Inhibition of FABP4 attenuates cardiac fibrosis through inhibition of NLRP3 inflammasome activation

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INTRODUCTION

Cardiac fibrosis is a pathological extracellular matrix (ECM) remodeling process. The condition is caused due to disrupting cardiac morphology, abnormal ECM accumulation, and impaired heart muscle function (1-3). Previous studies reported that ECM deposition could be a protective and beneficial mechanism for inflammation healing and tissue regeneration. However, excessive and spontaneous ECM deposition, specifically collagen type I (COL1A1) and collagen type III (COL3A1) in Ang II-infused mice. BMS309403 also reduced the number of α-SMA, MMP-2, MMP-9, and transforming growth factor-β (TGF-β) in ventricular tissue.

CONCLUSION: The inhibitory effect of BMS309403 on cardiac fibrosis might be associated with inhibition of NLRP3 inflammasome activation, which Ang II activated. Thus, our data speculated that inhibition of FABP4 could significantly induce cardiac fibrosis.

Introduction

Cardiac fibrosis is a pathological extracellular matrix (ECM) remodeling process. The condition is caused due to disrupting cardiac morphology, abnormal ECM accumulation, and impaired heart muscle function (1-3). Previous studies reported that ECM deposition could be a protective and beneficial mechanism for inflammation healing and tissue regeneration. However, excessive and spontaneous ECM deposition, specifically collagen type I secretion, may lead to impaired tissue function (4).

In cardiac fibrosis, inflammatory response plays a crucial role. Nucleotide-binding domain and leucine-rich repeat-containing family, pyrin domain-containing-3 (PYD-3), or Nod-like receptor protein 3 (NLRP3) inflammasome are an essential defining containing NLRP3 receptor, an apoptosis-associated speck-like protein consisting of a caspase recruitment domain (ASC) and precursor caspase-1 (5, 6). NLRP3 inflammasome is well characterized by its pathophysiological activities and clinical implications (7, 8). Unusually, it can be activated by different stimuli, including nigericin (Streptomyces hygroscopicus secreted, an antibiotic) or ATP released from damaged cells (7). However, the unregulated NLRP3 activation could lead to unconstrained infections, neurodegenerative diseases, autoimmune diseases, metabolic disorders, and other human disorders (9). Therefore, the interaction between NLRP3 and stimuli, and thus the mechanism by which NLRP3 is stimulated, remains unknown.

Fatty acid-binding protein 4 (FABP4) is a member of the FABP family and was first detected in mature adipocytes and adipose tissue, and is closely related to inflammation (10, 11). FABP4 plays a crucial role in insulin resistance, diabetes mellitus (type 2), gestational diabetes, and other metabolic syndromes (12-15). It has been further reported that FABP4 is associated with atherosclerosis and cardiovascular diseases (16, 17). Recent studies reported that FABP4 mRNA expression is higher in heart failure patients than in normal patients and is linked with heart failure severity. Thus, FABP4 is associated with heart failure (18). This evidence indicates that inhibition of FABP4 may attenuate cardiac fibrosis.

Our study identified FABP4 as a potential regulator of cardiac fibrosis because the expression of FABP4 was increased in the serum of cardiac fibrosis and Ang II-treated experimental mice. The results indicated that FABP4 inhibitor BMS309403 improved cardiac structure and function, attenuated cardiac and inflammatory response, and decreased collagen deposition and mRNA expression in Ang II-infused mice. BMS309403 also reduced the number of α-SMA+ cells and reduced the mRNA expression of α-SMA, MMP-2, MMP-9, and TGF-β in ventricular tissue. The above results speculated that FABP4 could be a possible target for the treatment of cardiac fibrosis as well as a diagnostic biomarker of cardiac fibrosis.
**Materials and Methods**

**Animals and treatment**

Male C57BL/6 mice (8-9 weeks old, 24-26 g) were used to establish the cardiac fibrosis model. This study was approved by the Animal Care and Use Committee of our Hospital. All experimental procedures were conducted following the Guide for the Care and Use of Laboratory Animals. Mice were infused with saline or Ang II to induce cardiac fibrosis by osmotic mini-pumps (2.8 mg/kg/day) for 4 weeks. For FABP4 inhibitor treatment, mice were intraperitoneally injected with either DMSO or BMS309403 (50 mg/kg, once a day) for 28 consecutive days. Mice were anesthetized with 2% isoflurane administration, mice underwent echocardiography (Vevo 3100 Imaging System) to assess the cardiac structure and function. Mice were anesthetized with 2% isoflurane and placed on a warming pad. Electrocardiogram (ECG) was used to monitor the heart rate and rhythm. Transthoracic echocardiography was performed, and Doppler echocardiographic images were taken at the mid-papillary level with a 30 MHz transducer. LV mass and LVID were measured using a two-dimensional M-mode in the parasternal short-axis view. Cardiac output (CO) was calculated as follows: cardiac output= (LVIDd - LVIDs)×heart rate, where LVIDd and LVIDs represent LV internal diameter in diastole and systole, respectively.

**Histology and immunohistochemistry**

Mice were anesthetized with pentobarbital sodium, decapitated, and the ventricular tissue was fixed with 4% paraformaldehyde, paraffin-embedded, and cut serially into sections (thickness 5 μm). Tissues were ascended on glass slides and deparaffinized. Slides were stained with hematoxylin-eosin for histology and Masson’s trichrome for collagen fiber. For immunohistochemistry, slides were incubated with rabbit antibodies against FABP4 (ab92501; Abcam, UK) or α-SMA antibody (ab5694; Abcam, UK) at 37 °C, followed by biotinylated HRP conjugated secondary antibody. The slides were washed and counter-stained. Images were photographed (×200 magnification). The collagen volume fraction was measured and expressed as the ratio of the connective tissue area to the total tissue area that was averaged from 10 images.

**Statistical analysis**

Data were expressed as the mean±SD and were analyzed using the SPSS software package (version 20.0, SPSS, Inc., USA). Comparisons among groups were analyzed using one-way ANOVA. A two-tailed t-test was used to compare two groups, and one-way ANOVA was used to compare three groups, followed by Student-Newman-Keuls tests. P<0.05 was considered statistically significant.

**Measurement of serum cytokines**

Enzyme-Linked Immunosorbent Assays (ELISA) determined the cytokine levels in the serum of mice. The blood sample was obtained from the caudal vein of mice after 4 weeks of Ang II infusion, and serum was isolated and stored at -80 °C. Levels of TNF-α (MTA00B), IL-6 (M6000B), and TGF-β (DB100B) were measured with commercial ELISA kits (R&D Systems, MN, USA).

**Western blot analysis**

Proteins were extracted from ventricular tissue and quantified. Total protein (50 μg) was separated by SDS-PAGE and transferred onto PVDF membranes. With 5% skimmed milk, the membranes were blocked and incubated with primary antibodies against FABP4 (1:500; ab92501; Abcam, UK), TGF-β (DB100B) were measured with commercial ELISA kits (R&D Systems, MN, USA). The 2-ΔΔCt method was used to calculate the mRNA levels of each gene after normalizing to GAPDH. The primer sequences were used in this study listed in Table 1.

**Real-time quantitative polymerase chain reaction (RT-qPCR)**

Total RNA was extracted from ventricular tissue with TRizol reagent (Invitrogen, USA) and was reversely transcribed into complementary DNA (cDNA), and the genomic DNA was digested with DNase I (TaKara Bio, China). mRNA was amplified by RT-qPCR using ABI PRISM 7500 Fast sequence detection system (Applied Biosystems, CA, USA). The 2-ΔΔCt method was used to calculate the mRNA levels of each gene after normalizing to GAPDH. The primer sequences were used in this study listed in Table 1.

**Table 1. List of oligonucleotide primers used in this study**

| Genes     | Forward primer (5′-3′)            | Reverse primer (5′-3′)          |
|-----------|-----------------------------------|---------------------------------|
| FABP4     | CATCACGTTAAATGGGAT                | TGGACTTCTCCATCCCGACCTC         |
| COL1A1    | TAAAGGCTCATCCTGTCCCTC            | GACCGCTGATGAGGAGCAC            |
| COL3A1    | CTGTAACATGAAAACCTGAACTGAA        | CCATAGCTGAAGCTGAAACCC          |
| α-SMA     | CTTGCCCTCCTATGCTCTGG             | AGGGCTGTAGATCCCTCCTG          |
| MMP-2     | TCAAGGCTGGAGGAAATACGC           | AGCTGTGAGAGGGAGGTGCC          |
| MMP-9     | AAGGGCTACAGCTGGTTCTGGT          | CTGGATGCCGGTGCTATGGTCT        |
| TGF-β     | AGCAACAAATTCCTGGGCAGTTACCTT     | CCGTATTCCCTGCTCCCTGTTGCAG     |
| GAPDH     | TGGTGGCCTGGTGTGGATGCAG          | TTGCGTGTGAAAGTCGAGGAG         |

FABP4: Fatty acid binding protein 4; α-SMA: α-smooth muscle actin; MMP-2: Matrix metalloproteinases-2; MMP-9: Matrix metalloproteinases-9; TGF-β: Transforming growth factor-β; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase
FABP4 inhibition suppresses cardiac fibrosis by NLRP3 pathway

FABP4 expression is elevated in cardiac fibrosis mice. Ang II significantly increased serum levels of TNF-α and IL-6. Ang II caused a significant increase in LV mass than the saline group, which decreased by BMS309403 (P<0.001). Ang II also increased left ventricular internal diameter (diastole) (LVIDd) and left ventricular inner diameter (systole) (LVIDs), and these changes were significantly reversed by BMS309403 (both P<0.05). Moreover, the cardiac function was also significantly changed, and the cardiac output (CO) was increased by Ang II but reduced by BMS309403 (P<0.05).

**Effect of FABP4 inhibitor on LV structure and function in Ang-II-infused mice**

Mice were infused with Ang II and intraperitoneally injected with FABP4 inhibitor BMS309403 (50 mg/kg, once a day) for 4 weeks. Echocardiography was performed to show the structure and function of the left ventricle (LV) (Table 2). Bodyweight (BW) and heart rate (HR) were not different between untreated saline and Ang II mice. BMS309403 did not alter body weight and heart rate. Ang II caused a significant increase in LV mass than the saline group, which decreased by BMS309403 (P<0.001). Ang II also increased left ventricular internal diameter (diastole) (LVIDd) and left ventricular inner diameter (systole) (LVIDs), and these changes were significantly reversed by BMS309403 (both P<0.05). Moreover, the cardiac function was also significantly changed, and the cardiac output (CO) was increased by Ang II but reduced by BMS309403 (P<0.05).

**FABP4 inhibitor suppressed Ang II-induced inflammatory response**

We stained the ventricular tissue with HE to determine whether the FABP4 inhibitor altered inflammation in the heart. Compared with saline-infused mice, Ang II–infused mice showed increased inflammatory cell infiltration in the ventricular tissue of mice. The inflammatory cell infiltration was further attenuated by BMS309403 treatment (Figure 2(a)). To ascertain whether inflammatory cell infiltration in ventricular tissue is associated with systemic inflammation, we additionally measured the serum levels of two proinflammatory cytokines, TNF-α and IL-6. Ang II significantly increased serum levels of TNF-α and IL-6. However, these changes were markedly attenuated by BMS309403 (both P<0.001) (Figure 2(b-c)).

**FABP4 inhibitor attenuated Ang II-induced cardiac fibrosis**

Cardiac fibrosis was evaluated by Masson's staining of ventricular tissue. Ang II leads to a significant increase in myocardial interstitial fibrosis, which could be markedly attenuated after BMS309403 treatment (Figure 3(a-b)).

**Results**

**FABP4 expression was elevated in the mouse heart after Ang II infusion**

We first determined whether FABP4 was expressed in the mouse heart after Ang II treatment. Mice were infused with Ang II (2.8 mg/kg/day) for 4 weeks. Immunohistochemistry assay showed higher FABP4 expression in the cytoplasm and intercellular matrix of ventricular muscle (Figure 1(a)). To investigate the transcription of FABP4, we measured FABP4 mRNA levels in the mice model. RT-qPCR showed increased FABP4 mRNA in the ventricular muscle of mice after Ang II infusion (P<0.001) (Figure 1(b)). To determine whether the protein expression of FABP4 is also changed, we performed Western blotting to determine the protein levels of FABP4 (Figure 1(c)). The FABP4 protein expression was significantly higher in Ang-II-infused mice's ventricular muscle than in the saline-infused mice (Figure 1(d)). These results indicate that Ang II regulates FABP4 expression at both mRNA and protein levels.

**Table 2. Characteristics and echocardiography measurements of mice after 4 weeks Ang II infusion**

| Groups          | Control   | BMS309403 | Ang II  | Ang II+BMS309403 |
|-----------------|-----------|-----------|---------|------------------|
| BW (g)          | 23.3±1.32 | 23.30±1.36| 23.85±1.02| 23.18±1.62       |
| HR (bpm)        | 452.63±53.40| 450.38±50.90| 442.38±25.85| 464.25±31.48     |
| LV mass (mg)    | 76.55±4.62 | 76.74±5.93 | 99.47±6.63***| 86.43±5.7##       |
| LVIDd (mm)      | 3.54±0.31 | 3.50±0.19  | 4.02±0.21## | 3.67±0.22##      |
| LVIDs (mm)      | 2.47±0.19 | 2.57±0.11  | 3.03±0.36* | 2.66±0.25*       |
| CO (ml/min)     | 14.60±1.67| 15.77±1.87 | 18.11±1.85** | 15.51±2.23##     |

Data are represented as mean±SD

**Results**

**FABP4 expression was elevated in the mouse heart after Ang II infusion**

We first determined whether FABP4 was expressed in the mouse heart after Ang II treatment. Mice were infused with Ang II (2000 ng/kg/min) for 4 weeks. Echocardiography was performed to show the structure and function of the left ventricle (LV) (Table 2). Bodyweight (BW) and heart rate (HR) were not different between untreated saline and Ang II mice. BMS309403 did not alter body weight and heart rate. Ang II caused a significant increase in LV mass than the saline group, which decreased by BMS309403 (P<0.001). Ang II also increased left ventricular internal diameter (diastole) (LVIDd) and left ventricular inner diameter (systole) (LVIDs), and these changes were significantly reversed by BMS309403 (both P<0.05). Moreover, the cardiac function was also significantly changed, and the cardiac output (CO) was increased by Ang II but reduced by BMS309403 (P<0.05).

**FABP4 inhibitor suppressed Ang II-induced inflammatory response**

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**FABP4 inhibitor attenuated Ang II-induced cardiac fibrosis**

Cardiac fibrosis was evaluated by Masson's staining of ventricular tissue. Ang II leads to a significant increase in myocardial interstitial fibrosis, which could be markedly attenuated after BMS309403 treatment (Figure 3(a-b)).
Consistent with this, the mRNA expression of fibrosis markers (collagen I and collagen III) was reduced in the hearts of mice treated with BMS309403 as assessed by RT-qPCR (Figure 3(c-d)).

**FABP4 inhibitor reduced the fibroblast activation and suppressed the TGF-β pathway**

To investigate if FABP4 modulates fibroblast activation, we performed immunohistochemistry to evaluate the number of fibroblasts in the intercellular matrix. FABP4 inhibitor markedly attenuated Ang II-induced increase in the number of α-SMA-positive cells, indicating that FABP4 inhibition can suppress the transformation of fibroblasts into myofibroblasts (Figure 4(a)). Moreover, RT-qPCR showed that Ang II infusion significantly increased the mRNA expression of α-SMA (Figure 4(b)), MMP-2 (Figure 4(c)), MMP-9 (Figure 4(d)), and TGF-β (Figure 4(e)), which were all significantly attenuated by BMS309403. This FABP4 inhibitor also reduced serum TGF-β concentration in Ang II-infused mice as assayed by ELISA (Figure 4(f)). The results indicated that the TGF-β pathway is involved in the inhibitory process of cardiac fibrosis by FABP4 inhibition.

**FABP4 inhibitor modulated the expression of proteins associated with NLRP3 inflammasome**

Western blot was performed to measure the protein level of NLRP3 inflammasome-related proteins (Figure 5a). FABP4 inhibitor reduces the FABP4 protein expression in ventricular tissue of Ang II-infused mice (Figure 5b). Ang II infusion increased protein expression in NLRP3, ASC, pro-caspase-1, and cleaved caspase-1, and this indicated that NLRP3 inflammasome activation is involved in the process of Ang II-induced cardiac fibrosis. FABP4 inhibitor treatment significantly reduced these protein expressions in Ang II-infused mice (Figure 5c-f).

**Discussion**

In this study, we explored the effect of FABP4 on cardiac fibrosis. The results showed that FABP4 expression was improved in the cardiac fibrosis patient’s serum and Ang II-infused mice, while inhibition of FABP4 reversed these effects. BMS309403 is a potent, targeted FABP4 inhibitor that interacts with fatty acid binding within a protein and inhibits endogenous fatty acid binding through competitive
inhibition. By modulating the macrophage-myofibroblast transition, BMS309403 suppresses FABP4 and reduces renal interstitial fibrosis (19). In the current study, BMS309403 potentially enhanced cardiac structure and function and reduced cardiac and inflammatory response, reduced collagen deposition and mRNA expression in Ang II-treated mice. In addition, BMS309403 decreased the number of α-SMA+cells and decreased the mRNA expression of α-SMA, MMP-2, MMP-9, and TGF-β in ventricular tissue. Thus, the results demonstrate that FABP4 could be a potential therapeutic target of cardiac fibrosis.

An important mediator of hypertension and heart failure (HF) is angiotensin II (Ang II), the main effector of the renin-angiotensin system (RAS). Chronic RAS activation is linked to the development of HF, and chronic Ang II leads heart’s structural and electrical remodeling (20). In this experiment, mice were treated with intraperitoneal injections of the FABP4 inhibitor BMS309403 (50 mg/kg, once a day) along with Ang II infusions. To visualize LV’s anatomy and function, echocardiography was used. Between untreated saline and Ang II mice, there were no differences in BW and HR. BMS309403 did not change the body mass index and pulse rate. Ang II significantly increased LV mass compared with the saline group, which had a BMS309403 (P<0.001) reduction. Both LVIDd (diastole) and LVIDs (systole) were considerably decreased in response to Ang II. The cardiac function was also considerably altered, and Ang II enhanced cardiac output (CO), but BMS309403 decreased it (P<0.05).

Cardiac fibrosis is a common pathological state in different cardiac diseases, characterized by abnormal accumulation of ECM and proliferation of cardiac fibroblasts in the heart (21-22). Cardiac fibroblasts are stimulated and segregated into myofibroblasts that migrate and accumulate in the inflamed areas of the myocardium. They can be expanded to the nearby in most cardiac pathologic conditions,
FABP4 inhibition suppresses cardiac fibrosis by NLRP3 pathway

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IRNP3 inflammasomes are required for our study showed that Ang II treated mice improved endoplasmic reticulum (ER) stress in macrophages (35). Accelerating atherosclerosis via enhancing FABP4-mediated inflammatory cytokine production. In contrast, inhibition of FABP4 improved cardiac structure and function, restrained cardiac and systemic inflammatory response, and attenuated collagen deposition and mRNA expression of COL1A1 and COL1A3 in Ang II-infused mice through inhibition of NLRP3 inflammasome activation. Our results suggested that FABP4 could be a potential therapeutic target for the treatment of cardiac fibrosis, as well as a diagnostic biomarker of cardiac fibrosis.

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Conclusion

FABP4 expression is increased in the serum of cardiac fibrosis mice, which exacerbates fibrosis and inflammatory cytokine production. In contrast, inhibition of FABP4 improved cardiac structure and function, restrained cardiac and systemic inflammatory response, and attenuated collagen deposition and mRNA expression of COL1A1 and COL1A3 in Ang II-infused mice through inhibition of NLRP3 inflammasome activation. Our results suggested that FABP4 could be a potential therapeutic target for the treatment of cardiac fibrosis, as well as a diagnostic biomarker of cardiac fibrosis.

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Authors’ Contributions

NZ designed and supervised the project and revised the manuscript. Zhu X and WY performed experiments and wrote the first draft of the manuscript. Zhang X, CX, and ZL analyzed the data and performed statistical analysis.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author upon reasonable request.

Conflicts of Interest

The authors declare no conflicts of interest with other people or organizations.

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