Original Article PPARα activation by fenofibrate protects against acute myocardial ischemia/reperfusion injury by inhibiting mitochondrial apoptosis

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Abstract: Peroxisome proliferator-activated receptor α (PPAR α) is highly expressed in heart, but its metabolic regulation effect in rats with myocardial ischemia/reperfusion injury is still a controversy. In this study, the cardioprotective effect of PPAR α activation in rats with myocardial ischemia/reperfusion injury and its relevant mechanism was investigated. Adult male Wistar rats were pretreated with fenofibrate (80 mg/kg) daily for a period of 7 days. After the treatment period, myocardial I/R injury model was made by left anterior descending coronary artery ligation for 45 min and reperfusion for 120 min. Myocardial damage was indicated by increased myocardial infarct size and serum lactate dehydrogenase and creatine kinase activities. Significant increases in myocardial cell apoptosis, malondialdehyde (MDA) level and cleaved-caspase9 protein expression level in myocardial tissue were also observed, along with reductions of PPAR α and uncoupling protein 2 (UCP2) mRNA levels in myocardial tissue. Impaired mitochondria were observed under electron microscopic. However, pretreatment of ischemia/reperfusion rats with fenofibrate brought the biochemical parameters and related genes expression levels to near normalcy, indicating the protective effect of fenofibrate against myocardial ischemia/reperfusion injury in rats. Histopathological findings of the heart tissues further confirmed the biochemical findings, indicating that fenofibrate confers protection against ischemia/ reperfusion-induced oxidative stress in the myocardium.

Keywords: PPARα, UCP2, acute myocardial ischemia/reperfusion injury, oxidative stress, apoptosis

Introduction

Acute myocardial ischemia/reperfusion (I/R) injury is a process that involves in several factors, with the specific mechanism has not been completely elucidated [1]. Oxidative stress is one of the characteristics of ischemia reperfusion injury, which is related to mitochondrial damage and apoptosis [2]. The excessively produced and accumulated reactive oxygen species (ROS) during the early stage of reperfusion gives rise to mitochondrial damage and the release of cytochrome c, then activates the cascade reaction of caspase, and causes mitochondrial apoptosis [3]. It is suggested that inhibition of myocardial cell apoptosis can alleviate myocardial I/R injury.

Peroxisome proliferator-activated receptor α (PPAR $\!\alpha\!$) is a ligand-activated transcription fac-

tor, belonging to the nuclear receptor superfamily. PPARα forms a heterodimer with the Retinoid X Receptor (RXR), then activates the transcription of various genes containing a PPAR response element (PPRE), and regulates lipid homeostasis and metabolism [4, 5]. PPAR α is abundantly expressed in heart and plays a key role in regulating cardiac energy metabolism under physiological and pathological conditions [6, 7]. However, the metabolic regulation effect of PPARα in myocardial reperfusion injury is still controversial [8, 9]. In addition to the direct effect on myocardial metabolism, the pleiotropic effects of PPAR α have been suggested [10]. Study on PPAR^{-/-} mice reveals that the expression and activity of superoxide dismutase 2 (SOD2) were decreased, while the oxidative stress induced myosin damage, lipid peroxidative damage, and myocardial systolic function disorder were increased [11], indicating that $PPAR\alpha$ plays a vital role in cardiac oxidation regulation.

It is reported that activation of PPAR α can lessen the infarct size induced by myocardial I/R injury, while the downstream targets are not clear. In this paper, we used fenefibrate to active PPAR α , and explored the nonmetabolic effect of PPAR α on rat with acute myocardial I/R injury and the potential mechanism involved.

Materials and methods

Animal

Male Wistar rats (160-220 g body weight), fed a standard diet and tap water ad libitum. All animals were kept under standard conditions with a constant 12 (light): 12 h (dark) cycle (lights on at 06:00 h) and temperature ($22^{\circ}C \pm 2^{\circ}C$) were employed. All of the animal procedures were approved by an institutional animal care and use committee and were performed according to the Guideline of the Ethical Committee of Harbin Medical University.

Myocardial I/R injury model

Wistar rats were randomly divided into four groups (n=8): 1) normal group (Normal); 2) sham group (Sham); 3) I/R group (I/R); 4) feno-fibrate pre-treatment +I/R group (FF+I/R). Fenofibrate (Sigma, USA) was suspended in 3% Gum acacia and administered for 7 days at the dose of 80 mg·kg⁻¹·day⁻¹ by gavage. In the rest three groups,rats were given a similar amount of the solvent (3% Gum acacia). The dose of fenofibrate and the concentration of Gum acacia were based on our previous study [12].

1 hour following the last intragastric administration, myocardial I/R model was performed. Briefly, rats were anesthetized by intraperitoneal injection of 10% chloral hydrate (3 ml/Kg). After the oral endotracheal intubation, the animals were mechanically ventilated with room air by a rodent ventilator (room air, rate 75 cycles/min, 3 ml/100 g tidal volume). ECG was recorded. Body temperature was maintained at $37^{\circ}C \pm 0.5^{\circ}C$ by a heated operation table. The heart was exposed through a left thoracotomy and the left anterior descending (LAD) coronary artery was ligated (1-2 mm region under the boundary pulmonary artery pyramid and left auricle of heart) with a 5-0 polyester suture. A small polyethylene tube was placed between the ligature and myocardial tissues. The rats in sham group underwent the same surgical procedures, except the suture was not fastened. After 45 min ischemia, the ligature was released to permit reperfusion for 120 min. The reperfusion was associated with hyperemia and the disappearance of the cyanotic color of the myocardium.

After 45 min of acute myocardial ischemia and 120 min reperfusion, 5 mL of blood was taken from abdominal aorta, standing at 4°C for 1 hour, then centrifuged at 10,000 rpm for 5 minutes. Serum was frozen at -20°C until LDH and CK activity were assessed. The heart was excised and the ischemic area of the left ventricle was removed and frozen in liquid nitrogen immediately or myocardial homogenate for further measurement.

Serum myocardial enzyme activity and the MDA level in myocardial tissue determination

The serum LDH and CK activity were measured by LDH assay kit (Jiancheng, China) and CK assay kit (Jiancheng, China).

The level of Malondialdehyde (MDA) in the myocardium was measured using a MDA assay kit (Beyotime Biotechnology, China), according to the manufacturer's protocol. Heart tissues were homogenated with lysis buffer and centrifuged at 1600 × g for 10 minutes at 4°C to get supernatant. The protein concentration of the supernatant was measured by Bicinchoninic acid kit (Beyotime biotechnology, China). Then the thiobarbituric acid (TBA) was put into the supematant, and the reaction products were measured with Varioskan Flash microplate reader at 532 nm emission wavelength and 450 nm emission as reference wavelength. The MDA level was expressed as ng/mg protein.

2,3,5-triphenyltetrazolium chloride and Evans blue staining

The infarct size (IS) and area at risk (AAR) size were evaluated by double staining with 2,3,5-triphenyltetrazolium chloride (TTC)/Evans blue and determined by a computerized planimetric method, as described earlier [13].

After 120 min of reperfusion, the LAD was religated, and 1 ml of 5% Evans blue was injected



Figure 1. The effect of pretreated with fenofibrate on myocardial infract size of rats with acute I/R. A. Representative slice of myocardium stained by TTC/Evans blue. The region with blue indicates the non-ischemic area, the red indicates the area at risk, and the white indicates the infracted area. Bar=5 mm. B. The quantitative analysis of area at risk, expressed as the percent of left ventricle. C. The quantitative analysis of infarct size, expressed as the percent of the area at risk. Data are presented as mean \pm SEM (n=8). Significant differences ***: *P*<0.001 compared with the sham group, ###: *P*<0.001 compared with the I/R group.

via the left femoral vein to delineate the ischemic myocardium (area at risk, AAR). The rats were euthanized with potassium chloride injection, the heart was rapidly excised and placed in -20°C for 10 min. The heart was cut into1-2 mm-thick slices perpendicular to the heart base-apex axis. The slice were photographed and then put into 1% 2,3,5-triphenyltetrazolium chloride (TTC) for 15 min at 37°C to identify the infarction area (INF). After an overnight incubation in 4% formaldehyde, the slices were photographed again. AAR, INF and the left ventricular area (LV) were determined by planimetry of computer images (Image pro-plus). The IS was expressed as the ratio of INF/AAR.

Myocardial apoptosis determination

The cardiomyocyte apoptosis was determined by using a cell death detection kit (Roche, USA). After 120 min reperfusion, heart tissue distal 2-3 mm to the ligature of left ventricular anterior wall was cut and trimmed into 5 mm³ and fixed in 4% (vol/vol) paraformaldehyde for 24 hours, then paraffin embedded. Sections of 5-µm thickness were mounted on gelatin-coated glass slides. And the serial sections were performed according to the manufacturer's recommendations. The terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) positive cell nuclei present as brown, the normal nuclei was blue. Five fields were selected from every slice, and the number of TUNEL-positive nuclei was calculated by Image Pro Plus software.

Electron microscopy

At the end of reperfusion, the tissue of cardiac apex was excised and trimmed into 1 mm³ and fixed overnight in 3% glutaraldehyde at 4°C. Trimmed tissues were post-fixed by 1% osmium tetraoxide for 2 h and prepared for thin sectioning. The thin sections were observed with a transmission electron microscope.

Immumohistochemical analysis

After deparaffinization and rehydration, the paraffin sections were performed in Immunohistochemical analysis. The primary antibodies used in Immunohistochemical analyses included anti-cleaved-caspase9 (Ruiying, China, 1:200). The secondary antibodies was (HRP)conjugated goat anti rabbit IgG (ZSGB BIO, China).

The analysis was performed by an optical microscope to evaluate the area occupied by



Figure 2. The effect of pretreated with fenofibrate on the activities of serum myocardial enzyme of rats with acute I/R. A. The serum CK activity. B. The serum LDH activity. Data are presented as mean \pm SEM (n=8). Significant differences, ***: P<0.001, compared with the sham group, *: P<0.05, compared with the sham group, ###: P<0.001, compared with the I/R group.

cell expression cleaved-caspass9 and their intensity. The latter was expressed as Integrated Optical density (IOD), as detected using a software for image analysis (Image Pro Plus 6.0).

Reverse transcription polymerase chain reaction (RT-PCR) and real-time quantitative PCR

Heart tissue were collected and frozen at -80°C for evaluating the expression of PPARa and UCP2 mRNA. The total RNA was extracted using Trizol reagent (Tiangen, China), and the reverse transcription of the total RNA to cDNA was carried out using an AccuPower RocketScript[™] RT PreMix Kit (Bioneer, South Korea) respectively following the instructions of manufacturer. The thermal cycle profile for reverse transcription was set for primer annealing at 37°C for 10 min, cDNA synthesis at 50°C for 60 min, heat inactivation at 95°C for 5 min. The real-time quantitive PCR was performed using AccuPower Plus DualStar[™] qPCR PreMix Kit (Bioneer, South Korea) in a Bio-Rad iQ5 optical module. The cycling conditions were set for pre-denaturation at 95°C for 10 min; 40 cycles of denaturation at 95°C for 10 sec; annealing at 58°C for 20 sec. The primer sequences are as follows: primer of PPARα forward: 5'-TGGCGTTCGCA-GCTGTTTTG-3' and reverse 5'-CTCGTGTGCCC-TCCCTCAAG-3'; primer of UCP2 forward: 5'-GC-AGTTCTACACCAAGGGCT-3' and reverse 5'-GG-AAGCGGACCTTTACCACA-3'; primer of β-actinforward: 5'-CACCCGCGAGTACAACCTTC-3' and reverse 5'-CCCATACCCACCATCACACC-3'.

Statistical analysis

All data were expressed as mean \pm SEM. A Student's t-test or one-way ANOVA on ranks was performed to compares the statistical difference between the groups with Graphpad Prim 5. *P*<0.05 was considered statistically significant.

Results

PPAR α activation protected against acute myocardial I/R injury

The infarct size and activity of serum myocardial enzyme were performed to assess acute myocardial I/R injury. Both the area at risk and infarct size were shown in **Figure 1A**. There was no difference in ratio of AAR to LV between the I/R group and the fenofibrate pretreatment group (I/R: 47.84 \pm 2.94% vs. FF+I/R: 49.15 \pm 2.32%, *P*>0.05; **Figure 1B**), indicating a similar tension and placement of the ligature among the groups. But fenofibrate pretreatment led to significant reduction in infarct size (IS/AAR, FF+I/R: 32.27% \pm 2.11% vs. I/R: 47.09 \pm 3.34%; *P*<0.05; **Figure 1C**) in comparison with the I/R group.

Compared to the I/R group, the serum CK activity (I/R: 8018.82 ± 183.42 vs. I/R+FF: 6336.12 ± 690.96 , *P*<0.05; **Figure 2A**) and LDH activity (I/R: 8018.82 ± 183.42 vs. I/R+FF: 6336.12 ± 690.96 , *P*<0.05; **Figure 2B**) were significantly decreased in fenofibrate pretreatment group, suggesting that PPAR α activation protects myocardium from acute I/R injury.



Figure 3. The effects of pretreated with fenofibrate on cardiomyocyte apoptosis of rats with acute I/R. A. Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL)-stained myocardium of rats from each group. Arrows indicate TUNEL-positive cells. Bar=100 μ m. B. The quantitative analysis of TUNEL-positive cells. Data are presented as mean ± SEM (n=8). Significant differences. ***: *P*<0.001 compared with the sham group, ###: *P*<0.001 compared with the I/R group.



Figure 4. The influence of pretreated with fenofibrate on the expression of cleaved-caspase-9 in myocardium of rats with acute I/R. A. Representative photomicrographs of immunohistochemistry staining for cleaved-caspase9. Bar=100 μ m. Arrow indicates cleaved-caspase9 positive staining. B. Quantitative analysis of cleaved-caspase9 positive staining in each groups. Data are presented as mean ± SEM (n=8). Significant differences, **: *P*<0.01 compared with the sham group; ##: *P*<0.01, compared with the I/R group.

PPAR α activation reduced cardiomyocyte apoptosis caused by acute myocardial I/R injury

TUNEL staining and caspase-9 activity were performed to measure the extent of apoptosis in ischemic myocardium. As shown in **Figure 3**, compared to the I/R group, fenofibrate pretreatment significantly reduced TUNNEL-positive cardiomyocytes (P<0.05). Furthermore, the casepase-9 activity, represented as the cleaved-caspase9 expression in myocardium, was obviously reduced in fenofibrate pretreat-

ed group compared to I/R group (P<0.01, **Figure 4**). These results suggests that the participation of anti-apoptosis effect of PPAR α in protecting against acute myocardial I/R injury.

PPARα activation decreased mitochondrial damage caused by acute myocardial I/R injury

Mitochondrial ultrastructure were observed by transmission electron microscopy (**Figure 5**). Results showed that I/R resulted in distinctive ultrastructure alterations, including disordered mitochondrion distribution with disarranged

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PPARa and mitochondrial apoptosis in myocardial ischemia/reperfusion injury



Figure 5. The influence of pretreated with fenofibrate on the ultrastructure of myocardial tissues of rats with acute I/R. The representative electron micrographs of cardiomyocytes of rats in each group. Bar=2 μ m.

and obscure crista and vacuoles within the matrix, accompanying by disrupted myofilament and sarcomere. Noticeably, fenefibrate pretreated alleviated these deleterious effects of mitochondrial ultrastructure induced by I/R injury.

PPAR activation alleviated oxidative stress induced by acute myocardial I/R injury

We measured the malondialdehyde (MDA) level in ischemic myocardium, and we found that the production of MDA significantly increased in I/R-treatment group, compared with sham group (Sham: 0.28 ± 0.06 vs. I/R: 1.21 ± 0.13 , P<0.001). While pretreatment with fenofibrate significantly reduced the elevated MDA production induced by I/R (I/R: 1.21 ± 0.13 vs. I/R+FF: 0.69 ± 0.09 , P<0.01, Figure 6).

The mRNA expression of PPAR α and UCP2 in myocardium

As shown in Figure 7, compared with the normal group, the expression of PPAR α mRNA



Figure 6. The effect of fenofibrate pretreated on MDA level in the myocardium of rats with acute I/R. Data are presented as mean \pm SEM (n=8). Significant differences, ***: P<0.001 vs. Sham group. *: P<0.05 vs. Sham group. ##: P<0.01 vs. I/R group.

(Normal: 0.79 \pm 0.04 vs. Sham: 0.85 \pm 0.04, *P*>0.05) and UCP2 mRNA (Normal: 0.94 \pm 0.05 vs. Sham: 1.01 \pm 0.07, *P*>0.05) in myocardium has no significantly difference in the sham group. Compared with the sham group, the expression of PPAR α and UCP2 mRNA significantly decreased (*P*<0.001, *P*<0.001) in the I/R group. Compared with the I/R group, pretreated with fenofibrate, significantly upregulated the expression of PPAR α mRNA (I/R: 0.51 \pm 0.02 vs. I/R+FF: 0.98 \pm 0.04, *P*<0.001) and UCP2 mRNA (I/R: 0.23 \pm 0.03 vs. I/R+FF: 0.39 \pm 0.03, *P*<0.01).

Discussion

We discovered in our previous research that pretreatment with fenofibrate could remarkably improve isoprenaline-induced acute myocardial ischemia injury. We explored the nonmetabolic effect of PPARα on rat with acute myocardial I/R injury and the potential mechanism involved in this paper. Consistent with the previous investigation, the results in this paper demonstrated that the expression of PPAR α mRNA decreased in the myocardial tissue of I/R rats. Short-term pretreatment with fenofibrate, a specific ligand of PPARa to activate PPARa, notably reduced the myocardial infarction size, decreased the activity of serum myocardial enzyme, and reduced the MDA level in myocardium, alleviated the cardiomyocyte mitochondria damage, decreased myocardial cell apoptosis, and displayed protective effect on acute myocardial I/R injury.



Figure 7. The effect of fenofibrate pretreated on expression of PPARα and UCP2 mRNA in the myocardial tissue of rats with acute I/R. The quantitative fold changes in mRNA expression were determined relative to β-actin mRNA levels in each corresponding group and were calculated using the 2^{-ΔΔCT} method. A. The expression of PPARα mRNA in myocardial. B. The expression of UCP2 mRNA in myocardial. Data expressed as mean ± SE. (n=8) ***: *P*<0.001 vs. Sham group. ###: *P*<0.001 vs. I/R group. #: *P*<0.05 vs. I/R group.

At the early stage of reperfusion, ROS is massively produced and accumulated, inducing polyunsaturated fatty acid oxidation and damaging the cell membrane directly through lipid peroxidation [14], as well as producing toxic metabolites, such as MDA [15]. MDA is a marker reflecting the degree of lipid peroxidation injury [16]. We found that the content of MDA was distinctly increased in ischemia reperfusion myocardial tissue, indicating remarkable oxidative stress injury, while activation of PPARα could reduce the MDA content in myocardial tissue from ischemia reperfusion rats, indicating that PPARα activation could alleviate ischemia-reperfusion induced oxidative stress injury.

The mechanism of anti-oxidant activity of PPAR $\!\alpha$ in heart remains unclear. Previous stud-

ies mostly focus on regulating the expression and activity of antioxidant enzymes, such as SOD and catalase (CAT) [17, 18]. PPARα activation in liver upregulates the expression of uncoupling protein 2 (UCP2), and relieves the oxidative stress induced injury [19]. UCP2 is one of the members of anion carrier protein family, and locates in the inner membrane of mitochondrial. By regulating the transfer of proton to the mitochondrial matrix, UCP2 dissipates the proton gradient in the inner mitochondrial membrane, decreases the mitochondrial inner memberane potential, and consequently reduces the production of ROS and alleviates oxidative stress response [20, 21]. The generation of mitochondrial ROS during I/R is related to a specific metabolic process [22]. The succinate that is massively produced and accumulated at stage of myocardial ischemia, is rapidly oxidized after reperfusion, which drives reverse electro transport at complex I, thus increasing the mitochondrial inner membrane potential and inducing the production of mitochondrial ROS that initiates I/R injury. UCP2 can mediate proton leak to dissipate the mitochondrial proton-motive force, so we detected the expression of UCP2 in myocardial tissue. We found that the mRNA level of UCP2 was decreased in the myocardial tissue suffered from I/R, while PPARα activation could apparently upregulate the UCP2, indicating that UCP2 may participate in the antioxidant effect of PPARα on acute myocardial I/R injury. In addition, we found that the expression of PPARα mRNA in myocardium has no difference between the sham group and the fenofibrate pre-treated group, but the mRNA expression of UCP2 failed to keep parallel to the expression of PPARa, indicating that there were other factors involved in regulating of UCP2. The role of UCP2 in myocardial I/R injury remains further investigation.

Apoptosis is the characteristic change of I/R injury, and inhibition of myocardial apoptosis can alleviate myocardial I/R injury [23]. Mitochondrion plays a core role in the apoptosis pathway [24]. Mitochondrion is a major source of free radicals after reperfusion, and vulnerable to oxidative damage [25, 26]. The excessive production and accumulation of ROS can aggravate mitochondrial injury, lead to mitochondrial membrane lipid peroxidation, and mitochondrial edema [27, 28], as well as reduced mitochondrial membrane potential, promoting the opening of mitochondrial permeability transition pores and the release of mitochondrial cytochrome c as well as apoptosis inducing factor (AIF), under the action of various pro-apoptotic signals [29]. Caspase-9 is the key to apoptosis pathway mediated by mitochondrion [30, 31]. The cytochrome c releases into the cytoplasm from the mitochondrion, binds to the apoptotic protease-activating factor and pro-caspase-9 and forms the apoptosis body, which renders the activation of caspase-9, giving rise to cascade reaction of caspase and inducing apoptosis [32]. In this paper, we observed that PPARa activation could alleviate the acute I/R induced injury of mitochondrial ultrastructure, reduce the release of mitochondrial cytochrome c, decrease the casepase-9 activity in myocardial tissue, and inhibit myocardial apoptosis. Consequently, PPARa activation might reduce the I/R induced myocardial infarction area by decreasing myocardial apoptosis, and such anti-apoptosis protective effect might be related to alleviating mitochondrial injury induced by oxidative stress.

To sum up, this research sheds new light on the protection effects of PPAR α activation on acute myocardial I/R injury, which may be achieved through alleviating oxidative stress induced mitochondrial apoptotic pathway; in addition, such protective effect may be related to the up-regulated expression of UCP2.

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Disclosure of conflict of interest

None.

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