Fenofibrate prevents myocardial inflammation and fibrosis via PPARα/IκBζ signaling pathway in rat autoimmune myocarditis

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Abstract. Fenofibrate is a peroxisome proliferator-activated receptor α (PPARα) agonist currently utilized clinically to reduce lipid levels. Experimental autoimmune myocarditis (EAM) is a T cell-mediated autoimmune myocarditis with histopathological evidence of extensive inflammatory cells infiltrated into the myocardium, resulting in myocardial fibrosis. Previously, the authors demonstrated that long-term fenofibrate could alleviate rat EAM; however, the underlying mechanism remains unclear. In the present study, EAM was induced in rats and fenofibrate was administered to evaluate its short-term effects. The CD4(+) T cells were purified from rats with EAM and PPARα (-/-) mice and determined the levels of IκBζ, pNF-κBp65 and IL-6. The findings of the present study revealed that fenofibrate treatment improved the progression of EAM in rats, as evidenced by a decreased ratio of the heart weight to body weight, reduced inflammatory cell infiltration, and relieved cardiac function. It also inhibited the transcription levels of T helper 17 (Th17)-related inflammatory cytokines, fibrosis-associated factors, and the expression of IκBζ, and dose-dependently downregulated IκBζ expression in differentiated Th17 cells from rats with EAM. The PPARα antagonist MK886 could reverse the effects in a dose-dependent manner. PPARα deficiency significantly upregulated IκBζ and pNF-κBp65 expression in the CD4(+) T cells of PPARα (-/-) mice. PPARα activation by three different PPARα agonists significantly inhibited IκBζ and IL-6 levels. The results suggested that IκBζ plays an important role in the pathogenesis of autoimmune myocarditis, and fenofibrate treatment ameliorates EAM by preventing myocardial inflammation and fibrosis, possibly through the PPARα/IκBζ signaling pathway.

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Introduction

Peroxisome proliferator-activated receptor α (PPARα) is a critical regulator of cardiac lipid metabolism and has a significant effect on various functions, such as glucose and lipid homeostasis, cardiac metabolism substrate conversion, and inflammation and autoimmune disease development (1). PPARα can also reduce the release of pro-inflammatory factors, inhibit the production of chemokines, and promote T cell differentiation (2). Fenofibrate lowers cholesterol and triglyceride levels and is a widely used as a PPARα agonist in clinical practice. It has multiple effects on the heart, including prevention of myocardial inflammation, attenuation of isoproterenol-induced acute myocardial ischemic injury, and inhibition of macrophage and T lymphocyte infiltration into the left ventricle (3). Experimental autoimmune myocarditis (EAM) is an autoimmune disease induced by CD4(+) T cells. Histopathology has shown that CD4(+) T cells infiltrate the myocardium in the acute phase, leading to severe myocardial damage and subsequent cardiac fibrosis (4-6). Fenofibrate treatment can alleviate EAM (7,8). It has been previously shown that PPARα plays a crucial role in T helper 17 (Th17) cell differentiation, and fenofibrate can alleviate EAM (9). Methyl-β-cyclodextrin, a specific cholesterol-depleting agent, also ameliorates EAM by suppressing myocarditis-induced apoptosis (10).

IκBζ is the most recently identified member of the IκB family and interacts with the NF-κBp50 subunit to positively regulate the expression of numerous inflammatory factors (11,12). IκBζ plays a central role in inflammatory diseases and various autoimmune diseases, making it a key Th17-associated factor (13,14). IκBζ mediates the inflammation response to TNF-α and IL-17, and its inhibition mediates toll-like receptor transcriptional responses (15,16). However, the role of IκBζ in EAM and the underlying mechanisms remain unclear.

In the present study, the authors investigated whether fenofibrate could regulate the IκBζ signaling pathway in rat EAM and explore its possible mechanisms. The results showed that fenofibrate treatment ameliorated EAM by preventing myocardial inflammation and fibrosis. It was also found that IκBζ is

a key factor in EAM, and the PPARα/IκBζ signaling pathway is involved in the pathogenesis of EAM. This suggested that IκBζ may be a new molecular target of fenofibrate for treating autoimmune myocarditis.

Materials and methods

Animals. Male Lewis rat aged 6-8 weeks (180-200 g) and PPARα (-/-) mice (129S4/SvJae) aged 8-10 weeks (20-25 g) were acquired from Beijing Vital River Lab Animal Technology Co., Ltd. and Jackson ImmunoResearch Laboratories, Inc., respectively. Male C57BL/6J mice as PPARα (+/+) wild-type (WT) mice were purchased from Shanghai SLAC Laboratory Animal Company, Ltd. Animal care and experiments were conducted in accordance with the procedures approved by the Animal Care and Use Committee of Xiamen University (approval no. XMULAC 20220111; Xiamen, China). Animals were provided free access to standard rodent chow and water and were housed in a SPF conditions at 23±2˚C and a relative humidity of 60±5%. Throughout the studies, all animals were treated in accordance with the guidelines for animal experiments of our institution.

Rat EAM model. EAM model was established as previously described (5,6). Throughout the studies, all the experiment operations were performed under the anesthesia environment to animal welfare consideration. The duration of the experiment was 21 days. On day 0 the rats were anesthetized with 2% isoflurane inhalation and immunized once by subcutaneous injection with a 0.2-ml emulsion containing 1 mg of cardiac myosin and an equal volume of complete Freud's adjuvant in both footpads; the morbidity and survival rate were 100% in rats immunized by this method (4). A total of 24 rats were divided into three groups (n=8 for each group). The control group rats received only complete Freund's adjuvant for immunization. The immunized EAM rats underwent daily oral gavage administration with fenofibrate or a solvent alone from day 14 to day 21 for 7 consecutive days. All the rats' health and behavior were monitored and body weight were measured every 3 days. It was previously reported that rat EAM cardiac inflammation occurred in the acute phase and peaked on day 14 to day 21, characterized by severe heart failure (5,6). On day 21 all the experimental rats were anesthetized with 2% isoflurane inhalation and euthanized through cervical dislocation, the area of the chest were sterilized with 75% alcohol, and an aseptic surgical knife was subsequently used to fully expose the heart. A total of 5 ml blood from the inferior vena cava were drawn out. After dissecting the heart, heart tissues were carefully harvested, washed, and weighted. Body weight (BW) and heart weight (HW) were measured to calculate the ratio of HW/BW.

Histopathological examination. Heart tissues were fixed in 10% formalin at room temperature for 3 days and 4% paraformaldehyde in PBS and embedded in paraffin wax. Sections were cut at 5-μm thickness for hematoxylin & eosin (H&E) staining and scored macroscopically as follows: i) 0, no inflammation; ii) 1, presence of a small discolored focus; iii) 2, presence of multiple small discolored foci; iv) 3, diffuse discolored areas not exceeding a total of 1/3 of the cardiac surface; and v) 4, diffuse discolored areas totaling >1/3 of the cardiac surface (5,9,10). Inflammatory cell infiltration was examined under a light microscope, the ratio of the area of inflammatory cell infiltration in each field to the area of the whole field was calculated, and its mean value was used for microscopic scoring as follows: i) 0, no inflammation; ii) 1,<25% of the heart section involved; iii) 2, 25-50%; iv) 3, 50-75%; and v) 4,>75% (5).

Cardiac function assessment. Echocardiography was performed on day 21 for all the experimental rats using a 14-MHz probe (Vivid 7; GE Healthcare). Wall thickness and left ventricular (LV) dimensions (including LV internal dimensions in systole, LV internal dimensions in diastole, and LV posterior wall of diastole), interventricular septal thickness at diastolic (IVS), and heart rate were measured. LV ejection fraction (LVEF) and LV fractional shortening (FS) were assessed as previously described (9,10).

CD4(+) T cell isolation. CD4(+) T cells were isolated and purified from rats with EAM and PPARα(-/-) mice spleens using microbeads [CD4(+) T cell isolation kit; MiltenyiBiotec, Inc.] as previously described (9). CD4(+) T cells from rats with EAM were induced Th17 cell differentiation and incubated with fenofibrate 20 μM (Abcam) and PPARα antagonist MK886 20 μM (Abcam) for 24 h for reverse transcription-quantitative PCR (RT-qPCR) analyses. CD4(+) T cells from PPARα(-/-) mice were activated using 1 μg/ml anti-CD3 (BD Biosciences) bound to plates and 2 μg/ml anti-CD28 (BD Biosciences) in solution for 3, 6, 12 and 24 h. CD4(+) T cells from PPARα(+/+) mice were added and incubated with three PPARα agonists, fenofibrate 100 μmol/l (Abcam), Wy14643 50 μmol/l (Abcam) or GW7646 1 μmol/l (Abcam) for 24 h. Cells were collected for western blotting and RT-qPCR analyses.

Western blot analysis. Cardiac ventricles from the EAM rats and cells were homogenized in a lysis buffer composed of 8 M urea, 1 mM dithiothreitol, 1 mM ethylenediaminetetraacetic acid (EDTA), and 50 mM Tris-HCl at pH 8.0. Following precipitation with trichloro-acetate and sodium deoxycholate, the protein samples were quantified using Lowry's method. SDS-PAGE (12.5%) was used to isolate 10 μg of proteins, which were subsequently transferred to a PVDF membrane, the membrane was blocked with 5% non-fat milk or 5% BSA (Beijing Solarbio Science & Technology Co.,Ltd.) for 1 h at room temperature and incubated overnight at 4˚C with antibodies against anti-vimentin (1:1,000; Thermo Fisher Scientific, Inc.), anti-collagen I (1:1,000; Abcam), anti-RAR-related orphan receptor gamma (RORγt; 1:1,000; BD Biosciences), anti-IκBζ (1:1,000; Cell Signaling Technology, Inc.), anti-pNF-κBp65 (1:1,000; Thermo Fisher Scientific, Inc.), anti-β-actin (1:2,000; Thermo Fisher Scientific, Inc.), anti-GAPDH (1:2,000; Thermo Fisher Scientific, Inc.) followed by incubation with goat anti-mouse IgG antibody (1:2,000; Cell Signaling Technology, Inc.) or goat anti-rabbit IgG antibody (1:2,000; Cell Signaling Technology, Inc.) at room temperature for 2 h. Membranes were eventually visualized with the ChemiDoc Touch Imaging System (Bio-Rad Laboratories, Inc.). Quantification of the resulting bands was achieved using densitometry software ImageJ (version 1.5.4; National Institutes of Health).

Table I. Echocardiographic parameters.

Parameters	Control	EAM	EAM + Feno
LVEDs (mm)	3.055±0.22	6.291±0.884[a]	3.904±0.604[c]
LVEDd (mm)	5.816±0.186	8.591±0.768[a]	5.753±0.931[c]
LVPW (mm)	1.347±0.18	1.732±0.475	1.513±0.185
IVS (mm)	1.208±0.137	2.520±0.285[a]	1.399±0.168
LVEF (%)	77.87±3.823	52.014±6.184[b]	54.319±8.652[c]
LVFS (%)	47.327±3.972	27.999±3.807[b]	30.297±5.603[c]
Heart rate (time/min)	294±13	458±24[b]	342±28[d]

All rats were sacrificed on day 21 (n=8 for each group). EAM vs. control, [a]P<0.01; [b]P<0.001. EAM + Feno vs. EAM, [c]P<0.05; [d]P<0.001. EAM, experimental autoimmune myocarditis; EAM + Feno, EAM and fenofibrate; LVEDs, left ventricular end-systolic internal diameter; LVEDd, left ventricular end-diastolic internal diameter; LVPW, left ventricular posterior wall at diastolic; IVS, interventricular septal thickness at diastolic; LVEF, left ventricular ejection fraction; LVFS, left ventricular fractional shortening.

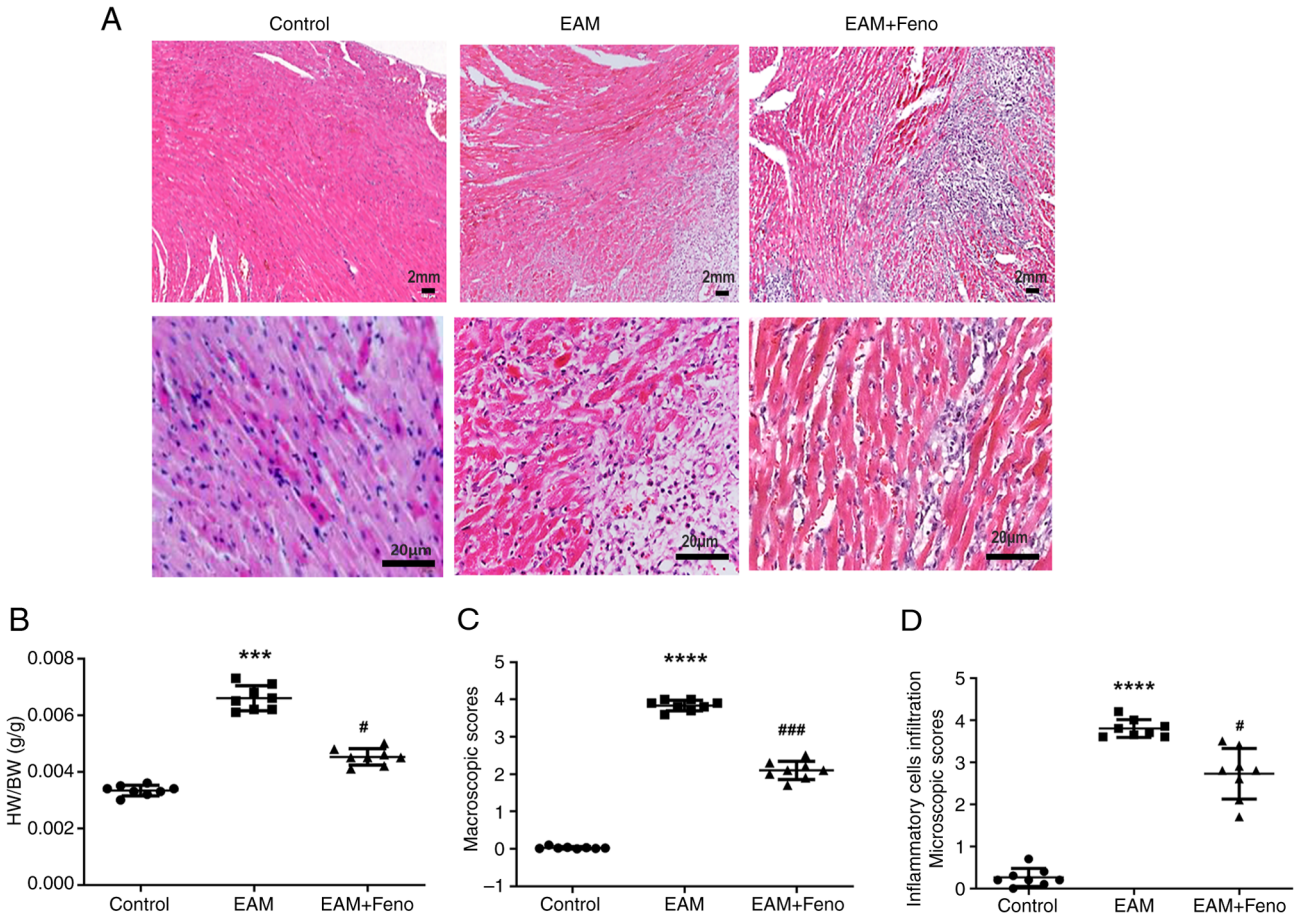

Figure 1. Feno alleviates rat EAM. (A) Representative hematoxylin & eosin staining of ventricular sections from control, EAM and feno-treated rats with EAM (upper images, 10x10; scale bar, 2 mm and lower images, 10x40; scale bar, 20 μm). (B) Ratio of heart HW/BW. (C) EAM macroscopic scores. (D) Microscopic scores (inflammatory cell infiltration) (n=8 for each group). EAM vs. control, ***P<0.001 and ****P<0.0001. EAM + Feno vs. EAM, $^{\#}$P<0.05 and $^{\#\#\#}$P<0.001. EAM, experimental autoimmune myocarditis; EAM + Feno, EAM and fenofibrate; HW/BW, heart weight to body weight.

RT-qPCR analysis. Total RNA was extracted from the rat hearts, spleen, and isolated CD4(+) T cells using TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.) in accordance with the manufacturer's protocol. Random primers and reverse transcriptase were employed to synthesize cDNA from 2 μg of the total RNA using the PrimeScript RT reagent Kit (Takara Bio, Inc.) according to the manufacturer's protocol. The reaction temperature and time were set according to the same protocol. qPCR was performed using SuperReal PreMix Plus (SYBR Green; Tiangen Biotech Co., Ltd.) on an ABI 7500 Fast Real-Time PCR Detection system (Applied Biosystems; Thermo Fisher Scientific, Inc.).Gene amplification was

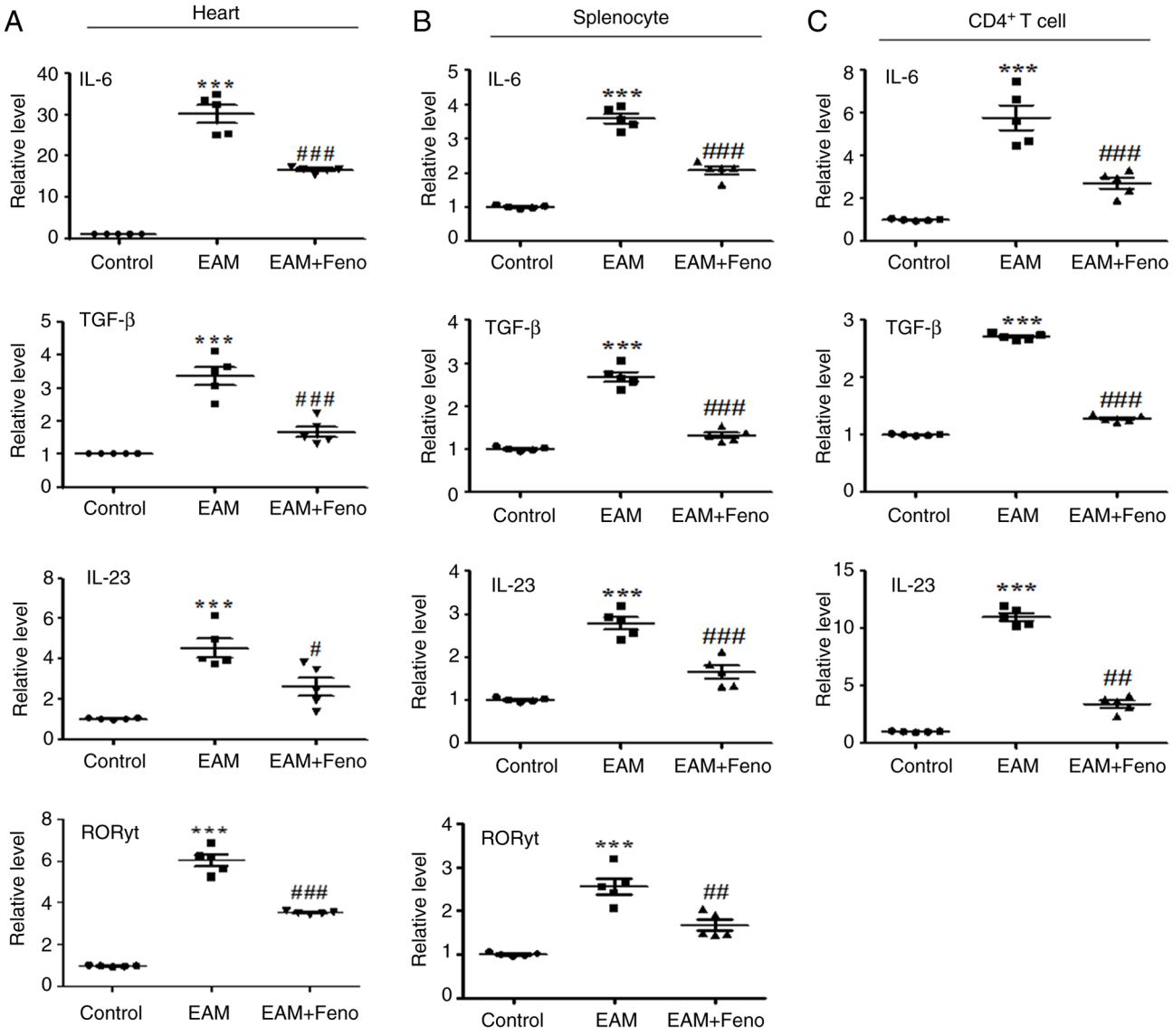

Figure 2. Feno inhibits IL-6, TGF-β, IL-23 and RORγt expression in the heart, splenocyte and CD4(+) T cells. (A) Heart, (B) splenocytes and (C) CD4(+) T cells were purified from the spleens of rats. Reverse transcription-quantitative PCR was employed to measure the relative transcript levels, with GAPDH serving as the internal reference for normalization (n=5 for each group). EAM vs. control, ***P<0.001. EAM + Feno vs. EAM, $^{\#}$P<0.05; $^{\#\#}$P<0.01 and $^{\#\#\#}$P<0.001. EAM, experimental autoimmune myocarditis; EAM + Feno, EAM and fenofibrate; IL, interleukin; TGF-β, transforming growth factor beta; RORγt, RAR-related orphan receptor gamma t.

performed using specific primer pairs (Table SI). The reaction program was determined as 3 min at 95˚C, followed by 40 cycles of denaturation at 95˚C for 30 sec, and annealing at 60˚C for 60 sec, and extension at 75˚C for 60 sec. Comparative analysis of qPCR results by utilizing the $2^{-\Delta\Delta Cq}$ method (17) was conducted to assess the relative levels of these molecules. This analysis was carried out after normalizing the results to GAPDH or β-actin expression.

Cytokine ELISA. The serum protein levels of IL-17 (eBioscience; Thermo Fisher Scientific, Inc.), IFN-γ (R&D Systems), and IL-4 (R&D Systems) in the control, EAM, and fenofibrate-treated EAM rats were measured using rat ELISA kit (Table SII) at day 21.

Immunohistochemistry staining (IHC). Deparaffinized cardiac tissue sections (5 μm) were heated in EDTA buffer, treated with 3% H_2O_2 in methanol for 10 min and blocked with 5% normal serum at room temperature for 30 min. These sections were incubated with the primary antibodies anti-RORγt (1:100; Abcam), anti-vimentin (1:100; Thermo Fisher Scientific, Inc.), and anti-IκBζ (1:100; Cell Signaling Technology, Inc.) at 4˚C overnight, followed by secondary antibodies rabbit anti-mouse IgG-HRP (1:5,000; Abcam) or goat anti-rabbit IgG-HRP (1:5,000; Abcam) (Table SII) for 1 h at room temperature. The sections were then visualized with DAB chromogen, counterstained with hematoxylin for 10 sec at room temperature, mounted with an antifade mounting medium (Applygen Technologies, Inc.) and analyzed using an Olympus fluorescent Microscope (IX51; Olympus Corporation) for the immunohistochemical examinations of vimentin, IκBζ, and RORγt.

Statistical analysis. The results are presented as the mean values with standard deviations. Statistical analysis was

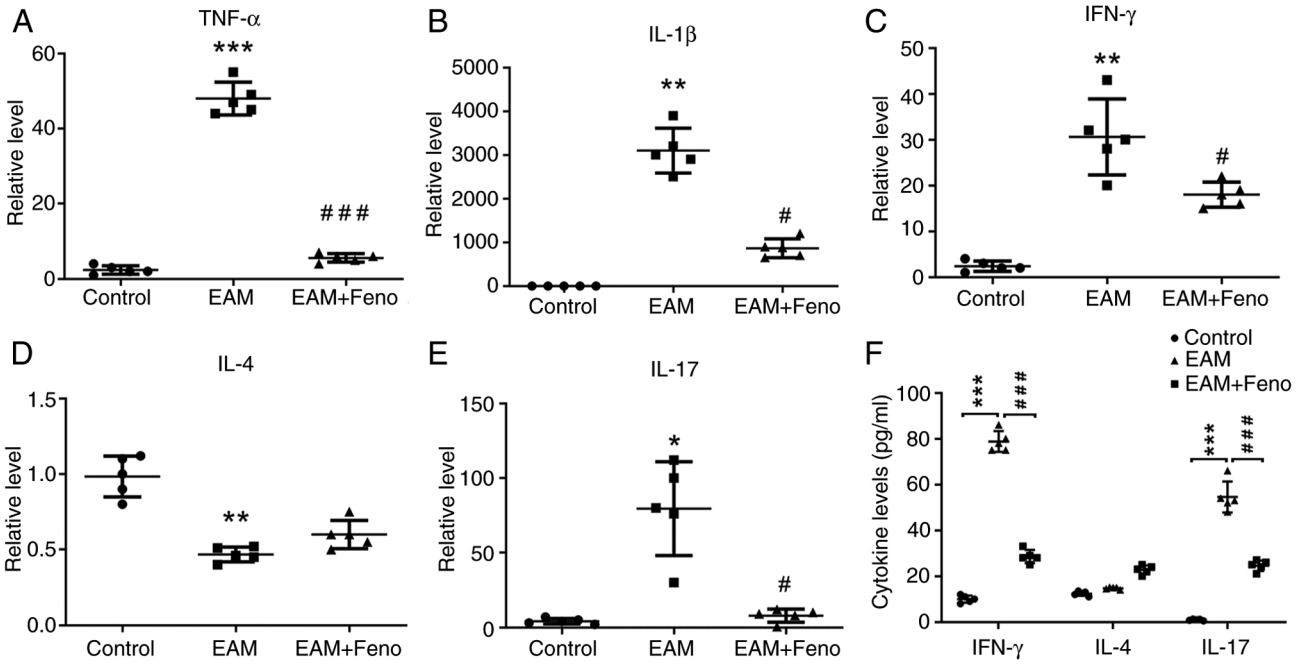

Figure 3. Feno inhibits the expression of Th17-related factors in the heart of rats with EAM. (A-E) TNF-α, IL-1β, IFN-γ, IL-4 and IL-17. Rat hearts were isolated and examined by reverse transcription-quantitative PCR analysis, with GAPDH serving as the internal reference for normalization (n=5 for each group). (F) Serum levels of IFNγ, IL-4 and IL-17 on day 21 of EAM rats were tested by ELISA (n=5 for each group). EAM vs. control, *P<0.05; **P<0.01 and ***P<0.001. EAM + Feno vs. EAM, $^{\#}$P<0.05 and $^{\#\#\#}$P<0.001. EAM, experimental autoimmune myocarditis; EAM + Feno, EAM and fenofibrate; Th17, T helper 17; TNF-α, tumor necrosis factor alpha; IL, interleukin; IFN-γ, interferon gamma.

conducted using one-way ANOVA, followed by Bonferroni's test for multiple comparisons. Statistical significance was established at P<0.05. All statistical analyses were performed using GraphPad Prism 7.0 software (GraphPad; Dotmatics).

Results

Fenofibrate treatment ameliorates rat EAM. Preliminary studies about the long-term effects of fenofibrate on EAM were conducted using different dosages from day 0 to day 21. The results showed that both 100 mg/kg and 200 mg/kg of fenofibrate ameliorated EAM while the dosage of 200 mg/kg had improved treatment effect compared with 100 mg/kg (Fig. S1). Therefore, fenofibrate was administered at a dosage of 200 mg/kg to evaluate its long-term effects in a previous study conducted by the authors (9). In the present study, fenofibrate (200 mg/kg) was used to evaluate its short-term effect. H&E staining of transverse cardiac ventricle sections revealed severe myocarditis in the rats with EAM, characterized by extensive inflammatory cells infiltration. Fenofibrate treatment significantly reduced inflammatory cells infiltration and mitigated EAM-induced myocardial inflammation (Fig. 1A). EAM in rats caused macroscopic and microscopic alterations with marked inflammatory cells infiltration and necrosis, and fenofibrate treatment ameliorated EAM as evidenced by a decrease in HW/BW, myocardial damage scores and inflammatory cell infiltrate scores (Fig. 1B-D). According to echocardiographic parameters, the rats with EAM showed enlarged LV, thick IVS and decreased LVEF leading to heart failure, Meanwhile, fenofibrate treatment improved the cardiac function as revealed by a reduction in LV end diameter at systole and diastole and an increase in LVEF and FS (Table I).

Fenofibrate inhibits the expression of Th17-related inflammatory cytokines. The experimental rats were euthanized after 21 days. Hearts, splenocytes and CD4(+) T cells were isolated. RT-qPCR quantified IL-6, TGF-β, IL-23 and RORγt expression levels, revealing that fenofibrate treatment suppressed the expression of these inflammatory cytokines in the hearts, splenocytes and $CD4^{+}$ T cells of the rats with EAM (Fig. 2A-C). Fenofibrate treatment also significantly reduced the expression of Th17-related factors TNF-α, IL-1β, IFNγ, IL-4 and IL-17 (Fig. 3A-E). ELISA confirmed that fenofibrate treatment suppressed the serum protein levels of IFNγ, IL-4 and IL-17 in the rats with EAM (Fig. 3F).

Fenofibrate inhibits the expression of fibrosis-associated factors. PPARα can suppress TGF-β-induced myocardial fibrosis (18). Therefore, the expression of fibrosis-associated factors TGF-β, tissue inhibitors of metalloproteinase (TIMP), fibronectin (FN) and galectin-3 (GAL3), and fibrosis markers vimentin and collagen type I (collagen I) were assessed in the hearts of rats with EAM. Fenofibrate treatment significantly reduced their expression (Fig. 4A-D). IHC and western blot analysis revealed that fenofibrate treatment significantly inhibited the expression of vimentin and collagen I (Fig. 4E and F).

Fenofibrate inhibits IκBζ expression in the heart of rats with EAM. IκBζ is critical in inflammatory and autoimmune diseases, and RORγt is essential for Th17 differentiation in EAM (19). IHC staining of transverse cardiac sections revealed the extensive infiltration of IκBζ-positive and RORγt-positive cells in the myocardium at EAM lesions, which were significantly reduced by fenofibrate treatment (Fig. 5A). Western

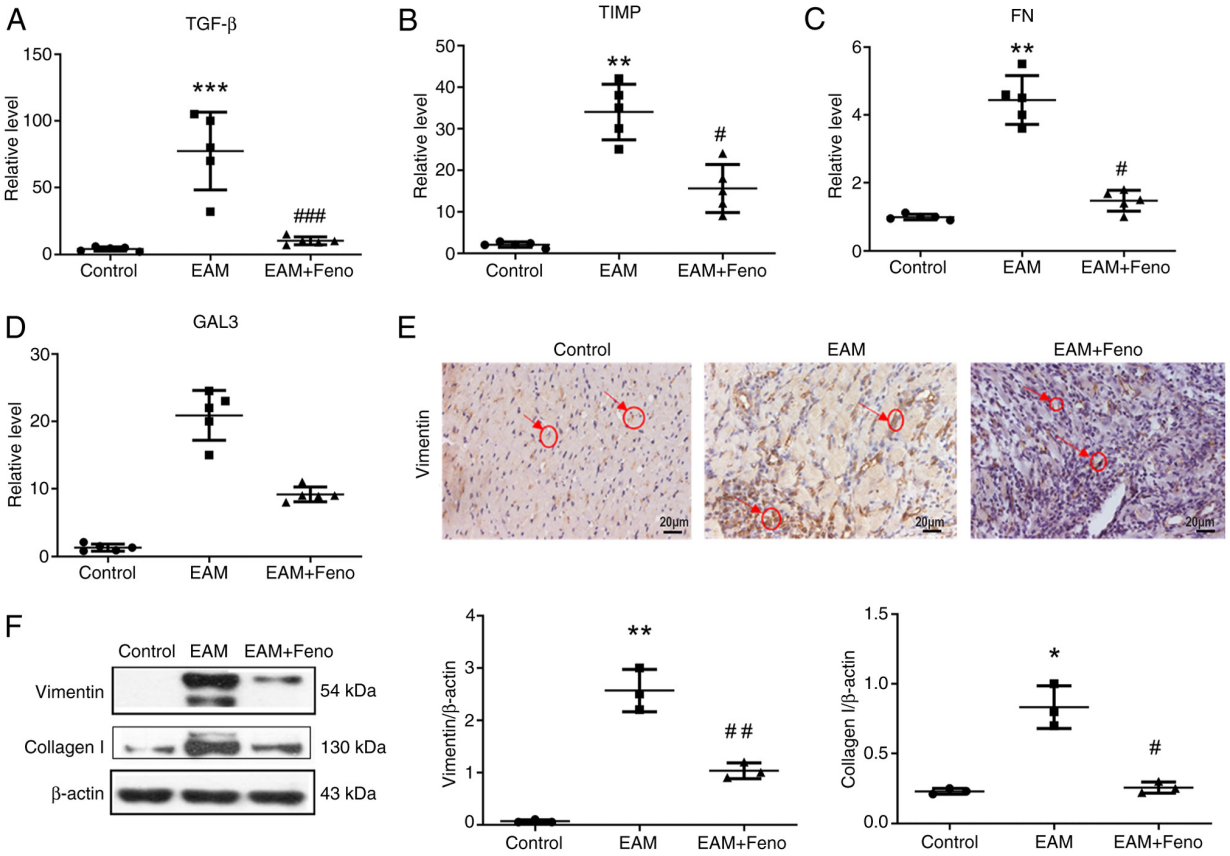

Figure 4. Feno inhibits the expression of fibrosis-associated factors in the heart of rats with EAM. mRNA levels of (A) TGF-β, (B) TIMPs, (C) FN and (D) GAL3 were determined using reverse transcription-quantitative PCR analysis with GAPDH serving as the internal reference for normalization (n=5 for each group). (E) Vimentin, representative immunohistochemistry image of ventricular sections. Scale bar, 20 μm. (F) Vimentin and collagen I protein expression in the heart examined by western blot analysis. β-actin serving as the internal reference (n=3). EAM vs. control, *P<0.05, **P<0.01 and ***P<0.001. EAM + Feno vs. EAM, $^{\#}$P<0.05, $^{\#\#}$P<0.01 and $^{\#\#\#}$P<0.001. EAM, experimental autoimmune myocarditis; EAM + Feno, EAM and fenofibrate; TGF-β, transforming growth factor beta; TIMPs, tissue inhibitors of metalloproteinase; FN, fibronectin; GAL3, galectin 3.

blot analysis confirmed that fenofibrate suppressed IκBζ and RORγt expression (Fig. 5B).

Fenofibrate inhibits IκBζ expression in the CD4(+) T cells from rats with EAM. The CD4(+) T cells were purified from the spleen of rats with EAM and induced Th17 cell differentiation using recombinant (r) IL-6 and rTGF-β. RT-qPCR analysis demonstrated that fenofibrate, at concentrations ranging from 0 to 20 μM, dose-dependently decreased IκBζ (Fig. 6A). MK886, which acts as a PPARα antagonist, displayed a dose-dependent reversal of these effects (Fig. 6B). These findings indicated that IκBζ is involved in Th17 differentiation during EAM development.

PPARα deficiency upregulates IκBζ and IL-6 expression. Spleen-derived CD4(+) T cells from PPARα(-/-) mice were exposed to anti-CD3 (1 μg/ml) and anti-CD28 (2 μg/ml) monoclonal antibodies and incubated for periods of 3, 6, 12 and 24 h. Western blot analysis demonstrated that IκBζ and pNF-κBp65 levels were significantly increased in the CD4(+) T cells of PPARα(-/-) mice compared with that in PPARα(+/+) mice (Fig. 7A and B). IL-6 is regulated by IκB-ζ and NF-κB activation. IκBζ controls Th17 differentiation by RORγt and IL-23 activation (20). It was observed that the levels of IL-6 and RORγt were higher at all tested time points in the CD4(+) T cells of PPARα (-/-) mice. However, the IL-23 expression did not change significantly (Fig. 7C-E).

Activation of PPARα inhibits IκBζ and IL-6 expression. CD4(+) T cells from PPARα(+/+) mice were added and incubated with three PPARα agonists (fenofibrate 100 μmol/l, Wy14643 50 μmol/l, GW7646 1 μmol/l) to study whether PPARα affects IL-6 expression. Western blot analysis revealed that these PPARα agonists suppressed IκBζ expression (Fig. 8A). ELISA results also indicated that these PPARα agonists significantly reduced IL-6 secretion (Fig. 8B). All these findings indicated that IL-6 mediates Th17 differentiation via the PPARα/IκBζ pathway.

Discussion

Myocarditis is an inflammatory cardiomyopathy that can lead to acute heart failure and dilated cardiomyopathy and currently has no specific treatment. EAM in rats is similar to human giant cell myocarditis, and recurrent forms can lead to dilated cardiomyopathy (4-6). In a previous study conducted by the authors, it was found that the peak of EAM cardiac inflammation occurred on days 14 to 21 and was characterized by infiltration of the CD4(+) T cells from the spleen into the myocardium. It was also revealed that Th17

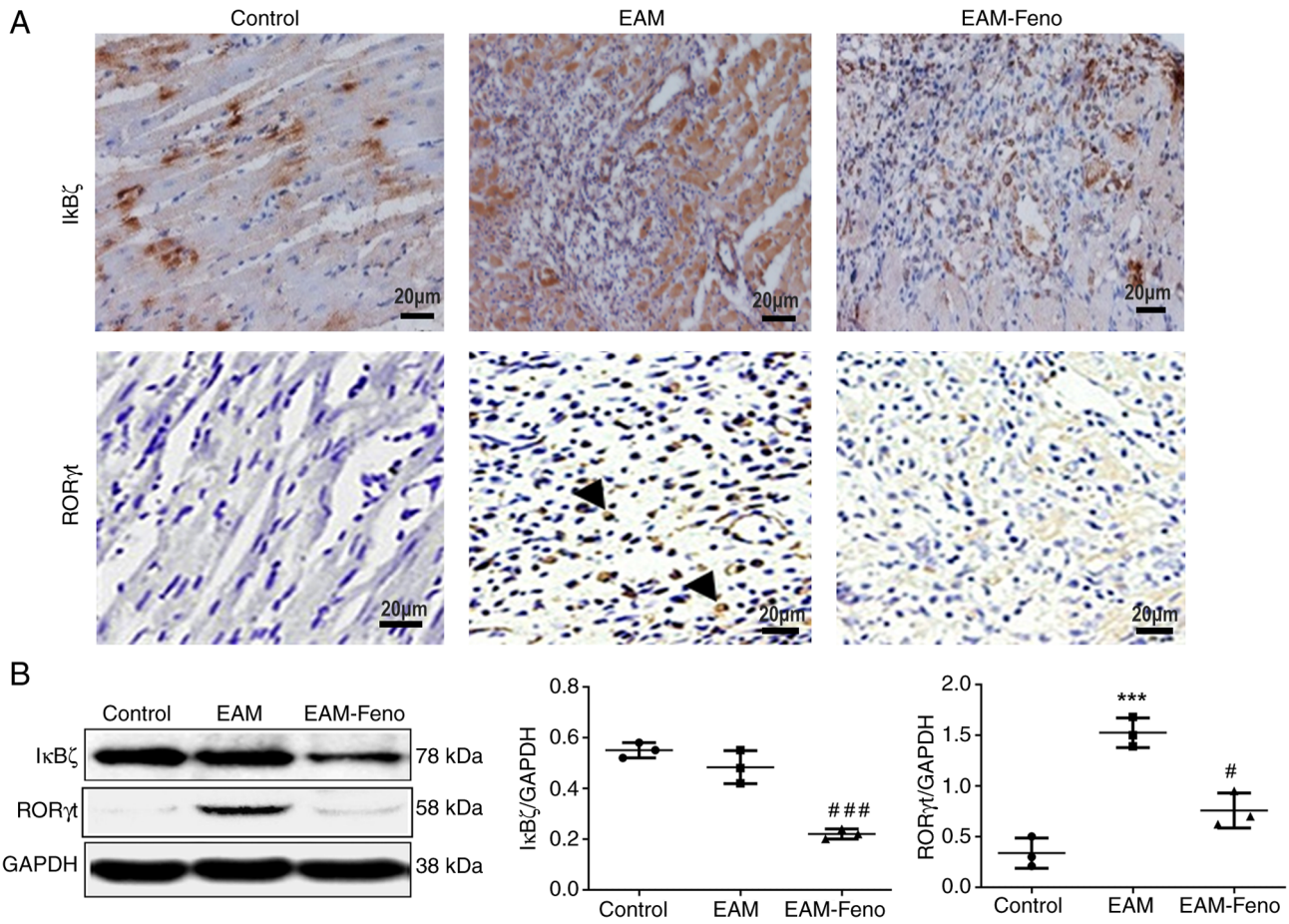

Figure 5. Feno inhibits IκBζ and RORγt expression in the heart of rats with EAM. (A) Representative immunohistochemistry image of IκBζ and RORγt in the ventricular sections. Scale bar, 20 μm. (B) Western blot and statistical analysis. GAPDH serving as the internal reference (n=3). EAM vs. control, ***P<0.001. EAM + Feno vs. EAM, $^{\#}$P<0.05 and $^{\#\#\#}$P<0.001. EAM, experimental autoimmune myocarditis; EAM + Feno, EAM and fenofibrate; RORγt, RAR-related orphan receptor gamma t.

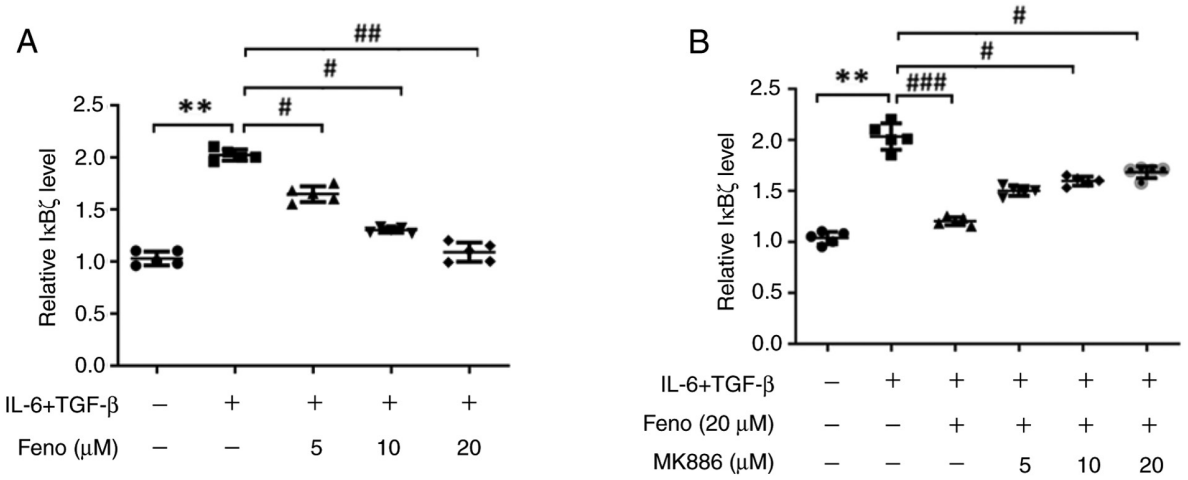

Figure 6. Feno inhibits IκBζ expression in the CD4(+) T cells from rats with EAM. (A and B) IκBζ expression in the CD4(+) T cells extracted from rats with EAM examined by reverse transcription-quantitative PCR. β-actin serving as the internal reference (n=5 for each group). CD4(+) T cells were obtained from EAM rat spleens and incubated with rIL-6 (40 ng/ml) and rTGF-β (10 ng/ml), and treated with either fenofibrate alone or a combination of fenofibrate and the PPARα antagonist MK886 for 72 h. **P<0.01, $^{\#}$P<0.05, $^{\#\#}$P<0.01 and $^{\#\#\#}$P<0.001. EAM, experimental autoimmune myocarditis; Feno, fenofibrate; r-, recombinant.

cells play an important role in the development of EAM and that fenofibrate can improve EAM by inhibiting Th17 differentiation (9).

In the present study, it was demonstrated that short-term administration of fenofibrate alleviated EAM. The expression levels of typical Th17-related factors including IL-6, TGF-β

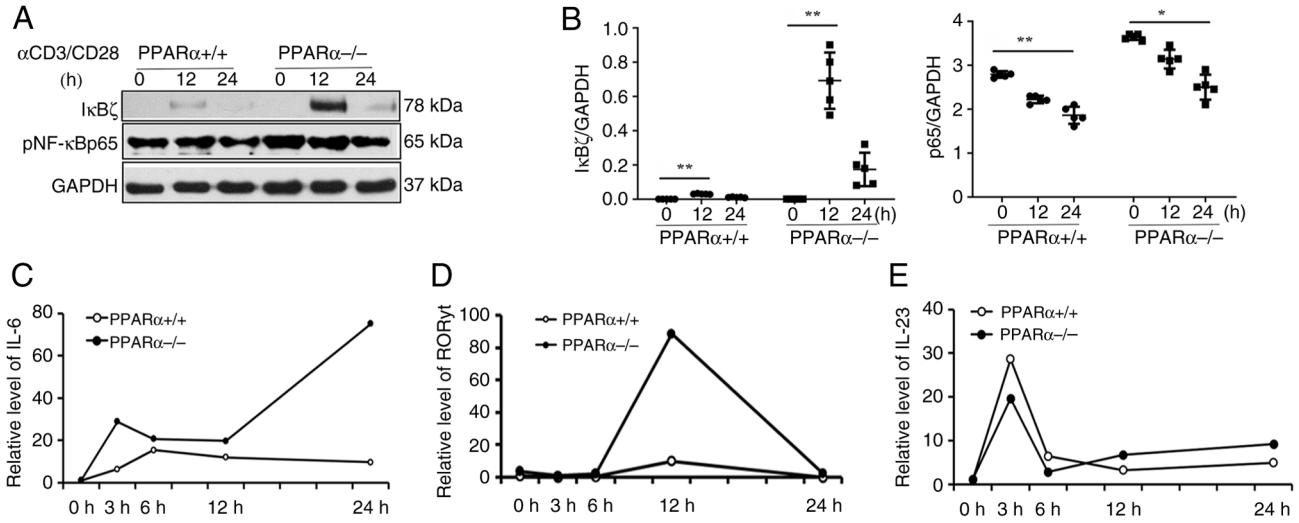

Figure 7. Time course expression of IκBζ-related factors in the CD4(+) T cells from PPARα(-/-) mice. (A and B) IκBζ and pNF-κBp65 protein levels determined by western blotting and statistical analysis. CD4(+) T cells were purified from the spleens of PPARα(+/+) and PPARα(-/-) mice, and incubated with anti-CD3 (1 μg/ml) and anti-CD28 (2 μg/ml) antibodies for 12 and 24 h. GAPDH serving as the internal reference (n=5). *P<0.05 and **P<0.01. (C-E) Reverse transcription-quantitative PCR analysis was employed to measure the relative transcript levels of IL-6, RORγt and IL-23 in the CD4(+) T cells. PPARα, peroxisome proliferator-activated receptor α; RORγt, RAR-related orphan receptor gamma t; IL, interleukin.

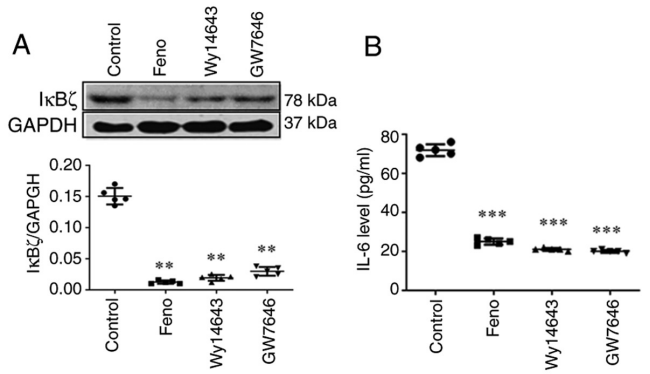

Figure 8. Activation of PPARα by three different PPARα agonists inhibits IκBζ and IL-6 expression. (A) IκBζ expression in the CD4(+) T cells determined by western blotting and statistical analysis; GAPDH was used as a control for internal comparison (n=5 for each group). (B) Production of IL-6 in the culture supernatants. Splenic CD4(+) T cells were isolated from PPARα wild type mice and incubated with three different PPARα agonists for 24 h (Feno 100 μmol/l, Wy14643 50 μmol/l and GW7646 1 μmol/l), and IL-6 levels in the culture supernatants were tested by ELISA analysis. (n=5 for each group). **P<0.01 and ***P<0.001. IL-6, interleukin-6; Feno, fenofibrate.

and IL-23 in the heart and splenic CD4(+) T cells from rats with EAM were significantly increased. The proinflammatory cytokines TNF-α, IL-1β, IFNγ and IL-17 were also significantly upregulated. The levels of these factors were reduced significantly by fenofibrate treatment. These findings suggested that fenofibrate attenuates cardiac inflammation and exerts anti-inflammatory effects by suppressing Th17-related inflammatory cytokines secretion. Thus, this provides a more comprehensive understanding of fenofibrate's therapeutic potential on autoimmune myocarditis. This result is consistent with the effect of fenofibrate on other inflammatory diseases (21,22).

In the pathological process of EAM, the infiltration of inflammatory cells into myocardium leads to myocardial fibrosis. Although cardiac fibrosis is beneficial for enhancing the structural stability of the heart, it can also lead to heart structure remodeling and impaired cardiac function. Therefore, improving cardiac fibrosis may help avoid further deterioration caused by EAM. The expression levels of types I, III and IV collagen, FN, matrix metalloproteinases, and TIMP are increased during the progression of myocardial fibrosis (23). PPARα activation can inhibit the TGF-β-induced cardiac fibrosis pathway. Fenofibrate can also reduce myocardial inflammation and collagen deposition by modulating the PPARα pathway and therefore, fenofibrate can relieve cardiac fibrosis and reverse cardiac dysfunction (24,25). In the present study, the expression levels of fibrosis-related factors TIMP, FN and GAL3 as well as fibrosis markers vimentin and collagen I, were detected by western blotting and RT-qPCR. Consistent with previous studies, the results also showed that these fibrosis-related factors were significantly upregulated in the hearts of EAM rats, and that fenofibrate treatment inhibited the levels of these factors and improved cardiac fibrosis.

IκBζ is a key Th17-related factor that plays an important role in the development of autoimmune diseases such as psoriasis (26,27). In mice with IkBz-deficiency, the development of psoriasis induced by IL-17, IL-23 and imiquimod was significantly inhibited (28,29). IL-17 and its family members, produced by CD4(+) T cells and various innate immune cells, are implicated in the pathogenesis of EAM (30); however, its role in EAM remains to be investigated. The results of the present study indicated that fenofibrate significantly inhibited the expression of IκBζ in the heart of rats with EAM. IL-6 plays an important role in EAM initiation through RORγt-mediated Th17 differentiation. The present study also revealed that fenofibrate treatment significantly inhibited the upregulation of RORγt expression in the hearts of rats with EAM. These findings suggested that IκBζ is a molecular target involved in Th17 differentiation in autoimmune myocarditis.

By stimulating CD4(+) T cells isolated from the spleen of EAM rats to induce Th17 cell differentiation, it was found that PPARα agonist fenofibrate upregulated IκBζ expression in a dose-dependent manner. This effect was reversed by PPARα antagonist MK886 in a dose-dependent manner. These results suggested that PPARα promotes Th17 cell differentiation through the IκBζ signaling pathway.

To further explore the potential mechanism of fenofibrate in treating EAM, CD4(+) T cells isolated from the spleen of PPARα-/- mice were activated and it was revealed that the mRNA and protein levels of IκBζ were upregulated in activated PPARα(-/-) mice CD4(+) T cells compared with those in PPARα(+/+) mice. IκBζ interacts with the NF-κBp50 subunit to positively regulate the expression of pro-inflammatory cytokines such as IL-6, IL-12 and CCL2 (31). The activation of NF-κB and stimulation of Toll-like receptor ligands and IL-1β are required to induce IκBζ expression (32). Previously, several studies have reported that IκBζ deficiency in LPS-induced macrophages prevents the production of the key pro-inflammatory cytokine IL-6 (33). In the absence of IκBζ, T cells exhibit serious defects in the development of Th17 cells (34). IκBζ is induced by IL-17R and collaboratively regulates IL-17 expression with RORγt. IL-6, which is a key factor in inducing Th17 cell differentiation, activates STAT3 and increases the expression of RORγt (35). Th17 cell differentiation and IL-6 secretion can be inhibited by PPARα agonists (36).

In the present study, the correlation between PPARα and IκBζ was confirmed. *In vivo,* it was shown that PPARα activation inhibited IκBζ expression on EAM. *In vitro*, it was revealed that PPARα deficiency upregulated IκBζ and IL-6 expression in the CD4(+) T cells from PPARα(-/-) mice. By conducting Chromatin immunoprecipitation (ChIP) assays, Muromoto *et al* (34,37) found that two different IκB-ζ promoter regions and STAT3 constitutively binds to the genomic promoter region of IκB-ζ TSS1. Luciferase reporter assays of the IκB-ζ promoter activity, revealed that catalytic activity of TYK2 and its substrate transcription factor STAT3, is required for IκB-ζ promoter activity. The limitation of the present study is the lack of direct molecular evidence of PPARα binding or transcriptional modulation with IκB-ζ. In a future study, the authors will provide further molecular evidences and focus on specific mechanisms regarding how PPARα and fenofibrate interacts with IκBζ and whether PPARα directly interacts with or binds the promoter regions of IκBζ gene. Luciferase reporter assays will be performed to evaluate the effects of fenofibrate on the PPARα/IκBζ pathway activation, and the promoter activity of IκBζ and mRNA stability will be examined using ChIP assay. Further mechanistic studies will be continued.

In summary, it was demonstrated that IκBζ contributes to the pathogenesis of autoimmune myocarditis, and fenofibrate treatment ameliorates EAM by preventing myocardial inflammation and fibrosis possibly through the PPARα/IκBζ signaling pathway. Thus, IκBζ may be a new molecular target for fenofibrate treatment in autoimmune myocarditis.

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Availability of data and materials

The data generated in this study may be requested from the corresponding author.

Authors' contributions

HC and ZQ designed the study, wrote and revised the manuscript. YWa and YWu conducted the experiments to collected data. SLS analyzed the data. HC and ZQ confirm the authenticity of all the raw data. All authors read and approved the final version of the manuscript.

Ethics approval and consent to participate

Animal care and experiments were conducted in accordance with the procedures approved by the Ethics Committee of Animal Care and Use of Xiamen University (approval no. XMULAC 20220111; Xiamen, China). All animals were treated in accordance with the principles of The Declaration of Helsinki and welfare considerations were taken to minimize the number of animals used and their suffering.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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