Diosgenin inhibits angiotensin II-induced extracellular matrix remodeling in cardiac fibroblasts through regulating the TGF-β1/Smad3 signaling pathway

HONG-TAO ZHOU[1], XUE-FANG YU[2] and GUI-MING ZHOU[3]

[1]Department of Ultrasound Room, Tianjin Medical University Metabolic Diseases Hospital, Tianjin 300070; Departments of [2]Cardiology and [3]Ultrasound Room, Tianjin Medical University General Hospital, Tianjin 300054, P.R. China

Received November 22, 2015; Accepted November 15, 2016

DOI: 10.3892/mmr.2017.6280

Abstract. The proliferation of cardiac fibroblasts (CFs) and deposition of extracellular matrix (ECM) proteins are pivotal in the development of cardiac fibrosis. Recent studies have indicated that diosgenin may inhibit high glucose-induced renal tubular fibrosis; however, to the best of our knowledge, no studies have focused on the effects of diosgenin on cardiac fibrosis. Therefore, the present study aimed to explore the effects of diosgenin on angiotensin II (Ang II)-induced ECM remodeling, and its possible mechanism in rat CFs. CFs were pre-incubated with diosgenin (1, 5 and 10 μM) for 24 h and were then stimulated with Ang II (100 nM) for 24 h. Cell proliferation was estimated using the MTS assay. The expression levels of α-SMA, fibronectin, collagen I, TGF-β1, in addition to phosphorylation of Smad3 were detected by western blotting. The results demonstrated that diosgenin inhibited Ang II-induced CF proliferation and the differentiation of CFs to myofibroblasts. In addition, diosgenin was able to inhibit Ang II-induced ECM expression in rat CFs. Furthermore, diosgenin inhibited Ang II-induced expression of transforming growth factor-β1 (TGF-β1) and Smad3 phosphorylation in CFs. Taken together, these results suggest that diosgenin may inhibit Ang II-induced ECM remodeling by suppressing the TGF-β1/Smad3 signaling pathway in rat CFs. Therefore, diosgenin may possess therapeutic potential for the treatment of cardiac fibrosis.

Introduction

Cardiac fibrosis is a common pathological alteration that occurs as a result of hypertension and myocardial infarction, and during valvular heart disease, which induces notable left ventricular hypertrophy (1). Cardiac fibroblasts (CFs) are the most abundant cell type in the myocardium, which serve an important role in the maintenance of myocardial structure and function (2). The abnormal proliferation of CFs, and deposition of extracellular matrix (ECM) proteins and collagens, results in the development of cardiac fibrosis, which then adversely affects cardiac performance (3). Previous studies have demonstrated that angiotensin II (Ang II), which is one of the most important renin-angiotensin system components, has a crucial role in the progression of cardiac fibrosis (4-6). Ang II has been reported to be aberrantly activated in patients with myocardial fibrosis (7). In addition, Ang II acts as a potent profibrotic molecule that promotes myofibroblast differentiation, and aberrant ECM production and degradation (8). Therefore, suppressing Ang II-induced ECM synthesis in CFs may provide a potential target for the prevention of cardiac fibrosis.

Diosgenin is a steroidal saponin present in various plants, including the *Solanum* and *Dioscorea* species. Numerous studies have indicated that diosgenin possesses important pharmacological activities, including anti-inflammatory, antiatherosclerotic, antitumor and antioxidant activities (9-12). Badalzadeh *et al* reported that diosgenin may exert cardioprotective effects against myocardial reperfusion injury via activation of mitochondrial K_{ATP} channels (13). A recent study reported that diosgenin inhibited high glucose-induced renal tubular fibrosis (14); however, to the best of our knowledge, no studies have focused on the effects of diosgenin against cardiac fibrosis. Therefore, the present study aimed to explore the effects of diosgenin on Ang II-induced ECM remodeling and its possible mechanism in CFs.

Materials and methods

Cell culture and treatment. The experimental protocol and associated animal handling procedures complied with the Guidelines for Animal Experiments from the Ethical Committee for Animal Research of Tianjin Medical University Metabolic Diseases Hospital (Tianjin, China). Primary cultures of neonatal rat CFs were prepared as previously described (15). Briefly, female Sprague-Dawley

Correspondence to: Dr Gui-Ming Zhou, Department of Ultrasound Room, Tianjin Medical University General Hospital, 154 Anshan Road, Tianjin 300054, P.R. China
E-mail: zhguimingtj@126.com

Key words: cardiac fibrosis, diosgenin, cardiac fibroblasts, extracellular matrix

rats (n=6, 180±200 g) were obtained from the Animal Experimental Center, Tianjin Medical University Metabolic Diseases Hospital. They were housed at 20-24˚C under 12 h light-dark cycles and were allowed free access to water and commercial pellets. Rats were anesthetized with 10% chloral hydrate (350 mg/kg, intraperitoneal; Sigma-Aldrich; Merck Millipore, Darmstadt, Germany). The right ventricles from the rats were then minced and digested in collagenase for 24 h at 37˚C (450 U/ml; Sigma-Aldrich; Merck Millipore). Cells were pelleted via centrifugation at 8,000 x *g* at 4˚C for 24 h, and were suspended in Dulbecco's modified Eagle's medium (DMEM; Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS; Invitrogen; Thermo Fisher Scientific, Inc.). CFs were cultured for 3-4 days and CFs in passages 9 to 20 were used.

Once the cells reached 80-90% confluence, the medium was replaced with serum-free DMEM 1 day prior to pretreatment with diosgenin (1, 5 and 10 μM; Sigma-Aldrich; Merck Millipore) for 24 h at 37˚C. The cells were then exposed to Ang II (100 nM; Sigma-Aldrich; Merck Millipore) at 37˚C for 30 min or 24 h.

Cell proliferation assay. Cell proliferation was detected with a colorimetric assay using the CellTiter 96 AQueous One Solution (MTS) reagent (Promega Corporation, Madison, WI, USA). CFs were added to 96-well plates at a density of $1x10^4$ cells/well. Following treatment for 24 h, 20 μl MTS reagent was added to each well and the CFs were incubated at 37˚C for a further 4 h. The optical density was measured at a wavelength of 490 nm. FBS (10%) was used as a positive control.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was isolated from CFs using TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.), and cDNA was generated from total RNA using the iScript cDNA synthesis kit (Bio-Rad Laboratories, Inc., Hercules, CA, USA) according to the manufacturer's protocol. The mRNA expression levels were analyzed by RT-qPCR using the SYBR-Green PCR Mix (Invitrogen; Thermo Fisher Scientific, Inc.) and run on the Bio-Rad iCycler system (Bio-Rad Laboratories, Inc.). The primers used were as follows: α-smooth muscle actin (α-SMA), forward 5'-GCT ATT CAG GCT GTG CTG TC-3', reverse 5'-GGT AGT CGG TGA GAT CTC GG-3'; transforming growth factor (TGF)-β1, forward 5'-CCA ACT ATT GCT TCA GCT CCA-3', reverse 5'-GTG TCC AGG CTC CAA ATG T-3'; and GAPDH, forward 5'-ACT CCC ATT CTT CCA CCT TTG-3' and reverse 5'-CCC TGT TGC TGT AGC CAT ATT-3'. The PCR cycling conditions were as follows: Initial denaturation at 94˚C for 4 min; 40 cycles of 94˚C for 20 sec, 55˚C for 30 sec and 72˚C for 20 sec; 2 sec for plate reading for 40 cycles; melting curve, 65-95˚C. GAPDH was used as a control for normalizing gene expression levels. The data obtained were analyzed using the $2^{-\Delta\Delta Cq}$ method (16).

Western blot analysis. Proteins were extracted from CFs using radioimmunoprecipitation assay lysis buffer (Invitrogen; Thermo Fisher Scientific, Inc.) supplemented with 1 mmol/l phenylmethylsulfonyl fluoride and phosphatase inhibitor (Pierce; Thermo Fisher Scientific, Inc.), and the protein concentration was quantified using the Bradford assay. A total of 20-30 μg protein was fractionated by 12% SDS-PAGE and was transferred to nitrocellulose membranes (Amersham; GE Healthcare Life Science, Little Chalfont, UK). Subsequently, the membranes were incubated in Tris-buffered saline (TBS) containing 5% non-fat dry milk to block non-specific binding at room temperature for 1 h. The membrane was then washed with TBS and incubated with primary antibodies against α-SMA (1:1,500; sc-53142), TGF-β1 (1:2,500; sc-130348), Smad3 (1:3,000; sc-101154), phosphorylated-Smad3 (1:3,000; sc-130218), fibronectin (1:2,000; sc-9068), type I collagen (1:1,500; sc-25,974) and GAPDH (1:3000; sc-367,714) (all Santa Cruz Biotechnology, Inc., Dallas, TX, USA) overnight at 4˚C. Subsequently, the membranes were washed extensively with TBS containing 0.1% Tween and were incubated with a horseradish peroxidase-conjugated secondary antibody (1:3,000; sc-516087; Santa Cruz Biotechnology, Inc.) for 1 h at room temperature. The signal was detected using Enhanced Chemiluminescence Plus (Thermo Fisher Scientific, Inc.). The optical densities of the bands were semi-quantified using Gel-Pro Analyzer v4.0 (Media Cybernetics, Inc., Rockville, MD, USA). GAPDH was used as the endogenous control.

Statistical analysis. All data are presented as the mean ± standard error of the mean. Statistical significance was assessed using one-way analysis of variance followed by Tukey's *post hoc* test for multiple-group comparisons. Differences between groups were compared using a paired-sample Student's *t*-test. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Diosgenin inhibits Ang II-induced proliferation of rat CFs. Ang II was revealed to induce the proliferation of CFs. To determine whether diosgenin may reverse the effects of Ang II on CF proliferation, rat CFs were treated with various concentrations of diosgenin prior to Ang II. As presented in Fig. 1, diosgenin significantly inhibited cell proliferation in Ang II-stimulated CFs in a dose-dependent manner.

Diosgenin reduces the differentiation of CFs to myofibroblasts. Treatment with Ang II was able to stimulate the differentiation of CFs to myofibroblasts, and α-SMA acted as a marker of myofibroblast differentiation. Therefore, the present study investigated the effects of diosgenin on the expression of α-SMA in Ang II-stimulated CFs. As presented in Fig. 2, the expression levels of α-SMA were significantly increased following treatment with Ang II; however, diosgenin reduced Ang II-induced α-SMA expression in CFs.

Diosgenin inhibits Ang II-induced ECM synthesis in rat CFs. It is widely accepted that ECM accumulation serves an important role in cardiac fibrosis; therefore, the present study aimed to determine whether ECM was regulated by diosgenin. As presented in Fig. 3, Ang II stimulation significantly induced the expression of fibronectin (FN), and this effect was reduced following treatment of CFs with diosgenin.

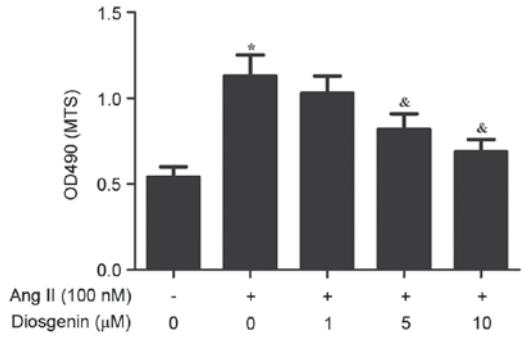

Figure 1. Diosgenin inhibits Ang II-induced proliferation of rat CFs. CFs were preincubated with diosgenin (1, 5 and 10 μM) for 24 h and were then stimulated with Ang II (100 nM) for 24 h. Cell proliferation was estimated using an MTS assay. Data are presented as the mean ± standard error of the mean of three independent experiments. *P<0.05 compared with the control group; &P<0.05 compared with the Ang II stimulation group. Ang II, angiotensin II; CFs, cardiac fibroblasts; OD, optical density.

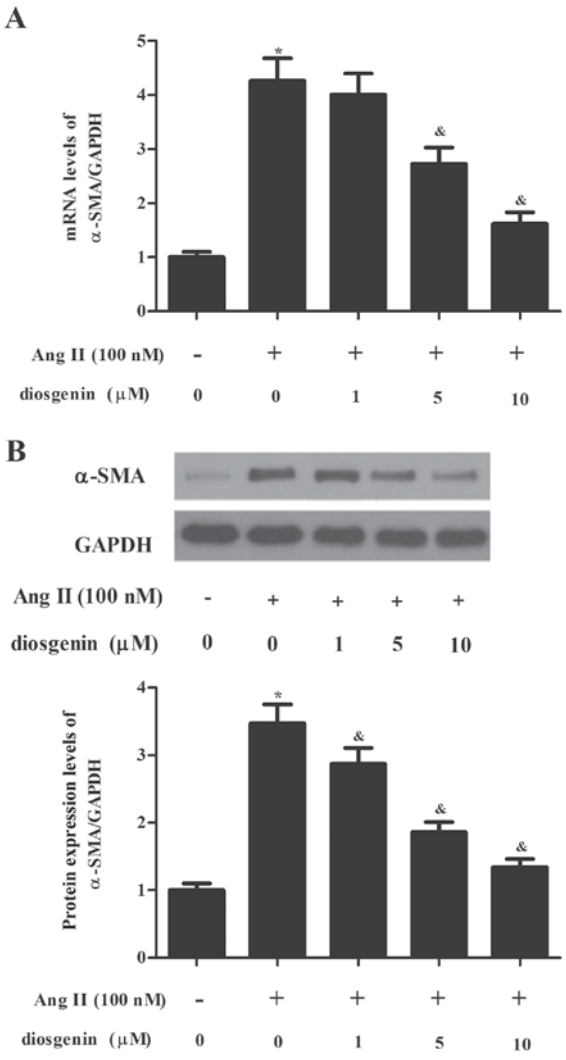

Figure 2. Diosgenin reduces the differentiation of CFs to myofibroblasts. CFs were preincubated with diosgenin (1, 5 and 10 μM) for 24 h and were then stimulated with Ang II (100 nM) for 24 h. (A) mRNA expression levels of α-SMA were detected by reverse transcription-quantitative polymerase chain reaction. (B) Protein expression levels of α-SMA were detected by western blotting. Data are presented as the mean ± standard error of the mean of three independent experiments. *P<0.05 compared with the control group; &P<0.05 compared with the Ang II stimulation group. Ang II, angiotensin II; CFs, cardiac fibroblasts; α-SMA, α-smooth muscle actin.

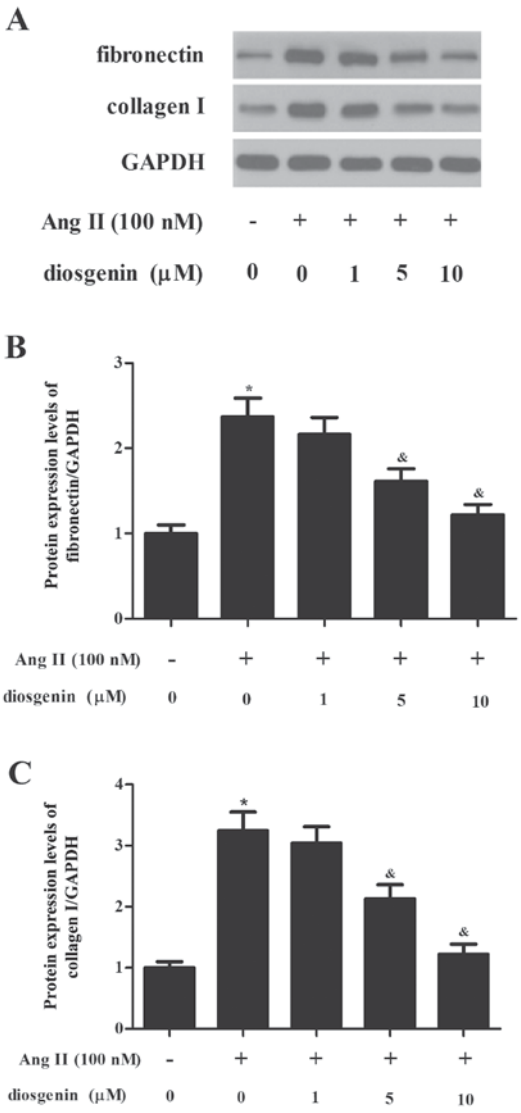

Figure 3. Diosgenin inhibits Ang II-induced extracellular matrix synthesis in rat CFs. CFs were preincubated with diosgenin (1, 5 and 10 μM) for 24 h and were then stimulated with Ang II (100 nM) for 24 h. (A) The protein expression levels of fibronectin and collagen I were detected by western blotting. Expression of (B) fibronectin and (C) collagen I proteins was analyzed using Gel-Pro Analyzer version 4.0 software and normalized to GAPDH. Data are presented as the mean ± standard error of the mean of three independent experiments. *P<0.05 compared with the control group; &P<0.05 compared with the Ang II stimulation group. Ang II, angiotensin II; CFs, cardiac fibroblasts.

Similarly, the Ang II-induced enhancement of type I collagen in CFs was significantly reversed by diosgenin.

Diosgenin inhibits Ang II-induced TGF-β1 expression. To investigate whether diosgenin has an effect of on TGF-β1 in CFs exposed to Ang II, CFs were treated with diosgenin prior to Ang II. As presented in Fig. 4A, Ang II treatment markedly increased the mRNA expression levels of TGF-β1 in CFs compared with the normal group. However, Ang II-induced TGF-β1 upregulation was prevented following treatment of CFs with diosgenin. Similarly, the western blot analysis demonstrated that diosgenin inhibited Ang II-induced TGF-β1 protein expression in CFs in a dose-dependent manner (Fig. 4B).

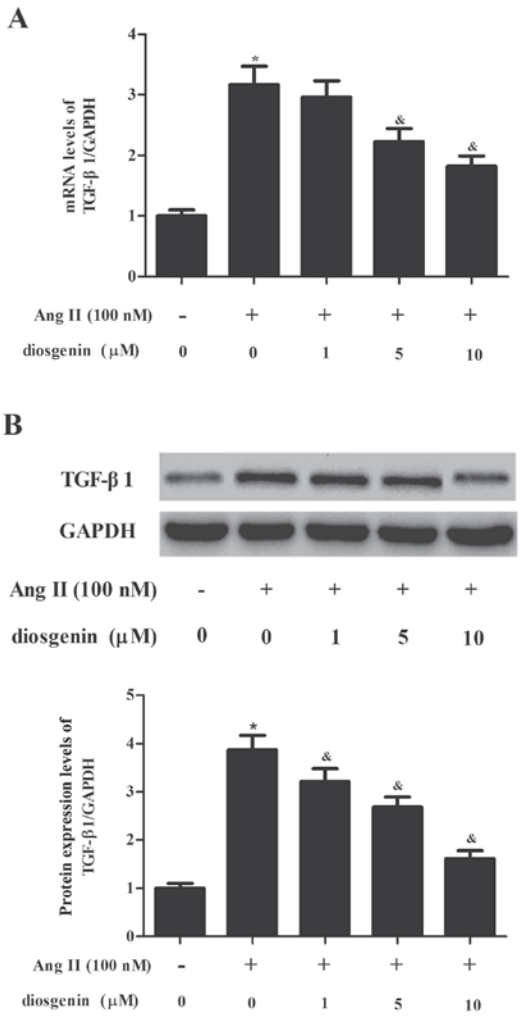

Figure 4. Diosgenin inhibits Ang II-induced TGF-β1 expression. CFs were preincubated with diosgenin (1, 5 and 10 μM) for 24 h and were then stimulated with Ang II (100 nM) for 24 h. (A) mRNA expression levels of TGF-β1 were detected by reverse transcription-quantitative polymerase chain reaction. (B) Protein expression levels of TGF-β1 were detected by western blotting. Data are presented as the mean ± standard error of the mean of three independent experiments. *P<0.05 compared with the control group; &P<0.05 compared with the Ang II stimulation group. Ang II, angiotensin II; CFs, cardiac fibroblasts; TGF-β1, transforming growth factor-β1.

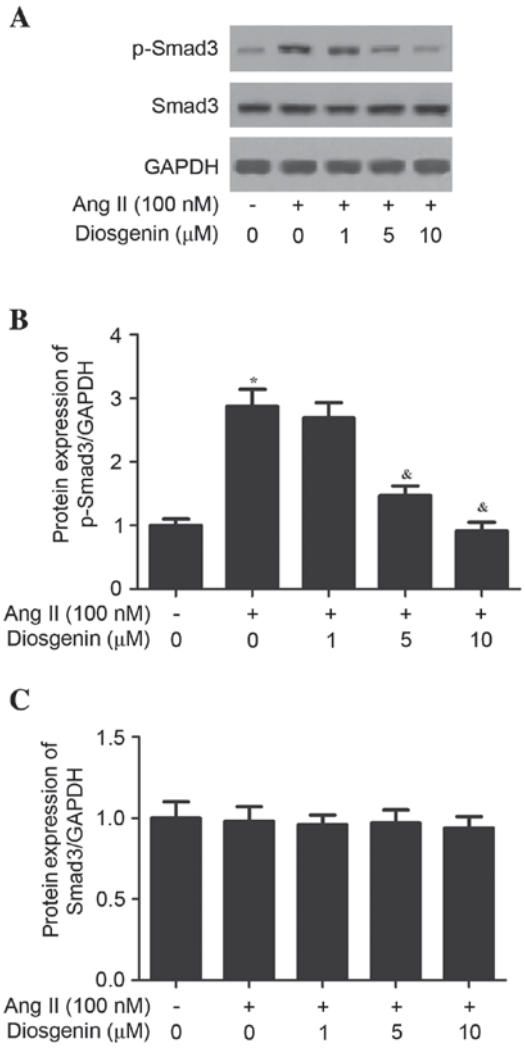

Figure 5. Diosgenin inhibits Ang II-induced TGF-β1/Smad3 pathway activation. Cardiac fibroblasts were preincubated with diosgenin (1, 5 and 10 μM) for 24 h and were then stimulated with Ang II (100 nM) for 30 min. (A) Phosphorylation of Smad3 was detected by western blotting using anti-p-Smad3 antibody. The protein expression levels of (B) p-Smad3 and (C) total Smad3 were analyzed. Data are presented as the mean ± standard error of the mean of three independent experiments. *P<0.05 compared with the control group; &P<0.05 compared with the Ang II stimulation group. Ang II, angiotensin II; p-Smad3, phosphorylated-Smad3.

Diosgenin inhibits Ang II-induced TGF-β1/Smad3 pathway activation. To explore the intracellular pathway that is associated with diosgenin-induced inhibition of TGF-β1 expression in Ang II-stimulated CFs, the present study examined the effects of diosgenin on Smad3 phosphorylation. As presented in Fig. 5, Ang II treatment was able to induce the phosphorylation of Smad3. Conversely, Ang II-stimulated Smad3 phosphorylation was markedly suppressed by diosgenin. These data indicated that diosgenin may inhibit the Ang II-mediated CF fibrotic response by suppressing the TGF-β1/Smad3 pathway.

Discussion

The major findings of the present study were: i) Diosgenin inhibited Ang II-induced CF proliferation and the differentiation of CFs to myofibroblasts; ii) diosgenin inhibited Ang II-induced ECM synthesis of rat CFs; and iii) diosgenin inhibited Ang II-induced expression of TGF-β1 and Smad3 phosphorylation in CFs.

Cardiac fibrosis, which is partially characterized by the proliferation of CFs, is a consequence of the cardiac remodeling process that is initiated by pathophysiological events (17). Previous studies have reported that Ang II serves as a pivotal positive regulator of CF proliferation (18-20). The results of the present study demonstrated that CF proliferation was significantly inhibited by treatment of Ang II-stimulated CFs with diosgenin in a dose-dependent manner. Therefore, it may be suggested that diosgenin has a role in cardiac remodeling via inhibiting the proliferation of CFs.

A critical event in the progression of cardiac fibrosis is the differentiation of CFs into myofibroblasts; myofibroblasts are highly active cells that are characterized by augmented expression of α-SMA (21). In addition, myofibroblast differentiation

is induced by several profibrotic factors, including Ang II (22), TGF-β (23), endothelin-1 and platelet-derived growth factor (24). The present study revealed that the expression of α-SMA was significantly increased following treatment with Ang II; however, diosgenin prevented Ang II-induced α-SMA expression in CFs. These data strongly indicated that diosgenin serves a crucial role in the phenotypic transformation of CFs to myofibroblasts.

Cardiac fibrosis is defined as a progressive accumulation of fibrillar ECM in the myocardium. FN is a key component of the ECM, which is upregulated in cardiac tissue during myocardial hypertrophy and failure (25). Collagen type I, which is excreted by CFs, is the major collagenous component of the cardiac interstitium and represents ~80% of total collagen (26). Furthermore, previous studies have demonstrated that collagen synthesis can be induced by Ang II (27,28). Consistent with these results, the present study demonstrated that Ang II stimulation significantly induced the expression of FN and type I collagen; however, diosgenin was able to inhibit Ang II-induced ECM synthesis in rat CFs. These findings suggested that diosgenin has a prominent role in regulating ECM expression during pathological cardiac remodeling.

The TGF-β/Smad signaling pathway serves an important role in the pathogenesis of cardiac fibrosis (29-31). As a main downstream signal transducer of TGF-β1, Smad3 can be phosphorylated by activated TGF-β1 type I receptor, after which it may form a complex with Smad4, which translocates into the nucleus, where it acts as a transcription factor to promote the expression of target genes, including type I and type III collagen (32). Furthermore, it has been reported that Ang II induces TGF-β1 upregulation and significant activation of the TGF-β1/Smad3 signaling pathway, thus resulting in a marked upregulation of type I and type III collagen expression (33). Similar to these previous results, the present study demonstrated that Ang II treatment induced TGF-β1 expression and Smad3 phosphorylation; however, diosgenin was able to inhibit Ang II-induced TGF-β1 expression and Smad3 phosphorylation. These results suggested that diosgenin may inhibit Ang II-induced ECM remodeling through suppressing the TGF-β1/Smad3 signaling pathway in rat CFs.

In conclusion, the results of the present study suggested that diosgenin inhibits Ang II-induced ECM remodeling through suppressing the TGF-β1/Smad signaling pathway in rat CFs. Therefore, diosgenin may be considered to possess therapeutic potential towards the treatment of cardiac fibrosis.

References

1. Khan R and Sheppard R: Fibrosis in heart disease: Understanding the role of transforming growth factor-beta in cardiomyopathy, valvular disease and arrhythmia. Immunology 118: 10-24, 2006.
2. Camelliti P, Borg TK and Kohl P: Structural and functional characterisation of cardiac fibroblasts. Cardiovasc Res 65: 40-51, 2005.
3. Krenning G, Zeisberg EM and Kalluri R: The origin of fibroblasts and mechanism of cardiac fibrosis. J Cell Physiol 225: 631-637, 2010.
4. Lee AA, Dillmann WH, McCulloch AD and Villarreal FJ: Angiotensin II stimulates the autocrine production of transforming growth factor-beta 1 in adult rat cardiac fibroblasts. J Mol Cell cardiol 27: 2347-2357, 1995.
5. Lim DS, Lutucuta S, Bachireddy P, Youker K, Evans A, Entman M, Roberts R and Marian AJ: Angiotensin II blockade reverses myocardial fibrosis in a transgenic mouse model of human hypertrophic cardiomyopathy. Circulation 103: 789-791, 2001.
6. Kawano H, Do YS, Kawano Y, Starnes V, Barr M, Law RE and Hsueh WA: Angiotensin II has multiple profibrotic effects in human cardiac fibroblasts. Circulation 101: 1130-1137, 2000.
7. Hara M, Sakata Y, Nakatani D, Suna S, Usami M, Matsumoto S, Sugitani T, Ozaki K, Nishino M, Sato H, *et al*: Renin-angiotensin-aldosterone system polymorphisms and 5-year mortality in survivors of acute myocardial infarction: A report from the osaka acute coronary insufficiency study. Int Heart J 55: 190-196, 2014.
8. Mehta PK and Griendling KK: Angiotensin II cell signaling: Physiological and pathological effects in the cardiovascular system. Am J Physiol Cell Physiol 292: C82-C97, 2007.
9. Hirai S, Uemura T, Mizoguchi N, Lee JY, Taketani K, Nakano Y, Hoshino S, Tsuge N, Narukami T, Yu R, *et al*: Diosgenin attenuates inflammatory changes in the interaction between adipocytes and macrophages. Mol Nutr Food Res 54: 797-804, 2010.
10. Park HJ, Byeon HE, Koo HJ and Pyo S: Anti-atherosclerotic effects of diosgenin in ApoE-deficient mice. FASEB J 25: 980-982, 2011.
11. Moalic S, Liagre B, Corbière C, Bianchi A, Dauça M, Bordji K and Beneytout JL: A plant steroid, diosgenin, induces apoptosis, cell cycle arrest and COX activity in osteosarcoma cells. FEBS Lett 506: 225-230, 2001.
12. Son IS, Kim JH, Sohn HY, Son KH, Kim JS and Kwon CS: Antioxidative and hypolipidemic effects of diosgenin, a steroidal saponin of yam (Dioscorea spp.), on high-cholesterol fed rats. Biosci Biotech Biochem 71: 3063-3071, 2007.
13. Badalzadeh R, Yousefi B, Tajaddini A and Ahmadian N: Diosgenin-induced protection against myocardial ischaemia-reperfusion injury is mediated by mitochondrial KATP channels in a rat model. Perfusion 30: 565-571, 2015.
14. Wang WC, Liu SF, Chang WT, Shiue YL, Hsieh PF, Hung TJ, Hung CY, Hung YJ, Chen MF and Yang YL: The effects of diosgenin in the Regulation of renal proximal tubular fibrosis. Exp Cell Res 323: 255-262, 2014.
15. Quintas LE, Pierre SV, Liu L, Bai Y, Liu X and Xie ZJ: Alterations of Na+/K+-ATPase function in caveolin-1 knockout cardiac fibroblasts. J Mol Cell Cardiol 49: 525-531, 2010.
16. Livak KJ and Schmittgen TD: Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. Methods 25: 402-408, 2001.
17. Schnee JM and Hsueh WA: Angiotensin II, adhesion, and cardiac fibrosis. Cardiovasc Res 46: 264-268, 2000.
18. Bouzegrhane F and Thibault G: Is angiotensin II a proliferative factor of cardiac fibroblasts? Cardiovasc Res 53: 304-312, 2002.
19. Van Kesteren CA, Van Heugten HA, Lamers JM, Saxena PR, Schalekamp MA and Danser AH: Angiotensin II-mediated growth and antigrowth effects in cultured neonatal rat cardiac myocytes and fibroblasts. J Mol Cell Cardiol 29: 2147-2157, 1997.
20. Hattori Y, Hattori S, Akimoto K, Nishikimi T, Suzuki K, Matsuoka H and Kasai K: Globular adiponectin activates nuclear factor-kappaB and activating protein-1 and enhances angiotensin II-induced proliferation in cardiac fibroblasts. Diabetes 56: 804-808, 2007.
21. Roy S, Khanna S, Rink T, Radtke J, Williams WT, Biswas S, Schnitt R, Strauch AR and Sen CK: P21waf1/cip1/sdi1 as a central regulator of inducible smooth muscle actin expression and differentiation of cardiac fibroblasts to myofibroblasts. Mol Biol Cell 18: 4837-4846, 2007.
22. Wu M, Han M, Li J, Xu X, Li T, Que L, Ha T, Li C, Chen Q and Li Y: 17beta-estradiol inhibits angiotensin II-induced cardiac myofibroblast differentiation. Eur J Pharmacol 616: 155-159, 2009.
23. Cucoranu I, Clempus R, Dikalova A, Phelan PJ, Ariyan S, Dikalov S and Sorescu D: NAD(P)H oxidase 4 mediates transforming growth factor-beta1-induced differentiation of cardiac fibroblasts into myofibroblasts. Circ Res 97: 900-907, 2005.
24. Leask A: Potential therapeutic targets for cardiac fibrosis: TGFbeta, angiotensin, endothelin, CCN2, and PDGF, partners in fibroblast activation. Circ Res 106: 1675-1680, 2010.
25. Reddy VS, Harskamp RE, Van Ginkel MW, Calhoon J, Baisden CE, Kim IS, Valente AJ and Chandrasekar B: Interleukin-18 stimulates fibronectin expression in primary human cardiac fibroblasts via PI3K-Akt-dependent NF-kappaB activation. J Cell Physiol 215: 697-707, 2008.
26. Brilla CG, Zhou G, Matsubara L and Weber KT: Collagen metabolism in cultured adult rat cardiac fibroblasts: Response to angiotensin II and aldosterone. J Mol Cell cardiol 26: 809-820, 1994.

27. Brilla CG, Zhou G, Rupp H, Maisch B and Weber KT: Role of angiotensin II and prostaglandin E2 in regulating cardiac fibroblast collagen turnover. Am J Cardiol 76: 8D-13D, 1995.
28. Chen K, Chen J, Li D, Zhang X and Mehta JL: Angiotensin II regulation of collagen type I expression in cardiac fibroblasts: Modulation by PPAR-gamma ligand pioglitazone. Hypertension 44: 655-661, 2004.
29. Li P, Wang D, Lucas J, Oparil S, Xing D, Cao X, Novak L, Renfrow MB and Chen YF: Atrial natriuretic peptide inhibits transforming growth factor beta-induced smad signaling and myofibroblast transformation in mouse cardiac fibroblasts. Circ Res 102: 185-192, 2008.
30. Yan W, Wang P, Zhao CX, Tang J, Xiao X and Wang DW: Decorin gene delivery inhibits cardiac fibrosis in spontaneously hypertensive rats by modulation of transforming growth factor-beta/Smad and p38 mitogen-activated protein kinase signaling pathways. Hum Gene ther 20: 1190-1200, 2009.
31. Liu X, Sun SQ, Hassid A and Ostrom RS: cAMP inhibits transforming growth factor-beta-stimulated collagen synthesis via inhibition of extracellular signal-regulated kinase 1/2 and Smad signaling in cardiac fibroblasts. Mol Pharmacol 70: 1992-2003, 2006.
32. Shi Y and Massagué J: Mechanisms of TGF-beta signaling from cell membrane to the nucleus. Cell 113: 685-700, 2003.
33. Chen K, Mehta JL, Li D, Joseph L and Joseph J: Transforming growth factor beta receptor endoglin is expressed in cardiac fibroblasts and modulates profibrogenic actions of angiotensin II. Circ Res 95: 1167-1173, 2004.