EXPERIMENTAL STUDY

Single-Stranded DNA-Binding Protein 1 Abrogates Cardiac Fibroblast Proliferation and Collagen Expression Induced by Angiotensin II

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Summary

Angiotensin II (Ang II), an effective component of renin-angiotensin system, plays a pivotal role in cardiac fibrosis, which may further contribute to heart failure. Single-stranded DNA-binding protein 1 (SSBP1), a DNA damage response protein, regulates both mitochondrial function and extracellular matrix remodeling. In this study, we aim to investigate the role of SSBP1 in cardiac fibrosis that is induced by Ang II. We infused C57BL/6J mice with vehicle or Ang II and valsartan using implanted osmotic mini-pumps. Moreover, heart function was examined by echocardiography and cardiac fibrosis was analyzed via picrosirus red staining. The expression of COL1A1, COL3A1, SSBP1, p53, Nox1, and Nox4 was analyzed via qRT-PCR and/or immunoblots. The SSBP1 expression was manipulated via SSBP1 shRNA and pcDNA3.1/SSBP1 plasmids, while the p53 expression was enhanced via AdCMV-p53 infection. The exposure to Ang II increased the mouse heart weight, systolic blood pressure, interventricular septal thickness diastolic (IVSTD) and left ventricular end posterior wall dimension diastolic (LVPWD), which were counteracted by valsartan. While cardiac fibrosis was induced with Ang II treatment, it was relieved using valsartan. Furthermore, Ang II treatment caused mitochondrial dysfunction, oxidative stress, and down-regulated SSBP1 expression. The knockdown of SSBP1 increased cardiac fibroblast proliferation, collagen expression, and decreased p53 expression, which was impeded via SSBP1 overexpression. Moreover, the forced expression of p53 abated the fibroblast proliferation and collagen expression that was induced by Ang II. To summarize, SSBP1 was down-regulated by Ang II and implicated in cardiac fibroblast proliferation and collagen expression partly via the p53 protein.

Key words: Heart failure, p53

During heart failure, there is an impairment of blood filling in or ejecting out of the ventricle. This cardiovascular disease affects a large number of people in China.1) Heart failure can be caused by ischemic heart disease, hypertension, cardiomyopathy, congenital heart disease, and other pathophysiological factors.2,3) To reduce hospitalization and mortality for patients, although obvious improvements have been made, additional effort is required to identify newer therapy targets for treating heart failure.

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Recently, researchers have identified that an imbalance in the synthesis and degradation of cardiac fibroblast collagen, an essential trigger for cardiac fibrosis, may ultimately result in structural and functional abnormalities for the heart.4,5) Several factors causing insidious collagen deposition in the heart, such as acute myocardial infarction, aging, pressure overload, volume overload, cardiomyopathy, toxic exposure, and metabolic diseases like diabetes, have been identified.6) Once cardiac fibroblasts are activated by these deleterious factors, they will further proliferate and differentiate into myofibroblasts.7) Moreover, myofibroblasts would express and secrete large amounts of pro-inflammatory factors, pro-fibrotic factors, matrix metalloproteinases, and collagen proteins.8) However, the activation of cardiac fibroblasts is regulated by multiple pro-fibrotic signaling pathways. Among them, the most well-known pathways are angiotensin II (Ang II) signaling and TGF-β signaling.9) Ang II is the effective component of the renin-angiotensin system (RAS) and plays a pivotal role in cardiac fibrosis and myocardial remodeling. Ang II functions through its two specific recep-
tors: Ang II type I receptor (AT1R) and Ang II type II receptor (AT2R), both of which are expressed within cardiomyocytes and fibroblasts.\(^ {9,10}\) Activation of AT1R induces vasoconstriction, inflammation, and proliferation, while activation of AT2R will cause vasodilation.\(^ {9}\) For cardiac fibroblast activation and differentiation, TGF-β and its downstream effectors can create an induction context. TGF-β signaling can be activated via Ang II and is responsible for extracellular matrix remodeling.\(^ {11,12}\) The non-myocyte activation of TGF-β signaling significantly contributes to the fibrosis in diabetic dysfunction and heart failure.\(^ {12}\) Furthermore, for TGF-β sensitivity in lung cancer cells\(^ {17}\) and blockage of SSBP1-induced mitochondria dysfunction and enhanced radiosensitivity in lung cancer cells\(^ {17}\) and blockage of SSBP1 acetylation-reduced HeLa cell viability in response to DNA damage by radiation or chemotherapy drugs.\(^ {18}\) Furthermore, in the higher expression of N-cadherin and fibronectin, SSBP1 has been reported to suppress TGF-β/Smad3 signaling pathway and its deficiency results, which indicates that SSBP1 might play a role for cardiac fibroblast activation.\(^ {19}\) To validate this possibility, in this study, we sought to explore the expression and role of SSBP1 in Ang II-treated animal models and cardiac fibroblasts in vitro to understand the mechanism of cardiac fibrosis.

**Methods**

**Animals:** Male C57BL/6j mice, aged 10 weeks, were purchased from the Shanghai SLAC Laboratory Animal Co., Ltd (Shanghai, China). These animals were housed at a constant temperature/humidity environment with a 12 hours/12 hours light/dark cycle. The animal experiments were performed in accordance with the guidelines of the Institute for Laboratory Animal Research at our hospital. The experimental procedures were approved by the Administrative Committee of Experimental Animal Care and Use of Nanfang Hospital, Southern Medical University.

**Establishment of the mouse model:** The C57BL/6j mice were subcutaneously implanted using an osmotic minipump. The mice in the model group (\( n = 11 \)) and valsartan group (\( n = 11 \)) were infused with Ang II with a dosage of 1.44 μg/g/day (diluted in 10 mM acetic acid) for 2 weeks. The mice of the sham group (\( n = 12 \)) were infused with an equal volume of acetic acid; moreover, the mice in the valsartan group were given valsartan diluted in water (40 mg/kg/day) after Ang II infusion, whereas the mice in the model group were fed with only water.

**Echocardiography:** After Ang II infusion, heart function of the mice in all three groups was examined using echocardiography. An echocardiographic system was used to measure heart rate, interventricular septal thickness diastolic (IVSTD), left ventricular end posterior wall dimension diastolic (LVPWD), fractional shortening (FS) and ejection fraction (EF), via a 12-MHz transducer. The systolic blood pressure of the mice was detected using tail-cuff methods.

**Picrosirus red staining:** After Ang II and valsartan treatment, the mice were sacrificed and the hearts were immediately harvested with the heart tissues being fixed in 4% paraformaldehyde. After decalcification and dehydration, the heart samples were embedded in paraffin and cut as 5-μm sections, and the sections were rehydrated and stained using picrosirus red dye (#36324ES60, Yeasen, Shanghai, China). The stained collagen was observed with an inverted microscope in a blinded manner, and the staining area was quantified by Image J software (NIH, Bethesda, MA, USA).

**Fibroblasts culturing:** The mouse adult cardiac fibroblasts (MCFs) were isolated based on a previous study.\(^ {20}\) Briefly, the hearts of the adult mice were minced and plated into gelatin-coated dishes and cultured for 14 days. The explants were cultured in Iscove’s Modified Dulbecco’s Medium supplemented with 20% fetal bovine serum (FBS). The migrated fibroblasts were harvested and cultured in Dulbecco’s Modified Eagle’s medium (DMEM) containing 10% FBS and 1% Penicillin-Streptomycin. The NIH 3T3 cells were provided by American Type Culture Collection and cultured in DMEM medium containing 10% FBS and 1% Penicillin-Streptomycin. The cells were passed for three passages, which were used for the following experiments. For Ang II treatment, the MCFs and 3T3 cells were treated 0.0, 0.5, and 1.0 μmol/L Ang II for 12 hours. To block the activity of AT1R, the MCFs and 3T3 cells were pretreated with 1.0 μmol/L valsartan for 12 hours before treatment with Ang II (1.0 μmol/L).

**Vectors’ transfection:** SSBP1 shRNA was synthesized by Genepharma (Shanghai, China) to silence the SSBP1’s expression in cardiac fibroblasts. The target sequence of SSBP1 shRNA was 5’-GCA CAG AAT ATC AGT GTT TCG-3’. The pcDNA3.1/SSBP1 plasmids were purchased from Genepharma (Shanghai, China) and the Ad-p53-GFP vector was provided by Vector Biolabs (#1260, Malvern, PA, USA). The fibroblasts were allowed to grow to 80% confluence before plasmid transfection. The transfection of pcDNA3.1/SSBP1 into fibroblasts was performed using lipofectamine 2000 using the manufacturer’s protocol (#11668027, Thermo Fisher). For SSBP1 shRNA infection, the lentivirus was packed, collected, and incubated with fibroblasts at a concentration of 8 μg/mL. However, for adenovirus infection, the cardiac fibroblasts were incubated with 5 MOI Ad-p53-GFP or control.

**Measurement of the activities of mitochondrial complexes:** Mitochondria were isolated and purified using a previously described method.\(^ {21}\) The heart tissue was homogenized in an isolation buffer (225 mmol/L mannitol, 75 mmol/L sucrose, 1 mmol/L EGTA, 5 mmol/L Hepes (pH 7.3), and 2 mg/mL fat-free BSA) and centrifuged at 1,000 g for 10 minutes. Then, the supernatant was placed with a layered Ficoll gradient (5 mL of 7.5% Ficoll medium, 5 mL of 10% Ficoll medium containing 0.3 mol/L sucrose, 50 μmol/L EGTA, and 10 mmol/L Hepes) and centrifuged at 79,000 g for 30 minutes. The isolated mitochondria were re-suspended in an isolation buffer. Using a microplate assay, complex I and complex III’s enzyme ac-
tivity was determined (#ab109721, Abcam).

**Determination of antioxidant enzymes:** The content of superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px) in the mouse hearts was examined using a biochemical analysis kit based on the manufacturer’s protocol (SOD, #S0109, and GSH-Px, #S0056, Beyotime Biotechnology). The experiment was repeated at least three times.

**Evaluation of fibroblast viability:** The viability of cardiac fibroblasts was evaluated using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (#13154, Thermo Fisher). Briefly, cardiac fibroblasts were placed in 24-well plates and treated. After this, the cells were incubated with 0.5 mg/mL MTT solution for 4 hours and the reaction ended with the addition of 1 mL dimethylsulfoxide (DMSO, #D8418, Sigma). Lastly, the absorbance of each well was detected using a microplate reader (490 nm).

**Real-time qPCR:** Using Trizol reagent (#15596026 Invitrogen, Carlsbad, CA, USA), the total RNA was extracted from heart tissues or cardiac fibroblasts. The RNA’s concentration and quality was detected by Nanodrop (Thermo Fisher). The membranes were blocked with 5% BSA, and then incubated with indicated primary antibodies, followed by horseradish peroxidase-conjugated secondary antibodies (1:10000, #58802, Cell Signaling Technology, Danvers, MA, USA). The primary antibodies included COL1A1 (1:1200, #NB600-408, Novus Biologicals, Littleton, CO, USA), COL3A1 (1:1200, #NB600-594, Novus Biologicals), Nox1 (1:1000, #NBPI-31546, Novus Biologicals), Nox4 (1:1000, #NB110-58849, Novus Biologicals), SSBP1 (1:1000, #NBPI-80720, Novus Biologicals), p53 (1:1000, #NB200-103, Novus Biologicals), and GAPDH (1:1200, #NB300-221, Novus Biologicals).

**Statistical analysis:** All our results are presented as Means ± SD. One-way ANOVA, followed by Tukey’s test was performed to compare the differences between three or more groups using the SPSS13.0 software (SPSS Inc., Chicago, IL, USA). When P < 0.05, the difference was considered statistically significant.

**Results**

**Functional alterations in mice with Ang II infusion:** The alterations in body weight, heart weight, and heart function in mice with different treatments were determined. Compared with the control (n = 12), the body weight of mice in the model group (n = 11) and valsartan group (n = 11) varied insignificantly; however, compared with control and valsartan group, the heart weight of the model group higher. Furthermore, there was no significant difference observed for the heart rate in mice of these three groups. In the model group, mice had a higher systolic blood pressure compared to the control, while valsartan treatment relieved the increase in systolic blood pressure. The IVSTD and LVPWD were the two critical parameters that had ventricular function. IVSTD and LVPWD increased in the mouse model and decreased by valsartan infusion; moreover, FS and EF were compared. However, there was no difference observed between the three groups of mice. These results showed that Ang II could induce morphological and functional alterations in the mouse hearts, while valsartan could relieve them (Table).

**Ang II infusion causes cardiac fibrosis in mice:** To investigate effects of Ang II on cardiac fibrosis, heart sections from control, model, and valsartan group were stained with picrosirius red. Figure 1A, B show that Ang II infusion enhanced collagen deposition in the mouse heart, while valsartan attenuated collagen deposition. Subsequently, the expression of COL1A1 and COL3A1 was
Figure 1. Angiotensin II (Ang II) induces cardiac fibrosis in the mouse model. A: Representative image of mouse heart sections stained by picrosiris red. Scale bar = 40 μm. B: Collagen staining areas were quantified Image J software. C: Collagen Type I Alpha 1 Chain (COL1A1) and Collagen Type III Alpha 1 Chain (COL3A1) mRNA expression was analyzed by qRT-PCR. D, COL1A1, and COL3A1 protein expression was analyzed by Western blot. *P < 0.05, **P < 0.01.
determined and Ang II enhanced COL1A1, COL3A1 mRNA, and protein expression, which was impeded using valsartan (Figure 1C, D). These results suggested induction cardiac fibrosis by Ang II infusion in mice.

SSBP1 expression in heart after Ang II infusion: Previously, mitochondrial dysfunction has been demonstrated to contribute to heart failure. Therefore, the function of cardiac mitochondria for mice infused with Ang II was examined. The results demonstrated that the activities of citrate synthase, complex I, and complex III were reduced after Ang II treatment (Figure 2A-C). However, valsartan alleviated the suppression of citrate synthase activity, complex I activity, and complex III activity (Figure 2A-C). Moreover, the mouse heart’s redox state was examined and the expression of Nox1 and Nox4 in mice was analyzed using qRT-PCR. Compared with the control, Ang II increased the mRNA expression of Nox1 and Nox4, while valsartan blocked the increase in Nox1 and Nox4 expression (Figure 2D). The two critical antioxidant enzymes were SOD and GSH are. Our results demonstrated that SOD levels and GSH-Px levels in Ang II-treated mice were decreased; however, they were augmented using valsartan (Figure 2E, F). In response to DNA damage caused by oxidative stress in mitochondria, SSBP1 is an essential protein. Furthermore, SSBP1 mRNA and protein expression was down-regulated by Ang II and restored by valsartan treatment (Figure 2G-I). These results indicated the disruption of mitochondrial function by Ang II in the mouse heart.

Ang II down-regulates SSBP1 expression in cardiac fibroblasts: To investigate Ang II’s effect on cardiac fibroblasts, mouse adult cardiac fibroblasts (MCFs) and mouse embryo fibroblast-NIH 3T3 cells were challenged with Ang II (0.0, 0.5, or 1 μmol/L) and valsartan (1 μmol/L). The viability of MCFs and 3T3 cells was promoted by exposure to Ang II for 24 hours, the effect of which was blocked by valsartan pretreatment (Figure 3A). In parallel, the expression of Nox1, Nox4, COL1A1, and COL3A1 was increased by Ang II and recovered by valsartan (Figure 3B, C). Importantly, for both cell lines, SSBP1 expression was down-regulated in fibroblasts with Ang II incubation and restored in cells exposed to valsartan (Figure 3B, C). These results indicate that Ang II can accelerate cardiac fibroblast proliferation and abate SSBP1 expression.

Involvement of SSBP1 in the regulation of cardiac fibroblast proliferation and collagen expression induced by Ang II: SSBP1 is involved in cancer cell transformation and negatively regulates cell migration. However, the role of SSBP1 in fibroblasts treated with Ang II is
elusive. In MCFs and 3T3 cells SSBP1 expression was silenced by SSBP1 shRNA transfection; moreover, the knockdown of SSBP1 expression remarkably increased cell viability of fibroblasts with Ang II exposure (Figure 4 A, B). In MCFs and 3T3 cells, SSBP1 shRNA reduced SSBP1 protein expression (Figure 4C, D). Simultaneously, the expression of COL1A1 and COL3A1 was increased after SSBP1 knockdown (Figure 4E, F); however, Nox1 and Nox4 expression was not affected via SSBP1 silencing (Figure 4E, F). In contrast, in these cells, SSBP1 vector transfection reduced fibroblasts’ viability (Figure 5A, B); moreover, SSBP1 vector transfection increased SSBP1 protein expression but reduced COL1A1 and COL3A1 expression (Figure 5C-F).

P53 mediates the function of SSBP1 in cardiac fibroblasts: P53, a transcription factor, plays an important role for maintaining normal mitochondrial function and affects cell proliferation and differentiation. Because SSBP1 modulates p53 downstream gene expression, we suspected that p53 mediates SSBP1 function in fibroblasts. Thus, once SSBP1 expression in fibroblasts was knocked down by SSBP1 shRNA, p53 protein expression was reduced while p53 mRNA expression remained stable (Figure 6A-D). Unlike SSBP1 knockdown, SSBP1 overexpression enhanced p53’s protein expression (Figure 6E, F). Furthermore, the overexpression of p53 using the Ad-p53-GFP recombinant virus in fibroblasts increased p53’s protein expression, reduced cell viability, and the collagen expression induced by Ang II (Figure 7A-F). These results suggest that p53 may mediate SSBP1’s function in fibroblast activation.

Discussion

In many studies, the activation of RAS resulting in severe heart failure has been documented. In mice, a long term low-dose Ang II treatment caused left ventricular dysfunction and heart failure. Ang II receptor’s blockade prevented cardiac hypertrophy and improved cardiac remodeling in experimental heart failure. Researchers have reached a consensus that, for the therapy of heart failure with reduced EF, inhibition of RAS activation by angiotensin receptor inhibitors is essential. Moreover, in the rat heart, blocking AT1R with valsartan attenuated
Figure 4. The effect of SSBP1 knockdown on fibroblast cell proliferation and collagen expression in the presence or absence of Ang II. A, B: Cell viability of MCFs and 3T3 cells with SSBP1 knockdown. C, D: Analysis of SSBP1 protein expression in MCFs and 3T3 cells after the transfection of SSBP1 shRNA. E, F: Evaluation of COL1A1, COL3A1, Nox1, and Nox4 protein expression in MCFs and 3T3 cells after the knockdown of SSBP1 expression.

*P < 0.05, **P < 0.01.

TGF-β/smad signaling and fibrosis-related protein expression. Our study demonstrated that Ang II altered heart weight, systolic blood pressure, IVSTD, and LVPWD in mouse models. The effect of Ang II was blocked by valsartan, indicating that Ang II exerted its influence on the mouse heart via AT1R. Previously, a study showed that heart rate augmentation was closely associated with increase in all-cause mortality; however, our study did not detect any alterations in heart rate for mice treated with Ang II.

Note that Ang II is an important pro-fibrotic factor implicated in adverse cardiac fibrosis, which may further develop into heart failure under pathophysiologic conditions. Thus, chronic exposure to Ang II activated cardiac fibroblasts and increased the expression and deposition of collagens. In this study, our results showed that Ang II promoted collagen deposition and fibrosis in the heart. Furthermore, COL1A1 and COL3A1 expression in heart
tissues was enhanced by Ang II, whereas cardiac fibrosis was relieved by AT1R blockade. Mitochondria play a central role for energy production, Ca\(^{2+}\) homeostasis maintenance, redox balance, and signaling transduction. The generation of mitochondrial superoxide modulated cardiac extracellular matrix remodeling and contributed to cardiac fibrosis. Also, the present study demonstrated that Ang II caused mitochondria dysfunction in mouse hearts, along with a higher oxidative stress in heart tissues. These results indicated that mitochondria dysfunction in myocytes was correlated with cell death and inflammation. However, for fibroblast proliferation and collagen expression, reactive oxygen species accumulation induced by mitochondria dysfunction served as an activator.

To some extent, Ang II exposure increased cardiac fibroblast proliferation and activation in vitro. In fibroblasts with Ang II incubation, the expression of NAD(P)H oxidases and collagens was prompted. In line with the results.
in vivo, in these fibroblasts, SSBP1 expression was reduced after Ang II incubation but was restored by valsartan. Normally, SSBP1 is supposed to be present in the mitochondrial compartment. However, under heat shock conditions, SSBP1 was observed to translocate into the nucleus and regulate downstream gene expression through HSF1 interaction. Furthermore, the Epstein-Bar virus infection caused the translocation of SSBP1 into the nucleus. Therefore, we suspected that Ang II may trigger SSBP1 translocation, resulting in cardiac fibroblast activation. When SSBP1 expression was knocked down, the viability of cardiac fibroblasts was increased despite the absence of Ang II. Moreover, the expression of collagens was promoted, while NAD(P)H oxidases were not affected after inhibition of SSBP1 expression. Our study demonstrated that the regulatory role of SSBP1 in collagen expression was induced by Ang II. Previously, a study had demonstrated a protective role of SSBP1 in p53 protein stability by affecting p53 acetylation. However, in this study, we showed that silencing SSBP1 expression re-
duced p53 protein expression. Note that SSBP1 inhibition did not produce any effect on p53 mRNA expression, suggesting that SSBP1 might not regulate p53 transcriptional activity. Also, the results of SSBP1 overexpression further supported that p53 protein expression could be regulated via SSBP1. In fact, the overexpression of p53 markedly impeded the effect of Ang II on cardiac fibroblasts. Ghosh, et al. reported that p53 repressed the expression of basal collagen and collagen stimulated by TGF-β; hence, p53 could act as one effector of SSBP1 and was involved in regulating cardiac fibrosis.

Overall, in the current study, we characterized the role of SSBP1 in cardiac fibrosis induced by Ang II. Moreover, SSBP1 partially modulated fibroblast proliferation and collagen expression via the p53 protein. In patients with heart failure, the expression and function of SSBP1 needs to be examined further. Importantly, our study may provide a potential target for alleviating cardiac fibrosis.

Figure 7. p53 expression affects fibroblast proliferation and collagen expression in the presence or absence of Ang II. A, B: Cell proliferation of MCFs and 3T3 cells with p53 overexpression by Ad-p53-GFP. C, D: Analysis of SSBP1 protein expression in MCFs and 3T3 cells after Ad-p53-GFP infection. E, F: Evaluation of COL1A1 and COL3A1 expression in MCFs and 3T3 cells with p53 overexpression. *P < 0.05, **P < 0.01.
fibrosis and treating heart failure.

Disclosures

Conflicts of interest: The authors declare there is no conflict of interest.

References