The effect of fenofibrate, a peroxisome proliferator-activated receptor α agonist, on cardiac damage from sepsis in BALB/c mice

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ABSTRACT

Cardiac dysfunction can be a fatal consequence of sepsis and lead to increased inflammatory responses or reduced fatty acid oxidation and final ATP depletion. Fenofibrate, which is an agonist of peroxisome proliferator-activated receptor α, has been used primarily in hypercholesterolemia and mixed dyslipidemia. Recent studies found that fenofibrate could alleviate energy metabolism and inflammation caused by cardiac damage during sepsis, and thus it had been paid great attention. This study was to investigate the possible protective roles of fenofibrate against cardiac *damage* in septic BALB/c mice. Methods: Forty male BALB/c mice aged 8 weeks old were divided randomly into four groups: control group; fenofibrate group; cecal ligation and puncture (CLP) group; and fenofibrate + CLP group. After administering fenofibrate or saline for 2 weeks, CLP was performed. Cardiac tissue and plasma were obtained 48 hours later. Plasma Troponin-T (Tnt), ATP, ADP and reactive oxygen species (ROS) levels were determined. PPARα and 53 protein levels were detected using western blotting. IL-6 and tumor necrosis factor-α (TNFα) were also assayed. We found that fenofibrate decreased plasma cTnT, ROS and increased the ratio of ATP/ADP. The elevations of IL-6, TNFα and P53 induced by sepsis were significantly suppressed by fenofibrate. Our results suggest that fenofibrate can regulate energy metabolism efficiently, which makes it a possible agent for treating sepsis-induced cardiac damage.

Introduction

Sepsis and septic shock have caused millions of deaths throughout the world every year. Cardiac damage induced by sepsis is a fatal complication of severe sepsis and septic shock (1). The increasing number of evidence suggests that elevating inflammatory cytokines can lead to cardiac damage, but the exact mechanisms underlying myocardial damage during sepsis remain unclear (1-3). In recent years, the relationship between sepsis and energy metabolism has been a popular research topic. Another important factor of the pathophysiology of sepsis is an energetic deficiency in organs, which is more critical for the heart because of its numerous energetic demands. Lipid oxidation is the main energy supply for the heart, which accounts for almost 70% of ATP production. The rest 30% comes from glucose oxidation. The existence of cardiac dysfunction decreases lipid oxidation and increases glucose oxidation for compensation of ATP (4, 5). In addition,

levels of cardiac transcription factors which are quite related to lipid oxidation, such as peroxisome proliferator-activated receptors (PPARs) and its coactivator PPARα, are lower during sepsis. Accordingly, cardiomyocyte-specific constitutive upregulation of PPARα induces lipid oxidation and improves cardiac function induced by sepsis (6-8). Interestingly, this improvement exists in spite of elevated inflammatory markers expression in the heart.

PPARα is abundant in metabolically active tissues. In addition to their metabolic roles in the heart, PPARα and its agonist fenofibrate have received great attention because of their effects related to cardiac damage inflammation and hypertrophy (9, 10). In cardiomyocytes, co-administration with fenofibrate inhibits the hypertrophic response induced by endothelin-1 by reducing cardiomyocyte surface area and decreasing protein synthesis (11). Fenofibrate also reduces the expression of transforming growth factor

(TGF-β1), macrophage infiltration and collagen deposition induced by AngII during the process of myocardial inflammation (12, 13).

In the present study, we investigated whether activation of PPARα with fenofibrate, a PPARα-specific agonist, modulates energy metabolism expression in cardiac damage induced by sepsis. Furthermore, we provide insights into a possible mechanism of how PPARα suppresses the progression of cardiac damage induced by sepsis: with energy metabolism involvement. Our study reveals a new role of fenofibrate in modulating the expression of proteins involved in energy metabolism and inflammation and provides a new potential mechanism for fenofibrate in treating cardiac damage induced by sepsis.

Materials and methods
Animals

The study procedures were approved by the Animal Studies Committee of the Affiliated Zhongshan Hospital of Dalian University. We used 8-week old male BALB/c mice, which were purchased from Dalian Medical University (Dalian, China). All mice were raised under constant conditions (temperature, 23-25°C; humidity, 40–60%; 12 h light/dark cycle).

Murine model of sepsis

Sepsis was induced by cecal ligation and puncture (CLP), as described previously (13). Briefly, under sodium pentobarbital (100mg/kg) anesthesia, laparotomy was operated and then two-thirds of the cecum was tightly ligated with a silk suture. One puncture of the cecum with a 21-gauge needle was performed. Few feces was exposed with gentle pressure and then the intestine was placed back in the peritoneal cavity. After that, the laparotomy site was closed. Sham-operated group mice underwent all the procedures except ligation and puncture. Forty male BALB/c mice aged 8 weeks old were divided randomly into four groups: (n=10/group): the control group, the fenofibrate (100 mg/kg/d; Sigma-Aldrich, St. Louis, MO, USA) treatment group, the CLP group, and the CLP with the fenofibrate treatment group. After 2 weeks of administration of fenofibrate or saline, all mice underwent CLP or sham operation. At 48 hours after the operation, mice were anesthetized by intraperitoneal injection of 4% chloral hydrate (0.1ml/10g). Blood samples were collected from

inferior vena cava of anesthetized mice and stored at -80°C until analysis. After cardiac tissues were obtained and coronal sections were fixed in formalin for histological evaluation, mice were sacrificed. The remaining part was then frozen in liquid nitrogen as soon as possible for mRNA or immune-histochemical use. All animal experiments were performed in accordance with the Guide for the Care and Use of Laboratory Animals. The study was approved by the ethical committee of the Affiliated Zhongshan Hospital of Dalian University.

Serum analysis

ATP, ADP, cTnT and ROS were quantified using a specific ELISA kit (Westang, Shanghai, China).

Hematoxylin-eosin staining (HE staining)

Myocardium was fixed in 10% formalin solution for 30 minutes and transferred in 75% ethanol for dehydration overnight. And then, it was embedded with paraffin. Continuous sections of 4-micrometer thickness were cut and stained with hematoxylin and eosin staining to analyze cardiac damage.

Morphologic analysis and Immunohistochemistry

We followed the methods of Pei et al. 2018 (14), immunohistochemistry was carried out using Histone Simple Stain Kit (Nichirei, Tokyo, Japan). In brief, paraffin-embedded sections were deparaffinised using xylene followed by a descending series of ethanol washes to rehydrate. Inactivation of endogenous peroxidases was conducted by treating sections with 3% H_2O_2 in methanol for 15 minutes. Then the sections were incubated with primary antibodies to PPAR-α (rabbit anti- PPAR-α antibody, 1:200; Proteintech, Wuhan, China) and P53 (rabbit anti-P53 antibody, 1:200; Proteintech) for 1 hour at room temperature. Observation of myocardium sections was carried out using a microscope (Olympus, Tokyo, Japan).

Real-time RT-PCR

We followed the methods of Pei et al. 2018[14], total RNA was extracted from cardiac tissues using the ISOGEN reagent (Nippon Gene, Tokyo, Japan) and reverse-transcribed into cDNA using a first-strand cDNA synthesis kit (SuperScript VILO cDNA Synthesis Kit; Life Technologies Carlsbad, CA,

USA). Then, real-time RT-PCR was performed using the fluorescent SYBR Green technology (Light Cycler; Roche Molecular Biochemicals). The relative amounts of target genes were calculated after normalized with β-actin levels as an internal control. Primer sequences have been shown in Table 1.

Table 1. Primer oligonucleotide sequences

Gene	Primer Sequence
TNF-α	F:5′-TCTCATGCACCACCATCAAGGACT-3′
	R:5′-ACCACTCTCCCTTTGCAGAACTCA-3
IL-6	F:5′-TACCAGTTGCCTTCTTGGGACTGA-3′
	R:5′-TAAGCCTCCGACTTGTGAAGTGGT-3′
β-actin	F:5′-CGATGCCCTGAGGGTCTTT-3′
	R:5′-TGGATGCCACAGGATTCCAT-3′

TNF-α, tumor necrosis factor-α; IL-6, interleukin- 6

Western blot Analysis

Isolation of whole-cell lysates from cardiac tissues was performed using radio immunoprecipitation assay buffer (P0013B; Beyotime, Shanghai, China) as described before (14). Proteins were separated by electrophoresis on 10% SDS-PAGE gel and transferred to polyvinylidene fluoride membrane (Immobilon, Millipore, Billerica, MA, USA). Then the membranes were blocked in Tris-buffered saline with 0.1% Tween-20 (TBS-T) containing 5% skim milk, and exposed to diluent (P0023A; Beyotime) of primary antibodies against PPARα (rabbit anti-PPAR-α antibody, 1:1000; Proteintech), P53 (rabbit anti- P53 antibody, 1:1000; Proteintech), anti-GAPDH (1:1000; Cell Signaling Technology), and gently shaken overnight at 4°C. Membranes were then exposed to secondary antibody (anti-rabbit Ig-G, 1:1000; Cell Signaling Technology) for one hour. This analysis was carried out independently three times. The intensities were analyzed using NIH Image J software and levels of protein were normalized to GAPDH.

Statistical Analysis

Results are presented as mean ± SEM. Statistical analysis was performed. One-way ANOVA followed by Tukey's test was conducted to analyze inter-group variance by using SPSS software version 23.0 (SPSS Inc., Chicago, IL, USA), with a P-value less than 0.05 being considered significant.

Results and discussion
Metabolic characterization

The metabolic data of four groups after different treatments are generalized in Figure 1. There was no significant difference in heart/body ratio among the four groups. The levels of cTnT and ROS were markedly increased in the CLP group as compared to the control group. While in Fen + CLP group, cTnT (P=0.001) and ROS (P=0.021) levels were significantly decreased. The level of ATP/ADP ratio was significantly increased (P=0.03) as compared with the CLP group. No difference was shown among Fen + CLP, control and Fen groups.
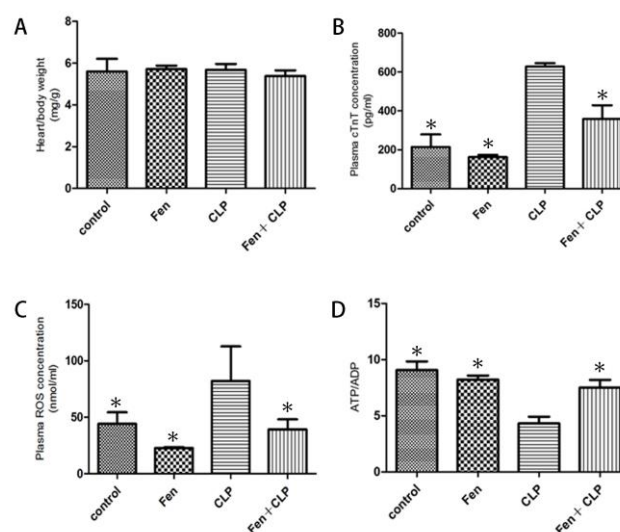
Figure 1. Metabolic data from the BALB/c mice of four groups after different treatments; Heart/body weights (A), cTnT (B), ROS (C), and ATP/ADP (D) of four groups after different treatments are presented; Data are means ± SEM; n=7-8 per group. *P<0.05 vs CLP group.

Fenofibrate decreased cardiac histopathological damage

To evaluate inflammatory cell infiltration into the cardiac tissue, haematoxylin and eosin staining were performed (Figure 2). The Fen + CLP group mice showed markedly reduced inflammatory cell infiltration in the cardiac tissue compared to CLP group mice. The results suggest that fenofibrate could decrease inflammatory cell infiltration in myocardial tissue of septic mice.

Fenofibrate upregulated PPARα expression in the cardiac tissue

Immunostaining of PPARα was conducted to assess its expression in myocardial tissues (Figure 3A-D). The CLP group showed significantly decreased PPARα expression in cardiac tissues in comparison

with the Fen + CLP group. Immunoblotting was performed to evaluate PPARα protein (Figure 3E). Expression of PPARα protein was significantly decreased (P=0.035) in the CLP group in comparison with Fen + CLP group (Figure 3F). The results suggested that fenofibrate upregulated the expression of PPARα in the CLP group.
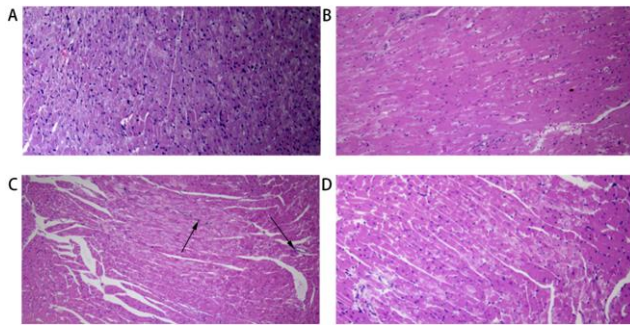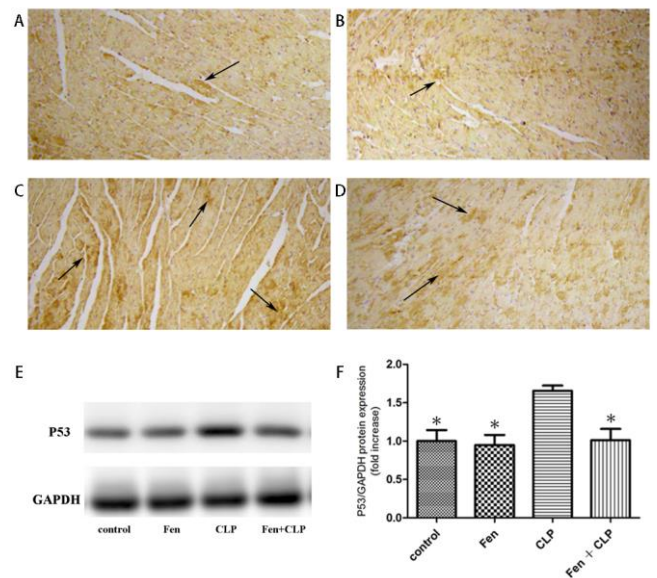

Figure 2. Inflammatory cell infiltration in the cardiac tissue from the BALB/c mice of the four groups after different treatment; Representative HE staining for morphology damage in cardiac tissue of mice with different diets (A). control group; (B)Fen group;(C)CLP group; (D)Fen + CLP group. Arrows indicate inflammatory cell infiltration area.
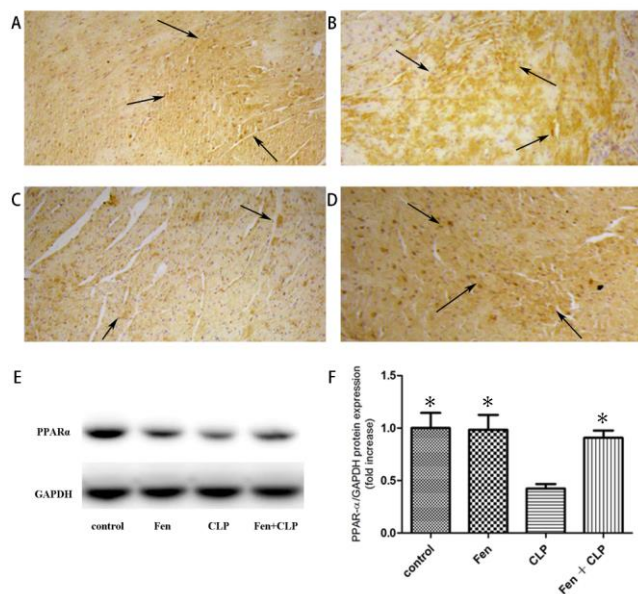
Figure 3. PPARα expression in the cardiac tissues from the BALB/c mice of the four groups after different treatment; Representative immunohistochemistry for PPARα in cardiac tissues, (A) control group; (B)Fen group;(C)CLP group; (D) Fen + CLP group. Arrows indicate PPARα protein expression positively stained cells. (E) Immunoblotting for PPARα in cardiac tissues. (F) Bar graph showing quantification of PPARα protein expression. Data are given as the means ± SEM; n=3-4 in each group. * P<0.05 vs. CLP group.

## Fenofibrate decreased expression of P53 in the cardiac tissue

Immuno-histochemical analysis was performed to obtain P53 staining to assess the expression of P53 in cardiac tissues (Figure 4A-D). In comparison with the CLP group, the Fen + CLP group showed significantly decreased expression of P53 in cardiac tissues. Immunoblotting for P53 protein was performed (Figure 4E). In comparison with the CLP group, the Fen + CLP exhibited markedly decreasing (P=0.028) P53 protein expression (Figure 4F). The results suggest that fenofibrate could decrease the expression of P53 protein in the CLP group.

Figure 4. P53 expression in the cardiac tissues from the BALB/c mice of the four groups after different treatment; Representative immunohistochemistry for P53 in cardiac tissues, (A) control group; (B)Fen group;(C)CLP group; (D)Fen+CLP group. Arrows indicate P53 protein expression positively stained cells. (E) Immunoblotting for P53 in cardiac tissues. (F) Bar graph showing quantification of P53 protein expression. Data are given as the means ± SEM; n=3-4 in each group. * P<0.05 vs. CLP group.

Fenofibrate decreased gene expression of IL-6 and TNFα in cardiac tissues

IL-6 and TNFα gene expression was measured by real-time PCR to evaluate the involvement of pro-inflammatory cytokines in cardiac tissues. (Figure 5). Upregulation of both IL-6 and TNF-α was exhibited in the CLP group, but the upregulation was attenuated in the Fen + CLP group (P=0.17, P=0.03 respectively).
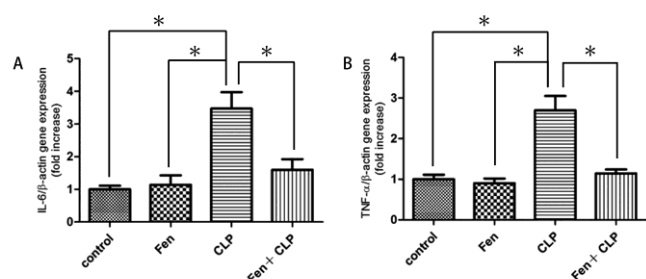
Figure 5. Expression of pro-inflammatory genes in the cardiac tissues from the BALB/c mice of the four groups after different treatment; Relative mRNA expression of (A) IL-6 and (B) TNF-α in the cardiac tissues of the four groups after different treatment; Data are given as the means ± SEM; n=6 in each group. * P<0.05 vs. CLP group.

In this study, we demonstrate that fenofibrate protects cardiac tissues against sepsis by increasing PP*AR*α and decreasing cTnT, ROS, P53 and pro-inflammatory cytokines.

In agreement with previous reports, plasma levels of cTnT and ROS are increased significantly in the CLP group as compared to that in the control group (15, 16). There is a significant decrease in serum levels of cTnT and ROS in the Fen + CLP group mice as compared to the CLP group mice. Sepsis results in cell apoptosis and organ failure, while ATP can reverse this phenomenon (17). Our results indicate that fenofibrate attenuates a decrease of ATP/ADP ratio after CLP, but the underlying mechanism is not yet clear. It is commonly recognized that the activation of numerous molecular signals by inflammatory mediators can produce suppressive cytokines, which finally leads to septic cardiomyopathy (18). Thus, an increase of leukocyte infiltration in the CLP group results in more inflammatory molecules than Fen +CLP group. All things considered, our study confirms that CLP can result in cardiac damage, which is significantly suppressed by fenofibrate.

The relationship between sepsis and energy metabolism is a popular research topic. Energetic deficiency induced by sepsis is another important reason for the disease's pathophysiology. PPARα, which is a ligand-activated nuclear receptor transcription factor, locates in the interface between metabolic and inflammatory pathways and regulates aspects of each (19). Specific fatty acids and their derivatives are endogenous PPAR ligands, and many exogenous PPAR agonists are used in the clinic. Fibrates, including fenofibrate, are a class of medicines that can activate PPARα, which is broadly distributed in the body (especially in the heart, kidney, and liver). PPARα functions as a regulator of immune inflammation and energetic metabolism.

A few clinical researches have demonstrated that the levels of PPARα protein are markedly reduced in the myocardium of patients with end-stage heart failure (20). With the same result, we analyzed PPARα expression in cardiac tissue with immunohistochemistry and immunoblotting. It is observed that PPARα expression is significantly downregulated after CLP, while this downregulation is alleviated markedly by fenofibrate.

p53, a transcription factor induced by stress, can be activated by a lot of stimuli including hypoxia, DNA damage and reactive oxygen species. Under these conditions, over-expression of P53 protein causes apoptosis or growth (21, 22). In sepsis, P53 expression was increased. The results in this study showed that the expression of P53 in cardiac tissues is significantly suppressed by fenofibrate.

As previously reported, pro-inflammatory genes, such as TNF- α and IL-6, were over-expressed and led to cardiac damage in sepsis (23-25). Our study demonstrated that fenofibrate can mitigate the over-expression of TNF- α and IL-6 in the CLP group.

Data provided in our study show that fenofibrate can help alleviate cardiac damage from sepsis by increasing PPARα and ATP/ADP ratio, and decreasing P53, cTnT and ROS. Our study also showed that fenofibrate reduced cardiac damage induced by sepsis via the energy metabolism pathway. These findings propose a new understanding of the mechanism and role of fenofibrate in cardiac damage induced by sepsis and put forward the possibility of a new therapeutic intervention for treating the progression of cardiovascular diseases.

Author Contributions

Interest conflict

The authors declare no conflict of interest.

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