

Betulinic acid prevents high glucose-induced expression of extracellular matrix protein in cardiac fibroblasts by inhibiting the TGF- β 1/Smad signaling pathway

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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)- β 1 were determined using reverse transcription-quantitative polymerase chain reaction analysis. The protein expression levels of α -SMA, collagen I, collagen III, fibronectin, TGF- β 1, 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- β 1/Smad signaling pathway. Thus, BA may offer therapeutic potential towards the treatment of cardiac fibrosis.

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 \pm 2°C) and 60% humidity in a holding facility under a 12-h light/dark cycle, with free access

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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 μ g/ml streptomycin (Sigma-Aldrich; Merck Millipore, Darmstadt, Germany). The CFs were maintained in growth media and incubated in humidified atmosphere of 5% CO₂ 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 1×10^4 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 μ M) for 24 h at room temperature, and exposed to high glucose (25 mM). After 24 h, 20 μ l 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 μ l 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 μ g) 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 μ l, which consisted of 5 μ l SsoFast™ EvaGreen Supermix (Bio-Rad Laboratories, Inc.), 1 μ l cDNA (1:50 dilution) and 2 μ l 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)- β 1 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 $2^{-\Delta\Delta C_q}$ 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 μ g) were separated via 12% SDS-PAGE and transferred onto nitrocellulose membranes (Amersham; GE Healthcare Life Sciences, Little Chalfont, UK). Following blocking

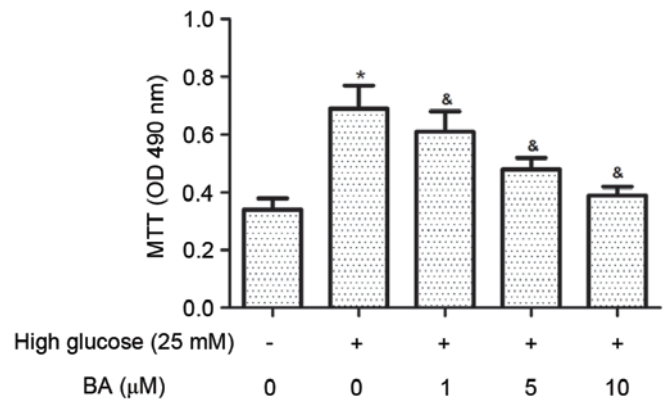


Figure 1. BA attenuates high glucose-induced CF proliferation. CFs were pre-incubated with BA (1, 5 and 10 μ M) for 24 h and then stimulated with high glucose (25 mM) for 24 h. Cell proliferation was evaluated using an MTT assay. Data are presented as the mean \pm standard deviation. The results were 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.

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- β 1 (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 horse-radish 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).

Statistical analysis. The results are expressed as the mean \pm 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).

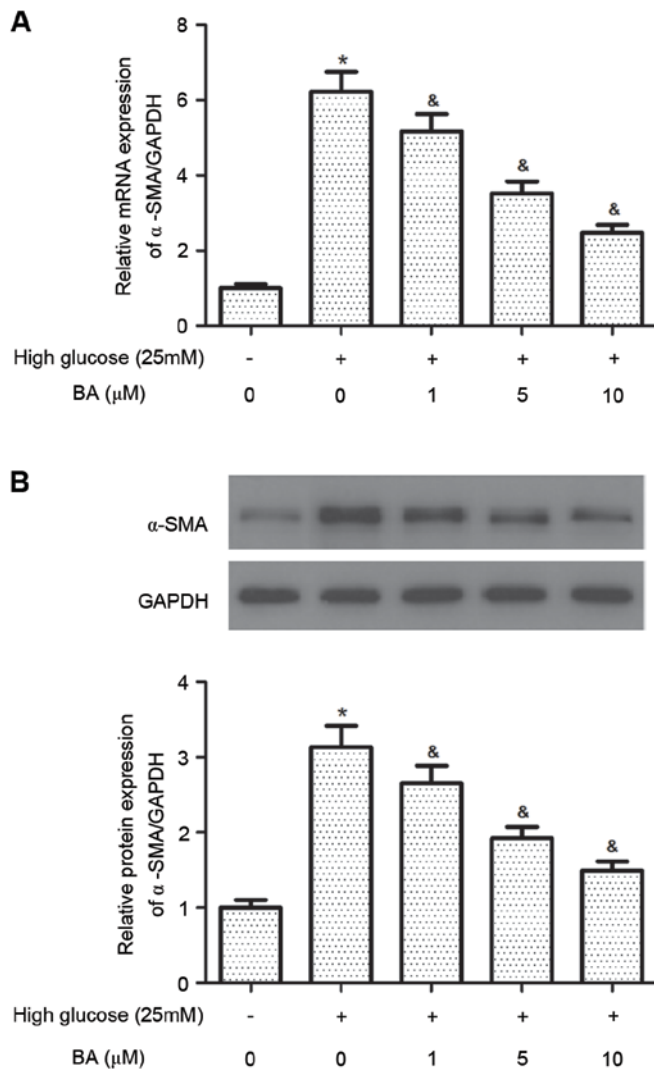


Figure 2. BA reduces the differentiation of CFs into myofibroblasts. CFs were pre-incubated with BA (1, 5 and 10 μM) for 24 h and then stimulated with high glucose (25 mM) for 24 h. (A) mRNA expression of levels of α-SMA were determined using reverse transcription-quantitative polymerase chain reaction 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.

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

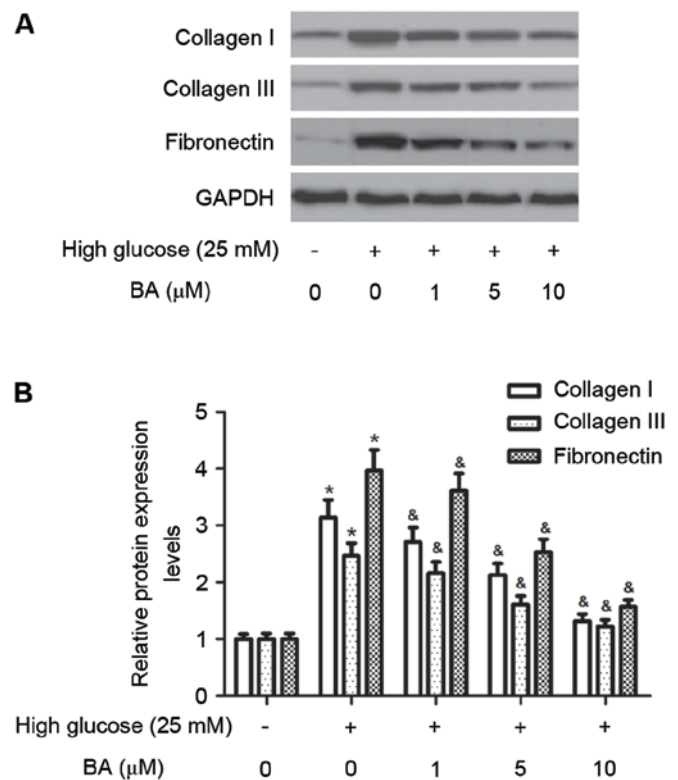


Figure 3. BA inhibits high glucose-induced expression of ECM in CFs. CFs were pre-incubated with BA (1, 5 and 10 μM) for 24 h and then stimulated with high glucose (25 mM) for 24 h. (A) Protein expression levels of collagen I, collagen III and fibronectin were determined using western blot analysis. (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.

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

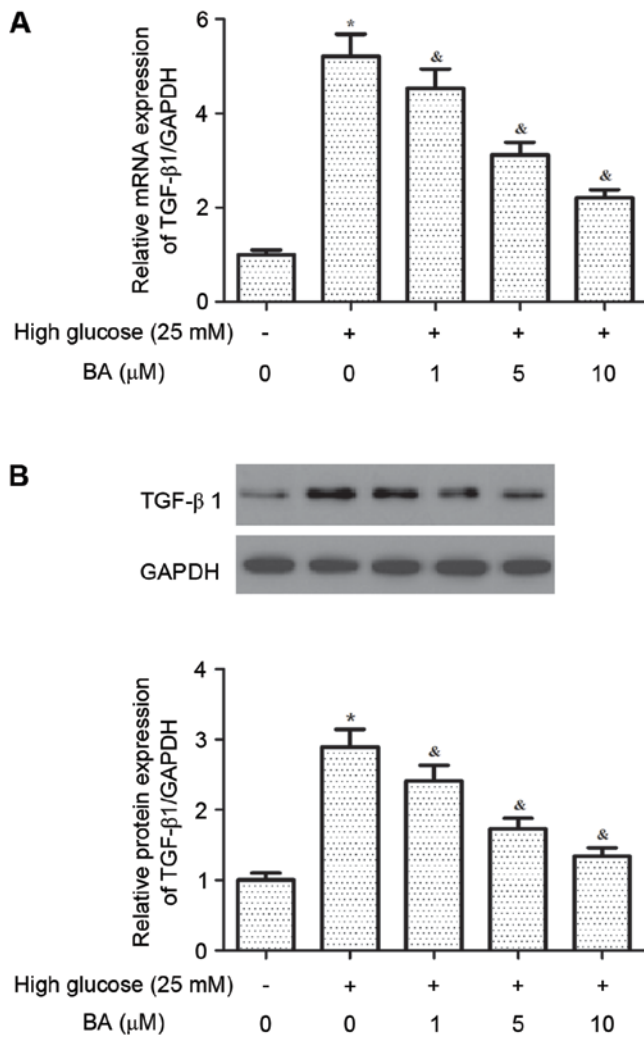


Figure 4. BA inhibits high glucose-induced expression of TGF- β 1 in CFs. CFs were pre-incubated with BA (1, 5 and 10 μ M) for 24 h and then stimulated with high glucose (25 mM) for 24 h. (A) mRNA expression levels of TGF- β 1 were determined using reverse transcription-quantitative polymerase chain reaction analysis. (B) Protein expression levels of TGF- β 1 were determined using western blot analysis. Data are presented as the mean \pm 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- β 1, transforming growth factor- β 1.

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

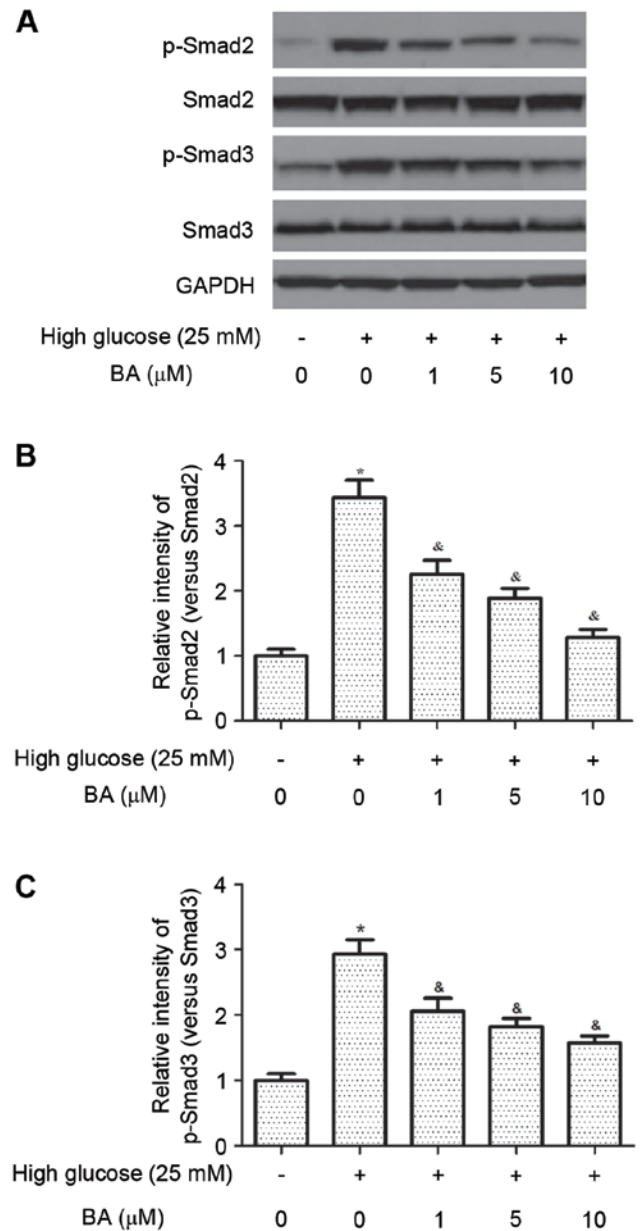


Figure 5. BA inhibits high glucose-induced activation of the TGF- β 1/Smad pathway in CFs. CFs were pre-incubated with BA (1, 5 and 10 μ M) for 24 h and then stimulated with high glucose (25 mM) for 1 h. (A) Expression levels of p-Smad2, p-Smad3 and Smad2/3 were detected using western blot analysis. 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 \pm 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- β 1, transforming growth factor- β 1; Smad, small mothers against decapentaplegic.

myofibroblast differentiation and the excessive expression of ECM involved the TGF- β 1/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- β 1 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- β 1 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- β 1 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- β 1 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- β 1. 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- β 1. 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- β 1 and induces activation of the TGF- β 1/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.

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