Betulinic acid prevents high glucose-induced expression of extracellular matrix protein in cardiac fibroblasts by inhibiting the TGF-β1/Smad signaling pathway

LING JIANG[1], FENG-XIA CHEN[2], SHU-TING ZANG[3] and QIAO-FANG YANG[4]

[1]Hypertension Department, [2]Department of Oncology, [3]Emergency Intensive Care Unit, [4]Department of Cardiology, Henan Provincial People's Hospital, Zhengzhou, Henan 450003, P.R. China

Received April 6, 2016; Accepted March 7, 2017

DOI: 10.3892/mmr.2017.7323

Abstract. The proliferation and differentiation of cardiac fibroblasts (CFs) is central to cardiac fibrosis. Betulinic acid (BA) is an active compound isolated from the bark of the birch tree *Betula* spp. (Betulaceae) and has been shown to attenuate hepatic fibrosis. However, the effect of BA on the high glucose-induced fibrosis response in CFs remains to be elucidated, therefore, the present study investigated the effect of BA on high glucose-induced CFs and examined the possible mechanism underlying the effect of BA on CF transdifferentiation. CFs were pre-incubated with various concentrations of BA for 24 h and then stimulated with high glucose (25 mM) for various times. Cell proliferation was evaluated using an MTT assay. The mRNA expression levels of α-smooth muscle actin (SMA) and transforming growth factor (TGF)-β1 were determined using reverse transcription-quantitative polymerase chain reaction analysis. The protein expression levels of α-SMA, collagen I, collagen III, fibronectin, TGF-β1, small mothers against decapentaplegic (Smad)2/3, phosphorylated (p)-Smad2 and p-Smad3 and were detected using western blot analysis. The data revealed that BA attenuated the CF proliferation and myofibroblast differentiation induced by high glucose. In addition, BA inhibited the expression of extracellular matrix (ECM) in the CFs induced by high glucose. It was also found that BA inhibited the high glucose-induced phosphorylation of Smad2/3 in the CFs. Taken together, BA suppressed the high glucose-induced increase in the proliferation of CFs and expression of ECM via inhibition of the TGF-β1/Smad signaling pathway. Thus, BA may offer therapeutic potential towards the treatment of cardiac fibrosis.

Correspondence to: Dr Qiao-Fang Yang, Department of Cardiology, Henan Provincial People's Hospital, 7 Weiwu Road, Zhengzhou, Henan 450003, P.R. China
E-mail: yangqfph@126.com

Key words: betulinic acid, cardiac fibrosis, cardiac fibroblasts, extracellular matrix

Introduction

Cardiac fibrosis is central to the development of cardiac dysfunction in a variety of cardiovascular diseases, including myocardial infarction, cardiac hypertrophy and heart failure (1). It is characterized by the proliferation of cardiac fibroblasts (CFs) and abundant accumulation of extracellular matrix (ECM) proteins in the myocardium (2). However, the molecular mechanisms underlying cardiac fibrosis remain to be elucidated.

CFs are the primary cardiac cells present in the myocardium. Accumulating evidence has suggested that the transdifferentiation of CFs into myofibroblasts is important in ECM deposition and cardiac fibrosis (3-5). High glucose or hyperglycemia is a factor, which promotes collagen deposition by inducing CF proliferation and activation *in vitro,* and can lead to cardiac fibrosis (6,7). Thus, intervention of high glucose-induced myofibroblast differentiation may be an effective method to improve and assist in curing cardiac fibrosis.

Betulinic acid (BA) is an active compound isolated from the bark of the birch tree *Betula* spp. (Betulaceae). Various biological and pharmacological effects of BA have been demonstrated, including anti-inflammatory, anti-viral, anti-oxidant and anti-tumor activities (8-10). For example, Xia *et al* (11) reported that BA prevents cardiomyocyte apoptosis and eventually improves cardiac function. In addition, BA attenuates hepatic fibrosis via suppressing thioacetamide-mediated increases in liver tissue hydroxyproline and α-smooth muscle actin (α-SMA) (12). However, the effect of BA on the high glucose-induced fibrotic response in CFs remains to be elucidated. Therefore, the present study investigated the effect of BA on high glucose-induced CFs and examined the possible mechanism underlying the effect of BA on CF transdifferentiation.

Materials and methods

Culture of cardiac fibroblasts. Neonatal Sprague-Dawley rats (age, 1-3 days; weight, 180-200 g) were obtained from the Animal Breeding Center of Henan Provincial People's Hospital (Zhengzhou, China) and were maintained at a constant temperature (21±2˚C) and 60% humidity in a holding facility under a 12-h light/dark cycle, with free access

to food and water. The CFs were isolated from neonatal rats as described previously (13). The cells were cultured in Dulbecco's modified Eagle's medium (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) containing 10% fetal bovine serum (Bio-Rad Laboratories, Inc., Hercules, CA, USA), 100 IU/ml penicillin and 100 μg/ml streptomycin (Sigma-Aldrich; Merck Millipore, Darmstadt, Germany). The CFs were maintained in growth media and incubated in humidified atmosphere of 5% CO_2 at 37˚C. All experimental protocols were approved by the Ethical Committee of Henan Provincial People's Hospital for Animal Care and Use (Henan, China).

Cell proliferation assay. Cell proliferation was measured using an MTT assay. Briefly, the CFs were seeded in 96-well culture plates at a density of $1x10^4$ cells per well. Following starvation in serum-free medium for 24 h, the CFs were pre-treated with various concentrations of BA (1, 5 and 10 μM) for 24 h at room temperature, and exposed to high glucose (25 mM). After 24 h, 20 μl of MTT solution (5 mg/ml; Sigma-Aldrich; Merck Millipore) was added to each well and incubated at 37˚C for 4 h, following which the culture medium was removed and 150 μl of DMSO (Sigma-Aldrich; Merck Millipore) was added. The absorbance was measured at 490 nm using a microplate spectrophotometer (Bio-Rad Laboratories, Inc.).

RNA isolation and reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis. Total RNA was isolated from the CFs using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.). Total RNA (~5 μg) was then subjected to TaqMan one-step reverse transcription (Applied Biosystems; Thermo Fisher Scientific, Inc.), followed by qPCR using an ABI PRISM 7700 sequence detection system (Applied Biosystems; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. RT-qPCR was performed in a final volume of 10 μl, which consisted of 5 μl SsoFast™ EvaGreen Supermix (Bio-Rad Laboratories, Inc.), 1 μl cDNA (1:50 dilution) and 2 μl each of the forward and reverse primers (1 mM). The primers used were as follows: α-smooth muscle actin (SMA) forward, 5'-GCTATT CAGGCTGTGCTGTC-3' and reverse, 5'-GGTAGTCGGTGA GATCTCGG-3'; transforming growth factor (TGF)-β1 forward, 5'-CCAACTATTGCTTCAGCTCCA-3' and reverse, 5'-GTG TCCAGGCTCCAAATGT-3'; and GAPDH forward, 5'-ACT CCCATTCTTCCACCTTTG-3' and reverse, 5'-CCCTGT TGCTGTAGCCATATT-3'. Thermocycling conditions were as follows: 94˚C for 2 min for initial denaturation; 94˚C for 20 sec, 59˚C for 15 sec, and 72˚C for 20 sec; 2 sec for plate reading (35 cycles); with a melt curve between 65 and 95˚C. GAPDH was used as an internal control. The relative expression levels of genes were calculated using control GAPDH mRNA and the $2^{-\Delta\Delta Cq}$ method (14).

Western blot analysis. The whole-cell proteins were collected in radioimmunoprecipitation assay lysis buffer (Beyotime Institute of Biotechnology, Shanghai, China) with protease inhibitor PMSF, and the protein concentration was quantified using a Bradford assay. Equal quantities of protein sample (20 μg) were separated via 12% SDS-PAGE and transferred onto nitrocellulose membranes (Amersham; GE Healthcare Life Sciences, Little Chalfont, UK). Following blocking with 5% non-fat dry milk at room temperature for 1 h, the membranes were incubated with primary antibodies against α-SMA (1:3,000; cat. no. PA5-19465; Invitrogen; Thermo Fisher Scientific, Inc.), TGF-β1 (1:2,000; cat. no. sc-146; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), Smad3 (1:2,500; cat. no. PA5-34774; Invitrogen; Thermo Fisher Scientific, Inc.), phosphorylated (p-)Smad3 (1:3,000; cat. no. 44-246G; Invitrogen; Thermo Fisher Scientific, Inc.) and GAPDH (1:3,000; cat. no. sc-25778; Santa Cruz Biotechnology, Inc.) overnight at 4˚C. The membranes were washed (3x5 min) in TBS containing 0.1% Tween-20 and incubated for 1 h at room temperature in the presence of horseradish peroxidase-conjugated secondary antibodies (1:5,000; cat. no. sc-2005; Santa Cruz Biotechnology, Inc.) diluted in blocking solution. Finally, the membranes were washed again with TBST, and the blots were examined using an enhanced chemiluminescence detection system (GE Healthcare Life Sciences, Uppsala, Sweden). The optical densities of the bands were quantified using Gel-Pro Analyzer version 4.0 (Media Cybernetics, Inc. Rockville, MD, USA).

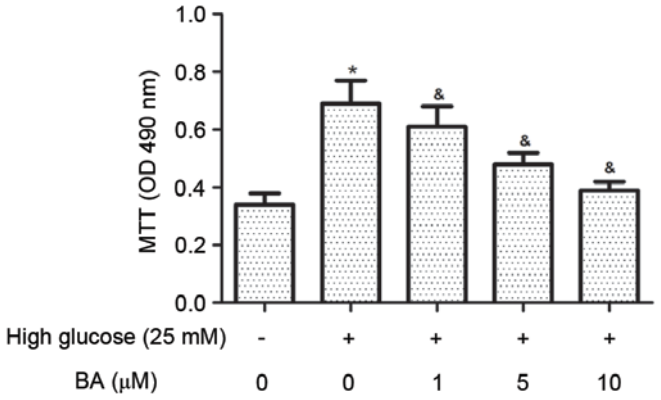

Figure 1. BA attenuates high glucose-induced CF proliferation. CFs were pre-incubated with BA (1, 5 and 10 μM) for 24 h and then stimulated with high glucose (25 mM) for 24 h. Cell proliferation was evaluated using an MTT assay. Data are presented as the mean ± standard deviation. The results were reproduced in three independent experiments. *P<0.05 vs. control group; &P<0.05 vs. high glucose group. CF, cardiac fibroblast; BA, betulinic acid; OD, optical density.

Statistical analysis. The results are expressed as the mean ± standard deviation of 3 independent experiments. Statistical analysis was performed using one-way analysis of variance followed by Tukey's multiple comparisons test using GraphPad Prism software version 5.01 (GraphPad software, Inc., San Diego, CA, USA). P<0.05 was considered to indicate a statistically significant difference.

Results

BA attenuates high glucose-induced CF proliferation. High glucose has been shown to promote the proliferation of CFs. The present study examined the effect of BA on CF proliferation induced by high glucose using an MTT assay. The results indicated that glucose at a high concentration significantly increased the proliferation of CFs, compared with glucose at a normal concentration. However, the high glucose induced-CF proliferation was significantly inhibited by BA treatment (Fig. 1).

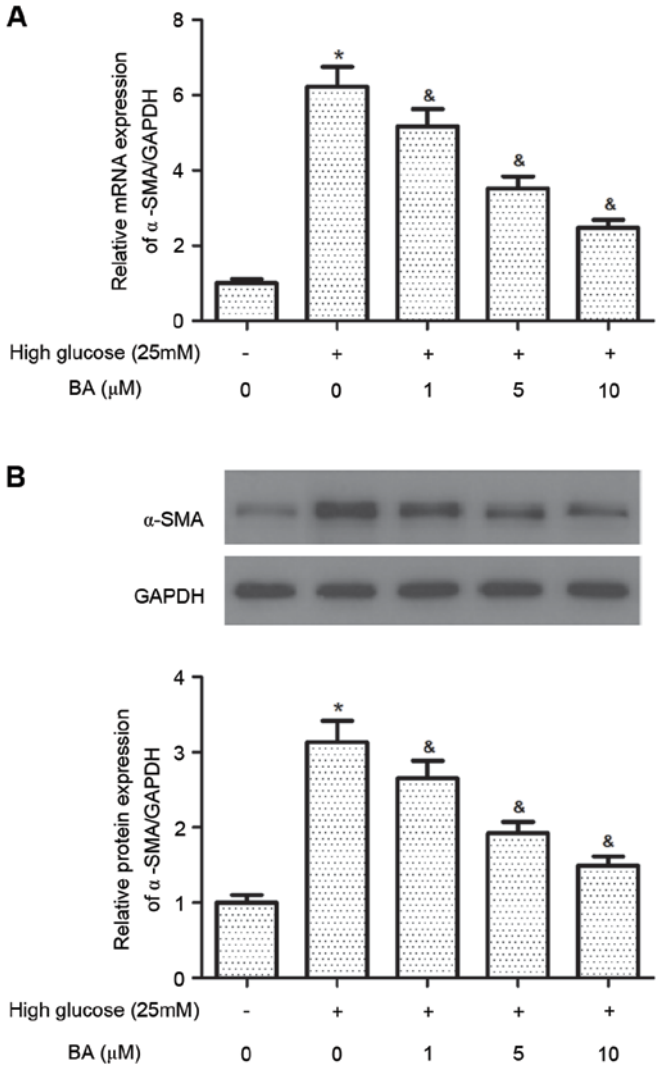

Figure 2. BA reduces the differentiation of CFs into myofibroblasts. CFs were pre-incubated with BA (1, 5 and 10 μM) for 24 h and then stimulated with high glucose (25 mM) for 24 h. (A) mRNA expression of levels of α-SMA were determined using reverse transcription-quantitative polymerase chain reaction analysis. (B) Protein expression levels of α-SMA were determined using western blot analysis. Data are presented as the mean ± standard deviation. The results were reproduced in three independent experiments. *P<0.05 vs. control group; &P<0.05 vs. high glucose group. CF, cardiac fibroblast; BA, betulinic acid; α-SMA, α-smooth muscle actin.

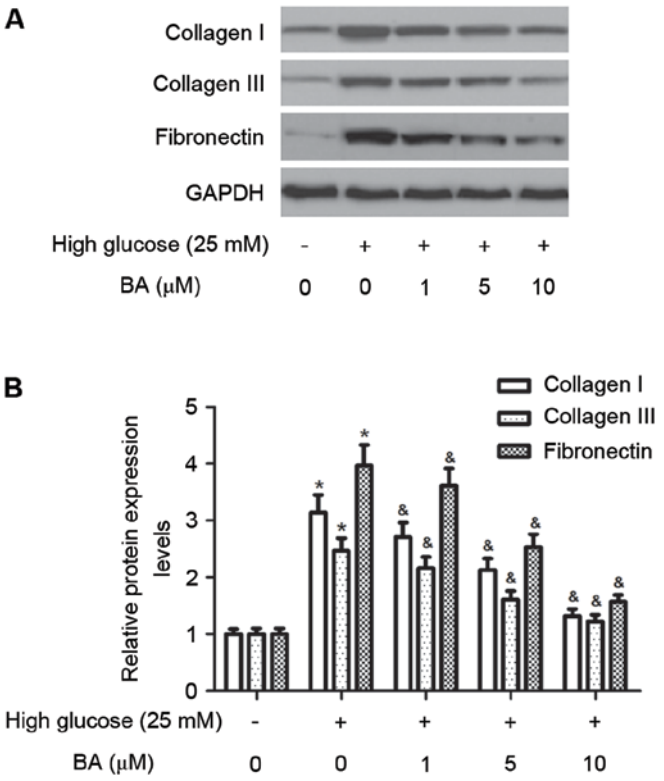

Figure 3. BA inhibits high glucose-induced expression of ECM in CFs. CFs were pre-incubated with BA (1, 5 and 10 μM) for 24 h and then stimulated with high glucose (25 mM) for 24 h. (A) Protein expression levels of collagen I, collagen III and fibronectin were determined using western blot analysis. (B) Relative expression levels of collagen I, collagen III and fibronectin were quantified using the Gel-Pro Analyzer v4.0 following normalization with GAPDH. Data are presented as the mean ± standard deviation. The results were reproduced in three independent experiments. *P<0.05 vs. control group; &P<0.05 vs. high glucose group. CFs, cardiac fibroblasts; BA, betulinic acid.

BA reduces the differentiation of CFs into myofibroblasts. The expression of α-SMA is a major morphological marker for myofibroblasts. Therefore, the present study evaluated the effect of BA on the expression of α-SMA in CFs stimulated by high glucose. The results of the RT-qPCR analysis demonstrated that the mRNA expression of α-SMA was significantly increased in the high glucose-treated CFs, compared with the control CFs. However, pre-treatment of the CFs with BA inhibited the high glucose-induced mRNA expression of α-SMA in the CFs (Fig. 2A). Consistent with the results of the RT-qPCR analysis, the results of the western blot analysis also indicated that BA inhibited the high glucose-induced increase of α-SMA in the CFs (Fig. 2B).

BA inhibits high glucose-induced expression of ECM in CFs. It has been reported that high glucose induces cardiac fibrosis by increasing the expression of ECM proteins, including collagen I, collagen III and fibronectin, therefore, the present study investigated the effect of BA on the expression of ECM proteins in CFs induced by high glucose. As shown in Fig. 3A and B, treatment of the cultured CFs with high glucose for 24 h caused significant increases in the protein levels of collagen I, collagen III and fibronectin. However, pre-treatment of the CFs with BA reduced the stimulatory effects of high glucose.

BA inhibits high glucose-induced expression of TGF-β1 in CFs. TGF-β1 can induce cardiac fibrosis by activating CFs. Thus, the present study investigated the effect of BA on the expression of TGF-β1 in CFs induced by high glucose. As shown in Fig. 4A and B, high glucose treatment significantly increased the expression of TGF-β1 at the mRNA and protein levels in the CFs, compared with the normal group. However, BA inhibited the high glucose-induced expression of TGF-β1 in the CFs in a dose-dependent manner.

BA inhibits high glucose-induced activation of the TGF-β1/Smad pathway in CFs. The TGF-β1/Smad signaling pathway is critical for the development of cardiac fibrosis. Therefore, the present study examined the effects of BA on

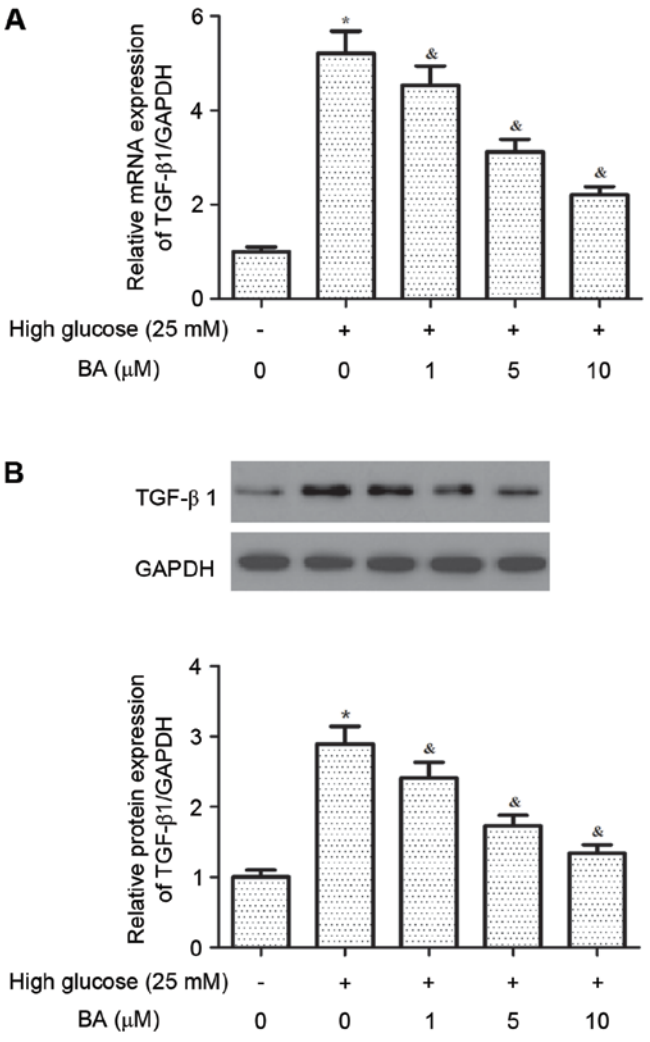

Figure 4. BA inhibits high glucose-induced expression of TGF-β1 in CFs. CFs were pre-incubated with BA (1, 5 and 10 μM) for 24 h and then stimulated with high glucose (25 mM) for 24 h. (A) mRNA expression levels of TGF-β1 were determined using reverse transcription-quantitative polymerase chain reaction analysis. (B) Protein expression levels of TGF-β1 were determined using western blot analysis. Data are presented as the mean ± standard deviation. The results were reproduced in three independent experiments. *P<0.05 vs. control group; &P<0.05 vs. high glucose group. CFs, cardiac fibroblasts; BA, betulinic acid; TGF-β1, transforming growth factor-β1.

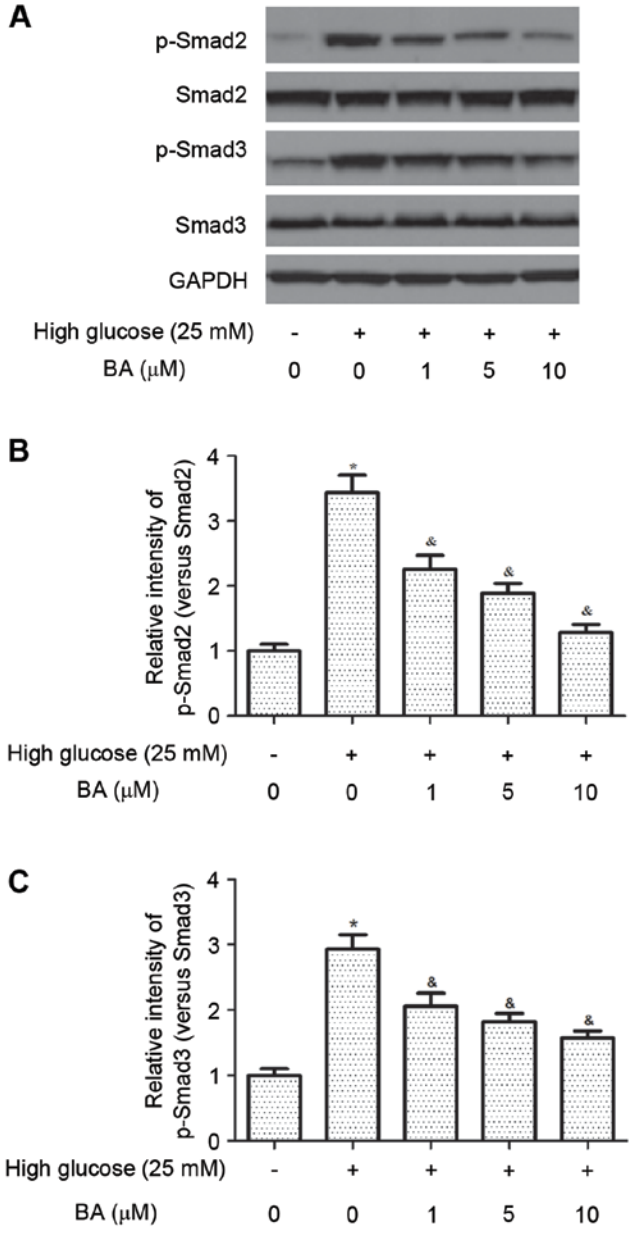

Figure 5. BA inhibits high glucose-induced activation of the TGF-β1/Smad pathway in CFs. CFs were pre-incubated with BA (1, 5 and 10 μM) for 24 h and then stimulated with high glucose (25 mM) for 1 h. (A) Expression levels of p-Smad2, p-Smad3 and Smad2/3 were detected using western blot analysis. Relative expression levels of (B) p-Smad2 and (C) p-Smad3 were quantified using the Gel-Pro Analyzer v4.0 following normalization with GAPDH. Data are presented as the mean ± standard deviation. The results were reproduced in three independent experiments. *P<0.05 vs. control group; &P<0.05 vs. high glucose group. CFs, cardiac fibroblasts; BA, betulinic acid; TGF-β1, transforming growth factor-β1; Smad, small mothers against decapentaplegic.

the levels of p-Smad2/3 in CFs. As shown in Fig. 5, the protein levels of Smad 2/3 were increased in CFs cultured in high glucose, which may facilitate the development of a pro-fibrotic phenotype. By contrast, BA inhibited the high glucose-induced phosphorylation of Smad2/3 in the CFs.

Discussion

It is well-known that hyperglycemia is an inducer of cardiac fibrosis, therefore, the present study cultured neonatal CFs with high glucose and observed the effect of BA on the high glucose-induced CFs, to elucidate the possible mechanism for BA on CF transdifferentiation. The results of the present study revealed that BA attenuated high glucose-induced CF proliferation and myofibroblast differentiation. In addition, BA inhibited the high glucose-induced expression of ECM proteins in the CFs. It was also found that the effect of BA on myofibroblast differentiation and the excessive expression of ECM involved the TGF-β1/Smad signaling pathway.

CF proliferation is the basic function in the response to pro-fibrotic stimuli, which can lead to excessive ECM production and subsequent cardiac fibrosis (15,16). Hyperglycemia was shown to promote the proliferation of CFs and these results are consistent with previous studies showing that CF proliferation was significantly increased by high glucose (17-19). In addition, the present study showed that BA significantly inhibited high glucose-induced fibroblast

proliferation. These data suggested that BA has a critical role in CF proliferation.

The differentiation of CFs into myofibroblasts and the exclusive deposition of ECM components, including α-SMA, collagen and fibronectin, are essential for the development of cardiac fibrosis (20). Previous evidence has suggested that the differentiation of CFs occurs in response to high glucose, TGF-β1 and angiotensin-II, which are important in this process (21,22). In the present study, it was found that the expression of α-SMA was significantly increased by high glucose. In addition, the results of the present study showed that BA prevented the high glucose-induced expression of α-SMA in the CFs. It was also found that BA inhibited the high glucose-induced levels of collagen I, collagen III and fibronectin in the CFs. These data suggested that BA is important in the phenotypic transformation of CFs into myofibroblasts.

TGF-β1 is known as a major stimulator of fibrous tissue deposition in the heart, and can induce cardiac fibrosis by activating fibroblasts and producing collagen. Inhibiting TGF-β1 effectively reverses CF transdifferentiation and reduces ECM deposition, and it may be a potential therapeutic target for cardiac fibrosis (23-25). In the present study, it was found that high glucose increased the protein levels of TGF-β1 in CFs. These observations are in agreement with those reported by Singh *et al* (26), in which neonatal rat CFs cultured in high glucose showed increased protein expression levels of TGF-β1. In addition, BA partially suppressed the high glucose-induced increases in the expression of ECM in the CFs. The Smad signaling pathway acts as a downstream mediator of TGF-β1. Smad2/3 translocate into the nucleus accompanied by Smad4. In the nucleus, this protein complex acts in conjunction with Sp1 and enhances the transcription of several genes, including collagen I, collagen III and fibronectin. In addition, it has been shown that high glucose increases the expression of TGF-β1 and induces activation of the TGF-β1/Smad signaling pathway, leading to upregulated expression of ECM in CFs (27). In accordance with these results, the results of the present study showed that the protein levels of Smad2/3 increased, whereas those of Smad7 were suppressed in CFs cultured in high glucose, which may facilitate the development of a pro-fibrotic phenotype. BA inhibited the high glucose-induced phosphorylation of Smad2/3 in the CFs. Thus, the findings of the present study suggested that BA suppressed the high glucose-induced increases in the proliferation of CFs and collagen synthesis, which may be associated with inhibition of the TGF-β/Smad signaling pathway.

In conclusion, the present study demonstrated that BA suppressed high glucose-induced increases in the proliferation of CFs and the expression of ECM via inhibition of the TGF-β/Smad signaling pathway. Thus, BA may offer therapeutic potential towards the treatment of cardiac fibrosis.

References

1. Kong P, Christia P and Frangogiannis NG: The pathogenesis of cardiac fibrosis. Cell Mol Life Sci 71: 549-574, 2014.
2. Krenning G, Zeisberg EM and Kalluri R: The origin of fibroblasts and mechanism of cardiac fibrosis. J Cell Physiol 225: 631-637, 2010.
3. Teunissen BE, Smeets PJ, Willemsen PH, De Windt LJ, Van der Vusse GJ and Van Bilsen M: Activation of PPARdelta inhibits cardiac fibroblast proliferation and the transdifferentiation into myofibroblasts. Cardiovasc Res 75: 519-529, 2007.
4. Fan D, Takawale A, Lee J and Kassiri Z: Cardiac fibroblasts, fibrosis and extracellular matrix remodeling in heart disease. Fibrogenesis Tissue Repair 5: 15, 2012.
5. Tian K, Liu Z, Wang J, Xu S, You T and Liu P: Sirtuin-6 inhibits cardiac fibroblasts differentiation into myofibroblasts via inactivation of nuclear factor κB signaling. Transl Res 165: 374-386, 2015.
6. Bugyei-Twum A, Advani A, Advani SL, Zhang Y, Thai K, Kelly DJ and Connelly KA: High glucose induces Smad activation via the transcriptional coregulator p300 and contributes to cardiac fibrosis and hypertrophy. Cardiovasc Diabetol 13: 89, 2014.
7. Tang M, Zhang W, Lin H, Jiang H, Dai H and Zhang Y: High glucose promotes the production of collagen types I and III by cardiac fibroblasts through a pathway dependent on extracellular-signal-regulated kinase 1/2. Mol Cell Biochem 301: 109-114, 2007.
8. Kasperczyk H, La Ferla-Brühl K, Westhoff MA, Behrend L, Zwacka RM, Debatin KM and Fulda S: Betulinic acid as new activator of NF-κB: Molecular mechanisms and implications for cancer therapy. Oncogene 24: 6945-6956, 2005.
9. Yogeeswari P and Sriram D: Betulinic acid and its derivatives: A review on their biological properties. Curr Med Chem 12: 657-666, 2005.
10. Pavlova N, Savinova O, Nikolaeva S, Boreko E and Flekhter O: Antiviral activity of betulin, betulinic and betulonic acids against some enveloped and non-enveloped viruses. Fitoterapia 74: 489-492, 2003.
11. Xia A, Xue Z, Li Y, Wang W, Xia J, Wei T, Cao J and Zhou W: Cardioprotective effect of betulinic acid on myocardial ischemia reperfusion injury in rats. Evid Based Complement Alternat Med 2014: 573745, 2014.
12. Wan Y, Wu YL, Lian LH, Xie WX, Li X, Ouyang BQ, Bai T, Li Q, Yang N and Nan JX: The anti-fibrotic effect of betulinic acid is mediated through the inhibition of NF-κB nuclear protein translocation. Chem-Biol Interact 195: 215-223, 2012.
13. Turner NA, Das A, Warburton P, O'Regan DJ, Ball SG and Porter KE: Interleukin-1alpha stimulates proinflammatory cytokine expression in human cardiac myofibroblasts. Am J Physiol Heart Circ Physiol 297: H1117-H1127, 2009.
14. 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.
15. Chen HN, Wang DJ, Ren MY, Wang QL and Sui SJ: TWEAK/Fn14 promotes the proliferation and collagen synthesis of rat cardiac fibroblasts via the NF-κB pathway. Mol Biol Rep 39: 8231-8241, 2012.
16. Driesen RB, Nagaraju CK, Abi-Char J, Coenen T, Lijnen PJ, Fagard RH, Sipido KR and Petrov VV: Reversible and irreversible differentiation of cardiac fibroblasts. Cardiovasc Res 101: 411-422, 2013.
17. Wang P, Li H, Wang Y, Chen H and Zhang P: Effects of recombinant human relaxin upon proliferation of cardiac fibroblast and synthesis of collagen under high glucose condition. J Endocrinol Invest 32: 242-247, 2009.
18. Neumann S, Huse K, Semrau R, Diegeler A, Gebhardt R, Buniatian GH and Scholz GH: Aldosterone and D-glucose stimulate the proliferation of human cardiac myofibroblasts in vitro. Hypertension 39: 756-760, 2002.
19. Zhou H, Zhang KX, Li YJ, Guo BY, Wang M and Wang M: Fasudil hydrochloride hydrate, a Rho-kinase inhibitor, suppresses high glucose-induced proliferation and collagen synthesis in rat cardiac fibroblasts. Clin Exp Pharmacol Physiol 38: 387-394, 2011.
20. Porter KE and Turner NA: Cardiac fibroblasts: At the heart of myocardial remodeling. Pharmacol Ther 123: 255-278, 2009.
21. Fedak PW, Bai L, Turnbull J, Ngu J, Narine K and Duff HJ: Cell therapy limits myofibroblast differentiation and structural cardiac remodeling basic fibroblast growth factor-mediated paracrine mechanism. Circ Heart Fail 5: 349-356, 2012.
22. Wang X, Gao G, Liu J, Guo R, Lin Y, Chu Y, Han F, Zhang W and Bai Y: Identification of differentially expressed genes induced by angiotensin II in rat cardiac fibroblasts. Clin Exp Pharmacol Physiol 33: 41-46, 2006.
23. Lim H and Zhu Y: Role of transforming growth factor-beta in the progression of heart failure. Cell Mol Life Sci 63: 2584-2596, 2006.

24. Bujak M and Frangogiannis NG: The role of TGF-beta signaling in myocardial infarction and cardiac remodeling. Cardiovasc Res 74: 184-195, 2007.
25. Kuwahara F, Kai H, Tokuda K, Kai M, Takeshita A, Egashira K and Imaizumi T: Transforming growth factor-beta function blocking prevents myocardial fibrosis and diastolic dysfunction in pressure-overloaded rats. Circulation 106: 130-135, 2002.
26. Singh VP, Baker KM and Kumar R: Activation of the intracellular renin-angiotensin system in cardiac fibroblasts by high glucose: Role in extracellular matrix production. Am J Physiol Heart Circ Physiol 294: H1675-H1684, 2008.
27. Liu J, Zhuo X, Liu W, Wan Z, Liang X, Gao S, Yuan Z and Wu Y: Resveratrol inhibits high glucose induced collagen upregulation in cardiac fibroblasts through regulating TGF-β1-Smad3 signaling pathway. Chem Biol Interact 227: 45-52, 2015.