Sciences (Shanghai, China). RPMI1640 medium was purchased from Beijing Solarbio Science & Technology Co., Ltd, China (Beijing, China). Fetal bovine serum (FBS) was purchased from Zhejiang Tian Hang Biological Technology Co., Ltd (Huzhou, Zhejiang province, China). The cells were cultured in RPMI1640 medium containing 10% FBS and incubated at 37°C in a humidified atmosphere containing 5% CO_2. Once the cells reached 80% confluence, they were used for the experiments.

Cell proliferation assay

The cells were seeded at 5,000 cells per well in a 96-well plate. After 24 h, the medium was changed to RPMI1640 medium with 0.1% FBS for 12 h. Then, the cells were incubated with fresh RPMI1640 medium containing 10% FBS or a combination of different concentrations of hesperidin (40- 200 μM). At 24 h, 36 h or 48 h, the cellular proliferation assay was performed by the addition of 10 μL CCK-8 solution to each well, followed by an incubation at 37 °C for 2 h. The absorbance (A) was measured at a wavelength of 450 nm using a microplate reader. All of the experiments were performed in quintuplicate. The cells without drug treatment were used as controls. The inhibition rate was calculated as follows:

$$\text{Inhibition rate (\%)} = \frac{A_{450}\text{ of control} - A_{450}\text{ of sample}}{A_{450}\text{ of control}} \times 100$$

Treatment with TGF-β1 and hesperidin

The cells were seeded at 50,000 cells per well in a 24-well plate. After 24 h, the medium was changed to RPMI1640 medium with 0.1% FBS for 12 h. Then, the cells were incubated with fresh RPMI1640 medium containing 10% FBS or a combination of 7.5 ng/mL TGF-β1 with different concentrations of hesperidin (0, 20, 40, and 60 μM) for 48 h.

Roundness assay of cellular morphology

At 48 h, the cells were assessed using a phase contrast microscope (Eclipse MV/TS 100LED-F MV Inverted microscope, Nikon, Tokyo, Japan), and images were captured in three arbitrary fields in a well by a digital camera. The images were saved on a computer and finally measured and analyzed for cellular shape in accordance with previous methods (Ren *et al*., 2015). Adobe Photoshop CS2 software (Adobe Systems Inc., San

Jose, CA, USA) was used for selecting, depicting shape and segmenting the cells in the images. Subsequently, Image Pro Plus 6.0 software (Media Cybernetics Inc., Rockville, MD, USA) was applied for calculating the roundness of the cells.

ELISA assay

After image acquirement, the medium was removed and centrifuged at 3000 rpm and 4 °C for 10 min. The cells were washed with PBS. Then, trypsin was added, and the cells were harvested with medium containing 10% FBS. After centrifugation of the cells with the medium at 1200 rpm and 4 °C for 5 min, the cell pellet was obtained and then lysed with 1% TritonX-100, 137 mmol/L NaCl, 20 mmol/L Tris-HCl,2 mmol/L EDTA, and 1 mmol/L PMSF. The lysates were centrifuged at 3500 rpm and 4 °C for 10 min, and the supernatant was stored at 4 °C. The supernatant was assayed for E-cad, Col-1a and α-SMA according to the manufacturer's protocol. The protein concentration in the supernatant was measured by a BCA kit.

Preparation of cytoplasm protein

The cells were plated at a density of 100,000 cells per well in a 60 mm dish. The cells were treated with 7.5 ng/mL TGF-β1 and various concentrations of hesperidin (0, 20, 40, and 60 μM) or 10 μM SB431542 as described above. After centrifugation, the cells were lysed with a Membrane and Cytosol protein extraction kit (Beyotime Biotechnology, Shanghai, China) after adding PMSF and were frozen at -70 °C. Next, the cells were thawed at room temperature. The freeze-thaw cycles were carried out 3 times to obtain up to a 90% broken cell ratio. Then, the lysates were centrifuged at 12000 g for 30 min at 4 °C, and the supernatant was extracted to obtain the total cytoplasm protein for the Western blotting assay.

Western blot analysis

The protein concentration in the supernatant was measured by a BCA kit. The total protein (30 or 50 μg) from a sample was separated by SDS-PAGE and transferred onto a PVDF membrane (Millipore Corp., Billerica, MA, USA). Then, the membranes were incubated in TBST containing 5% skim milk at room temperature for 2 h. After blocking, the membranes were incubated with specific primary antibodies at 4 °C overnight. The rabbit polyclonal anti-Smad 2, 3, 4, 7 and 2/3 antibodies (Boster Biological Technology Co., Ltd. Wuhan, Hubei province, China) were diluted 1:100. The rabbit polyclonal anti-p-Smad2/3 (Abcam, Cambridge, MA, USA) and rabbit polyclonal anti-GAPDH (Boster Biological Technology Co., Ltd. Wuhan, Hubei province, China) antibodies were diluted 1:400. The next day, the membranes were washed 3 times with TBST, followed by incubation in goat anti-rabbit IgG conjugated with horseradish peroxidase antibody (Boster Biological Technology Co., Ltd. Wuhan, Hubei province, China) for 2 h at room temperature. Finally, the membranes were washed another 3 times. The proteins were visualized with an ECL-chemiluminescent kit and were detected by the Chemi Doc MP Imaging system (Bio-RAD, Hercules, CA, USA). The expression of GAPDH was used as the loading control.

Statistical analyses

All the data are expressed as the mean ± standard error of the mean (SEM). The significance of the differences between groups was assessed by a one-way analysis of variance (ANOVA) using SPSS 16.0. Differences with a p-value of < 0.05 were considered statistically significant. The IC15 and IC50 values of hesperidin were calculated using GraphPad Prism5 software.

RESULTS

Hesperidin inhibited the proliferation of A549 cells

As shown in Figure 1, hesperidin had an anti-proliferative effect on A549 cells, with dose-dependent decreases in cell proliferation (40-200 μM). The inhibitory effect started at 24 h, was sustained for 48 h and was the strongest at 36 h. The IC50 values of hesperidin were 338.9±2.45 μM at 24 h, 153.3±1.76 μM at 36 h, and 216.8±2.02 μM at 48 h. The IC15 value at 48 h was 60.26 μM, and the approximate value of 60 μM was chosen as the highest dose in the following study.

Hesperidin inhibited the TGF-β1-induced epithelial to mesenchymal transition in A549 cells

To determine whether hesperidin acts as an EMT inhibitory compound in A549 cells, TGF-β1-induced EMT was carried out. The ELISA assay results showed that hesperidin inhibited the decreased E-cad protein level in a dose-dependent manner, and the 60 μM hesperidin treatment yielded a significant E-cad increase than the TGF-β1 control (Figure 2B). Hesperidin also reduced

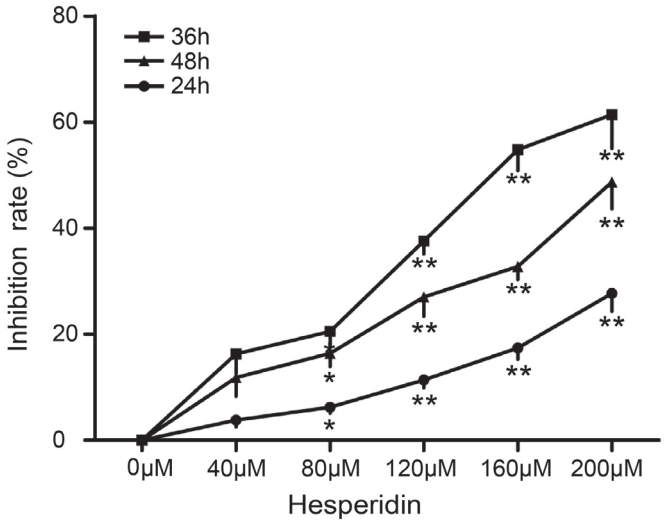

FIGURE 1 - Inhibition of hesperidin in A549 cell proliferation. The cells were treated with various concentrations of hesperidin for 24 h, 36 h and 48 h. Cells without hesperidin treatment were used as controls (0 μM hesperidin). *p < 0.05 and **p < 0.01 compared to the control group.

α-SMA and Col-1α levels in a dose-dependent manner, and the 40 and 60 μM hesperidin treatments yielded a significant decrease in α-SMA and Col-1α levels than the TGF-β1 control (Figure 2C; 2D). The inhibitory effect was confirmed by the subsequent Western blotting assay results (Figure 2E; 2F). However, hesperidin did not significantly inhibit the decrease in roundness. These data suggested that hesperidin had a partial inhibitory effect on EMT.

Hesperidin had no effect on Smad 2, 3, 4 and 7 protein synthesis in A549 cells stimulated by TGF-β1

Since the Smad family of proteins are key intracellular mediators of signal transduction in TGF-β1-induced EMT, the signal transduction efficiency is influenced by changes in Smad protein production in the cytoplasm. Therefore, we evaluated hesperidin efficacy on important Smad protein expression levels in the cytoplasm, including on Smad 2, 3, 4 and 7 expression levels, by a Western blotting assay. SB431542, a potent and specific inhibitor of the TGF-β1 type 1 actin receptor, was also used as a positive control for the inhibition of Smad activation. As shown in Figure 3, the A549 cells treated with hesperidin (20-60 μM) or SB431542 for 48 h showed no significant difference in the expression of these proteins compared to those with the TGF-β1 treatment. The results suggested that hesperidin might have no effect on the synthesis of these Smad proteins.

Hesperidin decreased p-Smad2/3 expression in TGF-β1-stimLuated cells

Previous reports indicated that Smad 2/3 phosphorylation in the cytoplasm is an essential step in signal transduction (Valcourt *et al*., 2005; Willis, DuBois, Borok, 2006). We therefore investigated whether hesperidin exerted an inhibitory effect on EMT via the modulation of Smad 2/3 phosphorylation. As expected, TGF-β1 treatment promoted Smad 2/3 phosphorylation in the cytoplasm, but SB431542 inhibited the phosphorylation. Hesperidin also inhibited the phosphorylation in a dose-dependent manner, with a 12.1%, 37.0% and 46.3% decrease in phospho-Smad2/3 at 20 μM, 40 μM, and 60 μM concentration, respectively, suggesting that the anti-EMT capacity of hesperidin was relative to the inhibition of Smad2/3 phosphorylation in the cytoplasm. Furthermore, the inhibition was not relative to Smad2/3 production in the cytoplasm because there was no obvious difference in Smad 2/3 protein expression in the cells (Figure 4).

DISCUSSION

More evidence supports the EMT of alveolar epithelial cells as playing a central role in the pathogenesis of pulmonary fibrosis (Derynck, Zhang, 2003; Gorowiec *et al*., 2012; Coward, Saini, Jenkins, 2010). Alveolar basement membrane disruption and alveolar injury lead to TGF-β1 activation and secretion, and, subsequently, TGF-β1 deteriorates EMT in alveolar epithelial cells (Coward, Saini, Jenkins, 2010). In vitro, TGF-β1-induced EMT in alveolar cells has been applied in many studies of IPF (Baek *et al*., 2016; Yang, Chen, Sun, 2013; Yu, *et al*., 2016b). In this study, A549 cells, stimulated by TGF-β1, showed an obvious EMT, including cellular morphology changes from cobblestone to spindle, an E-cad decrease and an α-SMA increase, which were similar to the results of previous studies (Baek, *et al*., 2016; Yang, Chen, Sun, 2013; Yu *et al*., 2016b).

Hesperidin, a flavonoid glycoside that is extracted from the fruit peels of the genus citrus, suppresses injury or fibrosis of some organs in vivo. These studies have mainly focused on anti-liver fibrosis, including the inhibition of dimethylnitrosamine-induced and CCl_4-induced fibrosis in rats (Elshazly, Mahmoud, 2014; Pérez-Vargas *et al*., 2014). The anti-liver fibrosis mechanism of hesperidin is complex and includes the Wnt/β-catenin pathway (Lin *et al*., 2015), the PTEN/AKT pathway (Li *et al.,* 2017), and the TGF-β1/Smad signaling pathway (Pérez-Vargas *et al*., 2014; Wu *et al*., 2015). Recently, some reports show the

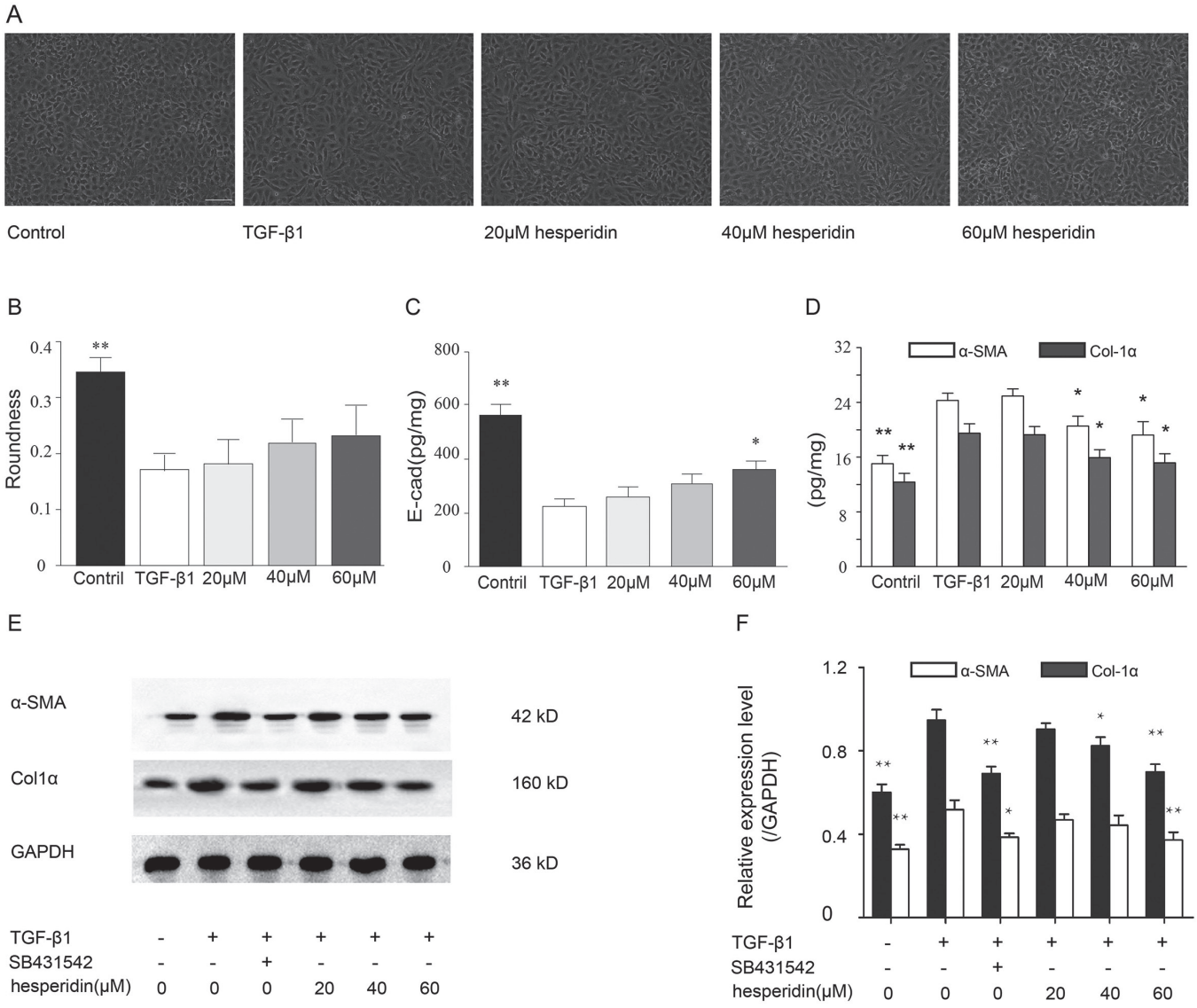

FIGURE 2 - Effect of hesperidin on the epithelial to mesenchymal transition. (A). The cells were treated with TGF-β1 (7.5 ng/mL) and various concentrations of hesperidin (0-60 μM) for 48 h. After TGF-β1 treatment, the cell morphology changed from a pebble-like shape to an elongated shape (magnification 100 ×). (B-D). Hesperidin did not significantly inhibit a decrease in the roundness value but significantly inhibited the downregulation of E-cad and the upregulation of α-SMA and Col-1α in a dose-dependent manner, as determined by ELISA assays. (E and F). The Western blotting assays evaluated the effect of hesperidin on Col-1α and α-SMA protein expression. Lanes 1, 2, 3, 4, 5 and 6 represent the normal control, TGF-β1 control, SB431542+TGF-β1, 20 μM heperidin+TGF-β1, 40 μM heperidin+TGF-β1 and 60 μM heperidin+TGF-β1 groups, respectively. The GADPH expression was the loading control. The quantitative data, expressing the corresponding proteins levels, were assessed using densitometry and are expressed in relative intensity arbitrary units in the panels. The bar represents the standard error of the mean (n = 5). *$p < 0.05$ and **$p < 0.01$ compared to the TGF-β1control group.

protective effect of hesperidin against pulmonary injury induced by endotoxin, intestinal ischemia/reperfusion or lipopolysaccharide in rats or mice (Bayomy *et al*., 2014; Liu *et al*., 2015; Yeh *et al*., 2007), suggesting the possible action of anti-pulmonary fibrosis of hesperidin. In one study, hesperidin increased E-cadherin and decreased α-SMA and Col-1α, thus appearing to inhibit EMT, which was similar to the result of our previous study (Yu *et al*., 2016a).

Exogenous TGF-β1 binds and activates TβRII and then forms heteromeric complexes of type I and type II serine/threonine kinase receptors, finally activating TβRI receptor kinases and the Smad signaling cascade. Smad proteins are mainly located in the cytoplasm and consist of a highly conserved family of signaling transducers. Smad proteins are divided into three subfamilies, including receptor-activated Smads (R-Smads), which include

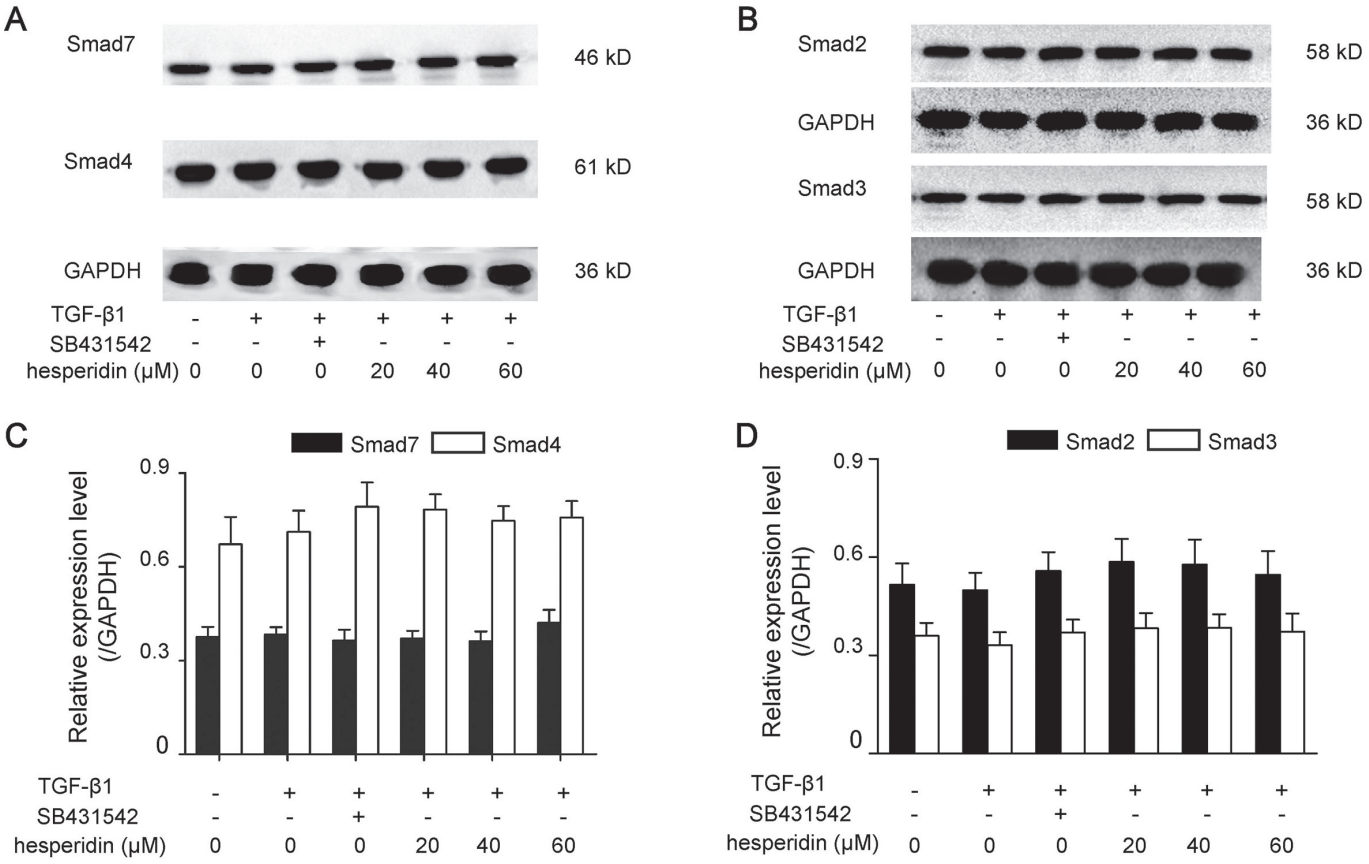

FIGURE 3 - Effect of hesperidin on the synthesis of Smad proteins in the cytoplasm. Lanes 1, 2, 3, 4, 5 and 6 represent the normal control, TGF-β1 control, 10 μM SB431542+ TGF-β1, 20 μM heperidin+ TGF-β1, 40 μM heperidin+TGF-β1 and 60 μM heperidin+TGF-β1 groups, respectively. The GADPH expression was the loading control. The quantitative data, expressing the corresponding Smad protein levels, were assessed using densitometry and are expressed in relative intensity arbitrary units in the panels. The bar represents the standard error of the mean (n = 5).

Smad1, Smad2, Smad3, Smad5, and Smad8, common mediator Smads (Co-Smads), such as Smad4, which oligomerizes with activates R-Smads, and inhibitory Smads (I-Smads), such as Smad6 and Smad7, which have a negative feedback effect by competing with R-Smads for receptor interaction and by marking receptors for degradation (Moustakas, Souchelnytskyi, Heldin, 2001). For the TGF-β/Smad signaling pathway in the cytoplasm, the key transducers are Smad 2, Smad 3, Smad 4 and Smad 7. Obviously, changes in these protein levels in the cytoplasm intervene in TGF-β/Smad signal transduction. Some reports show that the inhibition of Smad3 expression reduces the TGF-β-mediated induction of EMT, and small molecule inhibitors of Smad3 have clinical potential in the treatment of fibrotic diseases (Flanders, 2004; Liu, 2006). Interestingly, hesperidin reduces Smad 2 and Smad 3 mRNA and increases Smad 7 mRNA in PDGF-induced HSC-T6 cells (Wu *et al*., 2015). However, in this study, hesperidin did not obviously intervene in these protein levels, suggesting that the hesperidin inhibitory effect on TGF-β/Smad signal transduction in A549 cells may be related to another mechanism.

In TGF-β1/Smad signal transduction, p-Smad2/3 formation through the phosphorylation of the C-terminal serine residues in R-Smads by type I receptor kinases is a crucial step that is followed by the formation of an oligomeric complex with the Co-Smads and Smad4 (Moustakas, Souchelnytskyi, Heldin, 2001). The oligomeric complex translocates into the nucleus, binds to specific genes and results in protein expression changes. HDND-7, a derivative of hesperidin, significantly attenuates liver fibrosis and inhibits the activation and proliferation of PDGF-induced HSCs, including a decrease in the expression levels of p-Smad3 and Smad4, through not only the Wnt/β- catenin pathway but also the TGF-β/Smad pathway (Lin *et al*., 2015). In this study, the p-Smad2/3 protein expression level was increased 1.42-fold in the A549 cells stimulated by TGF-β1 compared to the unstimulated cells. Compared with the TGF-β1 stimulated cells, hesperidin decreased p-Smad2/3 in

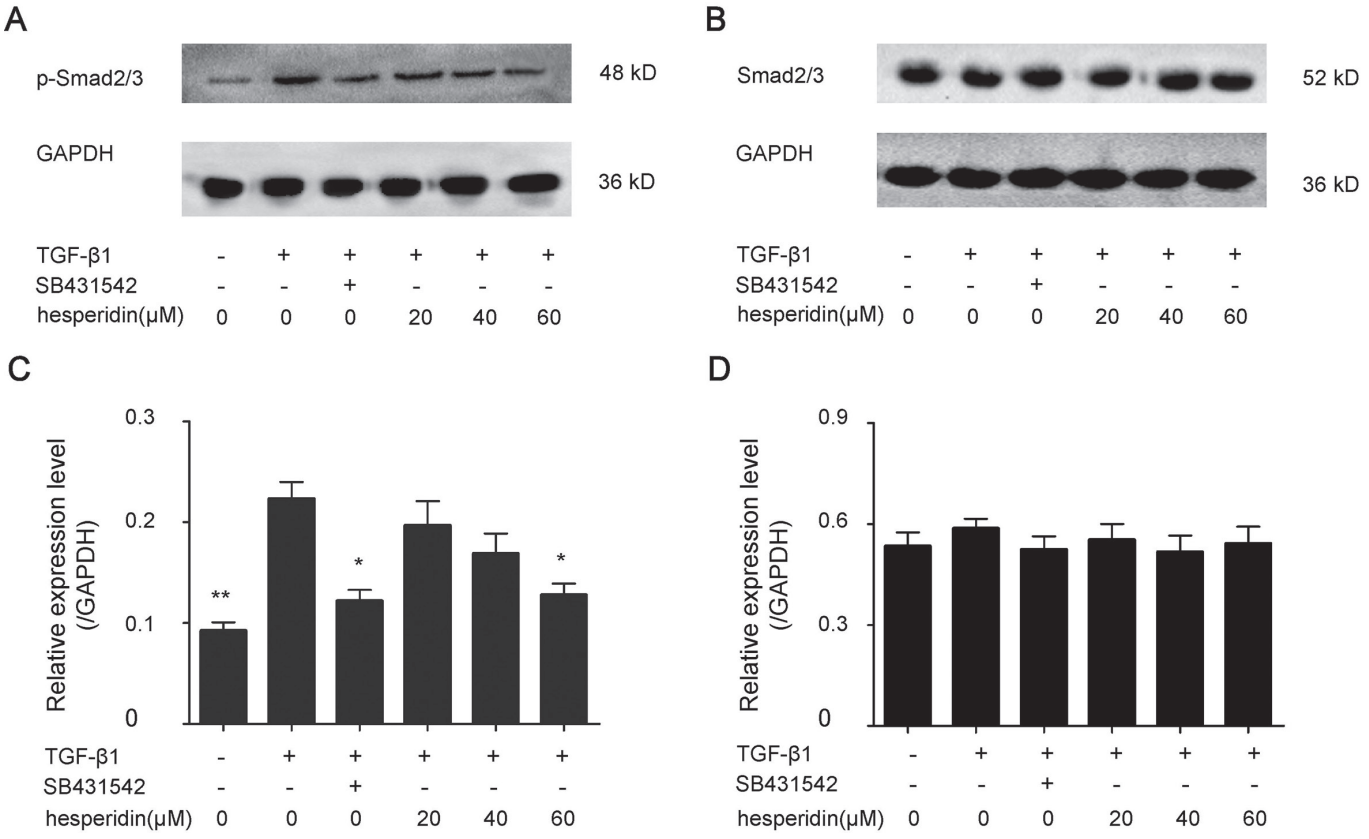

FIGURE 4 - Effect of hesperidin on Smad 2/3 phosphorylation in the cytoplasm. Lanes 1, 2, 3, 4, 5 and 6 represent the normal control, TGF-β1 control, SB431542+TGF-β1, 20 μM heperidin+TGF-β1, 40 μM heperidin+TGF-β1 and 60 μM heperidin+TGF-β1 groups, respectively. The GADPH expression was the loading control. The quantitative data, expressing the corresponding Smad proteins level, were assessed using densitometry and are expressed in relative intensity arbitrary units in the panels. The bar represents the standard error of the mean (n = 5). *$p < 0.05$ and **$p < 0.01$ compared to the TGF-β1control group.

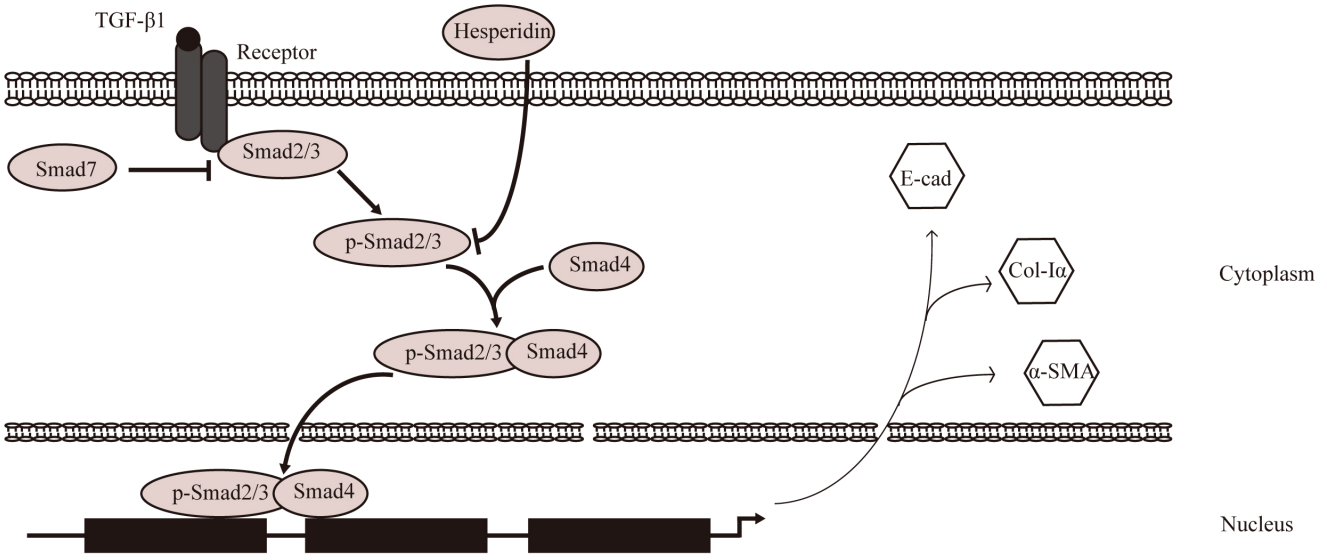

FIGURE 5 - Cartoon of the inhibitory effect of hesperidin on TGF-β1-induced EMT through the Smad pathway in the cytoplasm.

a dose-dependent manner. The results suggested that the inhibitory effect of hesperidin on TGF-β1 signal transduction might be associated with its suppressive effect on the phosphorylation of R-Smads or the formation of oligomeric complexes, such as p-Smad2/3.

Excessive alpha smooth muscle actin (α-SMA) expression is a hallmark of activated fibroblasts and myofibroblasts (Desmoulière, *et al*., 1993; Sanders, Kumbla, Hagood, 2007). These cells, in a high α-SMA expression state, have an excessive ability to synthesize and secrete extracellular matrix proteins, such as collagens (Col), resulting in the formation of fibrotic foci and abnormal structures in pulmonary tissue, which is one of the key pathological mechanisms in IPF. The TGF-β/Smad pathway partly regulates the synthesis and secretion of two proteins (Hu, Wu, Phan, 2003; Zhou *et al*., 2011). Hesperidin significantly decreases α-SMA and Col-1α proteins level, further supporting that the inhibition of the Smad pathway activity could be an important mechanism of hesperidin in anti-pulmonary fibrosis.

Collectively, our present study suggested that the anti-EMT mechanism of hesperidin was partly mediated by the inhibition of Smad2/3 phosphorylation of the TGF-β/Smad pathway in the cytoplasm. Thus, hesperidin may provide a potential new therapeutic strategy for the treatment of pulmonary fibrosis.

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