Effects of trimetazidine on mitochondrial respiratory function, biosynthesis, and fission/fusion in rats with acute myocardial ischemia

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Abstract

Objective: Myocardial ischemia affects mitochondrial functions, leading to ionic imbalance and susceptibility to ventricular fibrillation. Trimetazidine, a metabolic agent, is clinically used in anti-anginal therapy.
Methods: In this study, the rats were orally treated by gavage with trimetazidine 10 mg/kg/d for 7 days, and the effects of trimetazidine on mitochondrial respiratory function, biosynthesis, and fission/fusion in rats with acute myocardial ischemia were evaluated.
Results: It has been suggested that acute myocardial ischemia leads to a damage to mitochondrial functions. However, compared with ischemia group without trimetazidine administration, a significant reduction in the infarct size was observed in trimetazidine-treated ischemia group (31.24±3.02% vs. 52.87±4.89%). Trimetazidine preserved the mitochondrial structure and improved respiratory control ratio and complex I activity. Furthermore, trimetazidine improved mitochondrial biosynthesis and fission/fusion, as demonstrated by the promotion of peroxisome proliferator-activated receptor gamma (PPARγ) co-activator 1α (PGC-1α), mitofusins 1 (Mfn1), dynamin-related protein 1 (Drp1), and optic atrophy 1 (Opa1) expressions in rats with acute myocardial ischemia.
Conclusion: Taken together, it was suggested that in this rat model of myocardial ischemia, trimetazidine demonstrated cardioprotective effects attributing to the preservation of mitochondrial respiratory function, biosynthesis, and fission/fusion and, thus, could be considered as an agent for cardioprotection. *(Anatol J Cardiol 2017; 18: 175-81)*
Keywords: myocardial ischemia, trimetazidine, mitochondrial respiratory function, biosynthesis, fission/fusion

Introduction

Cardiovascular diseases are the most common cause of death in both industrialized and developing countries, and the incidence of ischemic heart disease is increasing. Following ischemic heart disease and heart surgery, myocardial ischemia/reperfusion (I/R) injury, a pathophysiological phenomenon is often observed (1, 2); elimination of this injury is urgently required. Conventional anti-ischemic agents such as beta-blockers, calcium channel blockers, and nitrates are usually used for patients with stable coronary artery disease. Unfortunately, these agents have side effects affecting heart rate and blood pressure. Conversely, trimetazidine [1-(2,3,4-trimethoxyl-benzyl) piperazine dihydrochloride] has a therapeutic potential in the management of myocardial ischemia without affecting the hemodynamic determinants of myocardial oxygen consumption. Thus, it is regarded as the ideal cellular anti-ischemic agent both in experimental conditions and in clinical trials (3, 4).

Few mechanisms have been proposed to explain the beneficial effects of trimetazidine in curing diseases. Previous studies revealed that the pharmacological mechanism of trimetazidine was involved in inhibiting oxidation, limiting acidosis and intracellular accumulation of sodium and calcium, reducing the utilization of fatty acids, protecting potassium permeability, and alleviating myocardial damage (5–7). Most notably, trimetazidine has been reported as having cardioprotective efficacy in several models of myocardial infarction (8). Trimetazidine was found to directly inhibit cardiac fibrosis via reduction of collagen accumulation, nicotinamide adenine dinucleotide phosphate-oxidase levels, reactive oxygen species (ROS) production, and connective tissue growth factor expression in cardiac fibroblasts (9). Trimetazidine exerts protective effects against cardiac I/R injury via cardiac

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miRNA-21 expression and via Akt and Bcl-2/Bax pathways (10) or adenosine 5'-monophosphate-activated protein kinase and extracellular regulated protein kinase signaling pathways (11). Trimetazidine also improved sarcolemma's mechanical resistance to edema-induced mechanical stress in an I/R animal model (12). To date, few studies have investigated endogenous molecules involved in mitochondrial functions in rats with acute myocardial ischemia following endogenous compounds trimetazidine administration. The current study was designed to investigate the effects of trimetazidine on mitochondrial respiratory function, biosynthesis, and fission/fusion in hearts of rats with acute myocardial ischemia.

Methods

Ethic Committee report

Adult male Wistar rats weighing 320–350 g used in this study were purchased from Experimental Animal Center, Military Medical Science Academy of Chinese people's Liberation Army [License No. SCXK (Army) 2012-0004]. The animal experiment was conducted according to the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. All animals received humane care in compliance with the Guide for the Care and Use of Experimental Animals (Animal Care Committee, 2002).

Treatment

Animals were divided into four groups (20 rats in each group): sham control (In), ischemia (I), sham control-trimetazidine (Tn), and ischemia-trimetazidine (T). The animals in trimetazidine-treated groups were randomly allocated to pretreatment with 10 mg/kg/d trimetazidine (Servier Laboratories, Neuilly, France), whereas those in non-trimetazidine-treated group received the same quantity of saline solution. Trimetazidine was daily administered orally by gavage for 7 days before the induction of ischemia. Sham-operated group received the same surgical procedure as the other groups without being subjected to the ischemia protocol. Trimetazidine solution was prepared daily; it was dissolved in saline [0.9% NaCl (w/v)] and appropriately warmed to body temperature before injection.

Surgical procedure

The technique of ischemia induction described by Kim et al. (13) was used in this study. The surgical procedure was performed 1 h after the last drug or saline administration, with the animals under general anesthesia induced using pentobarbital sodium (0.2 mL/100 g). After intubation and ventilation with room air for ischemia experiments, the sutured skin over the chest region was opened, a 5–0 suture loop was prepared, and an electrocardiogram was attached. After a 30-min ischemia, the animals were killed, and their hearts were immediately removed. Mitochondria were isolated according to the procedure described below.

Isolation of mitochondria

Rat heart mitochondria were isolated as described by Johnson et al. (14). Briefly, after the rats were killed, ischemic and non-ischemic areas of the myocardial tissue were rapidly excised and placed in a medium containing 250 mM of sucrose, 10 mM of Tris, and 1 mM of the chelator ethylene glycol-bis (β-aminoethyl ether)-N,N,N,N-tetraacetic acid (EGTA) with a pH of 7.8 at 4°C. The tissue was scissor minced and homogenized on ice using a Teflon Potter homogenizer. The homogenate was centrifuged at 600×g for 10 min (Sorvall RC 28 S). The supernatant was centrifuged for 5 min at 15000×g to obtain the mitochondrial pellet. The latter was washed with the same medium and centrifuged at 15000×g for 5 min. Then, the resulting mitochondrial pellet was washed with the same medium to omit EGTA and was centrifuged for 5 min at 15000×g to obtain a final pellet containing 50 mg of protein/mL. The protein content was determined using the method of Compton et al. (15). The mitochondrial suspension was stored on ice before performing mitochondrial respiration assay.

Respiratory Control Ratio (RCR) determination

O_2 consumption was measured using Oxygraph-2k (Oroboros, Schroecken, Vorarlberg, Austria) in a thermostat-controlled chamber. Mitochondria (300 µg) were added to 2.0 mL of the phosphate buffer containing 70 mM sucrose, 225 mM Mannitol, 1 mM EDTANa2, 10 mM KH2PO4, 10 mM K2HPO4, and 0.1% BSA (pH, 7.4). Mitochondrial respiration was initiated by adding 0.8 M malate and 1 M glutamate, and oxidative phosphorylation was started by the adding adenosine diphosphate (ADP) to a final concentration of 50 mM. O_2 consumption recordings allowed the calculation of RCR, which is ADP consumed divided by O_2 used in state 3 respiration.

Mitochondrial ultrastructure

Mitochondrial ultrastructure was observed using scanning electron microscopy. For this examination, ischemic and non-ischemia areas of myocardial tissue were excised, followed by fixation in 3% glutaraldehyde in phosphate buffer (pH, 7.4) for 2 h at 4°C. Further fixation was done in 1% osmium tetroxide buffer for 1.5 h, and dehydration was done using solutions with ascending alcohol concentrations (70% alcohol solution was saturated by uranyl acetate). Next, the tissue was embedded in Epon-812 epoxy resin. Serial ultrathin sections were made using a Leica ultramicrotome (Leica Microsystems Inc, LKB-II, Germany), followed by lead staining according to Reynolds. The data were viewed and photographed using an H-12 electron microscope (Hitachi, Tokyo, Japan). Final images were scanned at 1200 and 2400 dpi resolutions.

Mitochondrial respiratory chain complex I activity

Enzymatic activities of mitochondrial respiratory chain complex I were spectrophotometrically determined using fresh mitochondria isolated from rat myocardial tissue as

previously described (16, 17). Mitochondrial respiratory chain complex I was calculated as the rotenone-sensitive rate of nicotinamide adenine dinucleotide (NADH)-oxidation (ε=6180 M–1 at 340 nm).

Western blot analysis

For Western blot analysis, proteins in the mitochondria isolated from rat myocardial tissue were separated using 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and electrophoretically transferred to polyvinylidene fluoride membranes. Then, the membranes were blocked in 5% non-fat milk for 2 h at room temperature and incubated at 4°C overnight with polyclonal anti-PGC-1α and mitochondrial transcription factor A (Tfam). After overnight incubation, the membranes were washed and immunoblotted with horseradish peroxidase-conjugated anti-rabbit IgG antibody (Cell Signaling Technology, MA, USA) at 37°C for 1 h. The membranes were then developed using an enhanced chemiluminescence substrate (Millipore, Molsheim, France) and exposed to an X-ray film. β-tubulin (Abcam, Massachusetts, USA) was used to ensure adequate sample loading for all western blots. Polyclonal antibodies against PGC-1α and Tfam used in this study were purchased from Abcam, Massachusetts, USA. Band density was quantitated using Image J software (Image J 1.35, National Institute of Mental Health, Maryland, USA).

Gene expression analysis

mRNA expression of mitochondrial fusion-related genes Drp1, Mfn1, Mfn2, and Opa1 in mitochondria isolated from rat myocardial tissue was determined at indicated times by RNA preparation and quantitative reverse transcription-polymerase chain reaction (RT-PCR). Briefly, total RNA was isolated from tissue using TRIZOL reagent following the manufacturer's instructions (TaKaRa, Osaka, Japan). RNA quality was assessed using agarose gel electrophoresis, and complementary DNA (cDNA) was synthesized with random hexamer (TaKaRa, Osaka, Japan). The real time RT-PCR analysis was performed using the QuantiTect SYBR Green RT-PCR Kit (Qiagen, Valencia, CA) under the ABI Prism 7500 Sequence Detector (Applied Biosystems, Foster City, CA, USA) according to the manufacturers' instructions. The reaction run at 1 cycle of 94°C for 10 min, 95°C for 15 s and 57°C for 30 s, followed by 40 cycles of 95°C for 15 s, 60°C for 1 min, and 95°C for 15 s. β-actin (Santa Cruz Biotechnology, CA, USA) was used as the control. Specific primer sequences (Invitrogen, Carlsbad, CA, USA) are shown in Table 1.

Statistical analysis

All data were expressed as mean±standard deviation (n=6 for each group). One-way analysis of variance (ANOVA) using SPSS 19.0 software (IBM, Armonk, NY, USA) was performed for comparison of group data. If ANOVA was significant, multiple comparisons were performed using the Newman–Keuls test; $p<0.05$ was considered to be significant.

Table 1. Primers used in this study

Gene	Product (bp)	Sequence (5′-3′)
β-actin	150	F: CCCATCTATGAGGGTTACGC
		R: TTTAATGTCACGCACGATTTC
Drp1	167	F: TGGAGATGGTGGTCAGGAAC
		R: CACAATCTCGCTGTTCTCGG
Mfn1	150	F: TTGTCGCCTGTCTGTTTTGG
		R: GCATTGACTTCACTGGTGCA
Mfn2	203	F: ACCGCCATATAGAGGAAGGC
		R: GCACAGCTTGTCACAGTTCA
Opa1	237	F: TACCACAGTCCGGAAGAACC
		R: GTGTTGGCAGTGATAGCGAG

Drp1 - dynamin-related protein 1; Mfn1 - mitofusins 1; Mfn2 - mitofusins 2; Opa1 - optic atrophy 1

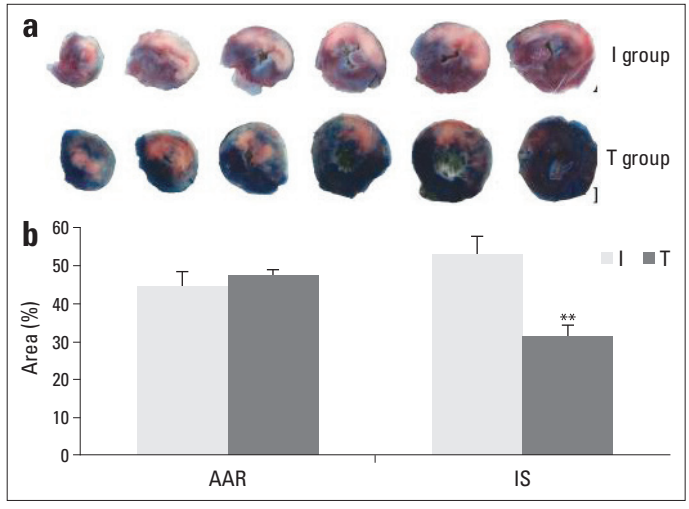

Figure 1. Isolated hearts (a) were used to determine AAR and IS (b) of heart in rats with acute myocardial ischemia

AAR - area at risk; IS - infarct size. Results were presented as means±SD. *$P<0.05$ compared with control

Results

Effects of trimetazidine on the infarct size (IS) of heart in rats with acute myocardial ischemia

Hearts of ischemic rats and trimetazidine-treated ischemic rats were isolated (Fig. 1a), and the area at risk (AAR) and IS of hearts were determined. Results presented in Figure 1b indicate that the percentage of AAR changed a little between the ischemic group and ischemic-trimetazidine group (44.42±4.15% vs. 47.12±1.94%), indicating that there was no difference in ischemic area between the two groups ($p>0.05$). However, compared with the ischemic rats without trimetazidine administration, a significant reduction in IS percentage was observed in the trimetazidine-treated ischemic rats (31.24±3.02% vs. 52.87±4.89%) ($p<0.05$). It was suggested that trimetazidine reduced IS in hearts of rats with acute myocardial ischemia.

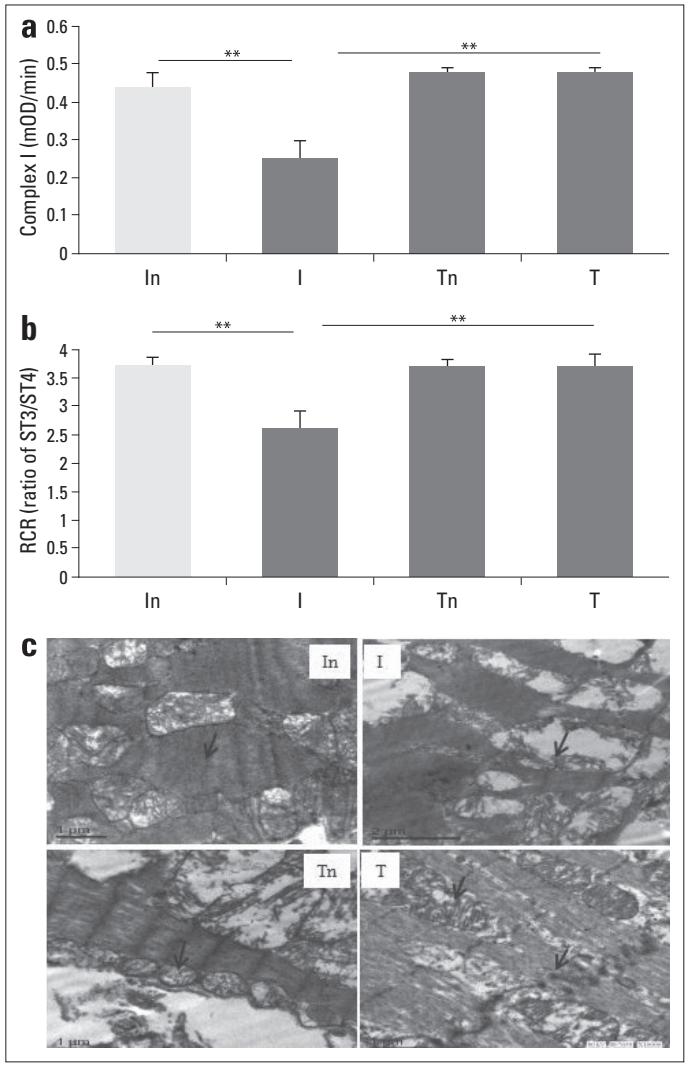

Figure 2. Complex I activity (a), RCR (b), and ultrastructure (c) of mitochondria were tested in experimental groups

RCR - respiratory control ratio. Results were presented as means±SD. *P<0.05 compared with control

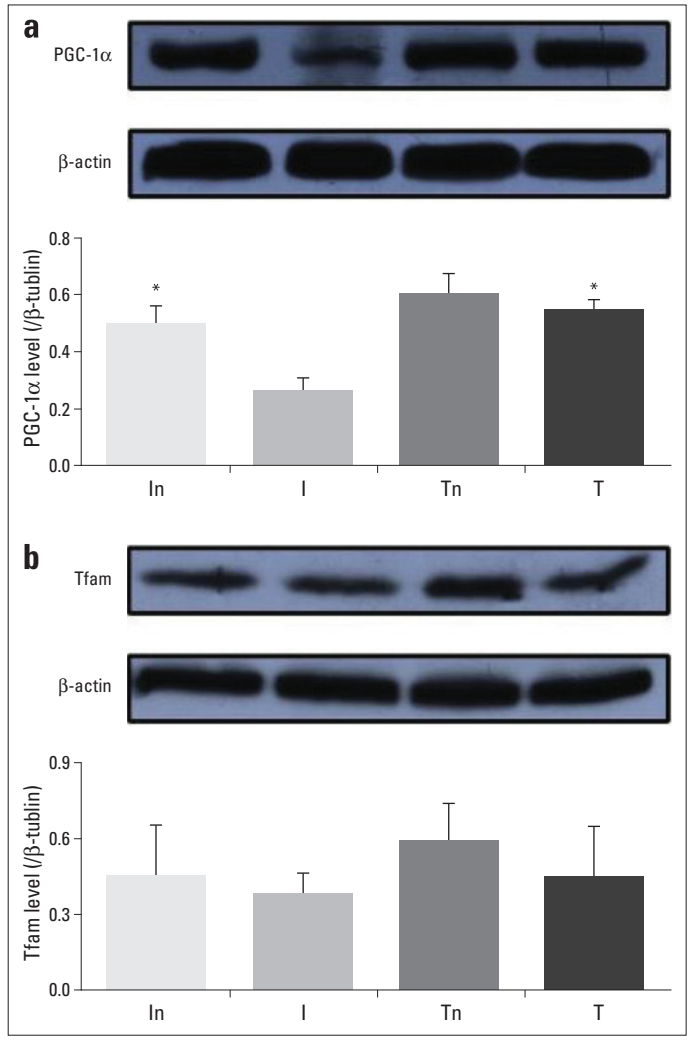

Figure 3. The protein expression level of PGC-1α (a) and Tfam (b) was determined by Western blot analysis in experimental groups

PGC-1α-peroxisome proliferator-activated receptor gamma (PPARγ) co-activator 1α; Tfam-mitochondrial transcription factor A. Results were presented as means±SD. *P<0.05 compared with control

Effects of trimetazidine on mitochondrial respiratory function in rats with acute myocardial ischemia

The comparison of mitochondrial respiratory function in rats at the end of ischemia showed that complex I activity (Fig. 2a) and RCR (Fig. 2b) in ischemia group were significantly decreased compared with those in the normal group ($p<0.05$); RCR and respiratory complex I activity did not significantly differ between the sham control and sham control-trimetazidine groups ($p>0.05$). However, trimetazidine restored the ischemia-induced decrease in RCR and complex I activity to the normal level ($p<0.05$).

In addition, after the isolation of rat mitochondria, mitochondrial ultrastructure was observed on electron microscopy (Fig. 2c). It was found that the mitochondria in sham control group were intact and showed a round or oval shape. Furthermore, the mitochondria were orderly arranged, and the cristae were closely connected. In ischemia group, mitochondrial morphology was significantly damaged; the majority of mitochondria were swollen and their cristae fragmented. In contrast, in ischemia-trimetazidine group, mitochondria were intact with clear and visible cristae, and only a few mitochondria were slightly swollen. Thus, the results of complex I activity, RCR, and mitochondrial structure analyses provided the evidence that trimetazidine promoted mitochondrial respiratory function in rats with acute myocardial ischemia.

Effects of trimetazidine on biosynthesis of mitochondria in rats with acute myocardial ischemia

Biosynthesis-related proteins including PGC-1α and Tfam were determined using western blot analyses. As shown in Figure 3a, compared with sham control group, the PGC-1α protein level was significantly decreased in ischemia group ($p<0.05$), but it was slightly increased in sham control-trimetazidine group ($p>0.05$). Moreover, after ischemic rats were treated with trimetazidine, the decreased PGC-1α protein expression was highly res-

Table 2. Effects of trimetazidine on the expression of mitochondrial fission/fusion-related factors

Groups	Drp1	Mfn1	Mfn2	Opa1
In	1.00±0.00	1.00±0.00	1.00±0.00	1.00±0.00
I	0.34±0.07**	0.37±0.16**	1.08±0.05	0.26±0.15**
Tn	0.95±0.02	1.09±0.30	3.77±0.29	0.85±0.08
T	0.52±0.08#	0.95±0.25##	3.41±0.26##	0.65±0.06#

In - sham control; I - ischemia; Tn - sham control-trimetazidine; T - ischemia-trimetazidine. Data are expressed as mean±SD; n=6 per group. Drp1 - dynamin-related protein 1; Mfn1 - mitofusins 1; Mfn2 - mitofusins 2; Opa1 - optic atrophy 1; *P<0.05 vs. In group; #P<0.05 vs. I group

tored (0.51±0.09 vs. 1.06±0.08, p<0.05). There were no differences in PGC-1α expression between sham control-trimetazidine and ischemia-trimetazidine groups (p>0.05). Results presented in Figure 3b prove that Tfam expression showed no difference between four treatments (p>0.05). Thus, it was suggested that trimetazidine heavily stimulated mitochondrial biosynthesis in rats with acute myocardial ischemia.

Effects of trimetazidine on fission/fusion of mitochondria in rats with acute myocardial ischemia

Mitochondrial fission/fusion is primarily regulated by Drp1, Mfn1/2, and Opa1. The effects of trimetazidine on mitochondrial fission/fusion in rats with acute myocardial ischemia are shown in Table 2. The expression of Drp1, Mfn1, and Opa1 was heavily suppressed by ischemia compared with non-ischemia treatment (p<0.05). Trimetazidine alone did not affect the expression of Drp1, Mfn1, and Opa1 in sham control rats (p>0.05). Conversely, when ischemic rats were treated with trimetazidine, the expression of Drp1, Mfn1, and Opa1 was significantly enhanced to the normal level (p<0.05). In addition, there was no difference in Mfn2 expression between sham control and ischemia groups (p>0.05). Trimetazidine promoted Mfn2 expression both in ischemic and non-ischemic rats (p<0.05). Collectively, our findings revealed that trimetazidine maintained fission/fusion in rats with acute myocardial ischemia via upregulating the expression of Mfn1, Opa1, and Drp1.

Discussion

In this study, we found that trimetazidine reduced IS in hearts of rats with acute myocardial ischemia and further protected the mitochondrial respiratory function, biosynthesis, and fission/fusion. Owing to its limited hemodynamic effects, trimetazidine alone or as a part of a combination regimen has been used to protect from I/R injury including heart, kidney, intestine, and liver (18). Researchers have provided new evidence suggesting that trimetazidine played an important role in cardiovascular disease. For instance, trimetazidine may inhibit pressure overload-induced cardiac fibrosis and myocardial remodeling (19). Trimetazidine was beneficial in patients with ischemic dilated cardiomyopathy in a South Asian population (20, 21) via improving left ventricular function; reducing T-wave amplitude, QT interval, and number and duration of arrhythmias; and restoring oxidation–phosphorylation coupling (22). By increasing sarcolemmal resistance, trimetazidine protected cells from potential apoptosis and the resultant ventricular dysfunction (7, 23).

In the present study, we found that trimetazidine promoted RCR and complex I activity in ischemic rats and restored mitochondrial structure to normal. Mitochondria constitute about 45% of myocardial volume and are key factors in energy production and life cycle in cells. Furthermore, mitochondria are the key to I/R protection (24). RCR is thought to be a particularly valuable measure of mitochondrial function because it is responsive essentially to all changes in the functionality of the mitochondrial electron transport chain (ETC) (25). Using a rabbit model of non-ischemic heart failure, Dedkova et al. (26) evaluated the cellular mechanisms of the cardioprotective action of trimetazidine and suggested that trimetazidine protected heart failure via attenuation of ROS generation by ETC and uncoupled mitochondrial nitric oxide synthase. Additionally, trimetazidine inhibited the elevated electron leak at the level of mitochondrial ETC complex II and improved the impaired activity of mitochondrial complex I. Specifically, we measured the respiratory function of the mitochondria by evaluating RCR ratio at state 3 (maximum metabolic rate) to state 4 (basal metabolic rate), mitochondrial complex I activity, and mitochondria structure.

We speculated that trimetazidine protected heart mitochondria against the deleterious effects of ischemia via upregulating the expression of PGC-1α. As part of our efforts to explore the regulated functions of trimetazidine in mitochondrial biosynthesis in hearts of rats with acute myocardial ischemia by measuring PGC-1α and Tfam protein levels. PGC-1α and its downstream factor Tfam are crucial regulators of mitochondrial biogenesis and energy metabolism. Furthermore, PGC-1α is a pivotal co-activator protein related to numerous transcription factors and increases their ability to induce expression of their cognate target genes (27). Deregulation of PGC-1α mRNA levels was observed in obesity and several other diseases (28). A main attribute of PGC-1α is its ability to promote oxidative metabolism and enhance mitochondrial biogenesis (29). Consequently, we also provided the evidence that PGC-1α was downregulated in rats with myocardial ischemia and this effect was heavily changed by trimetazidine. Previous studies indicated that trimetazidine used clinically could improve energy metabolism during the process of myocardial ischemia (30, 31). Using isolated cardiac mitochondria prepared from rat hearts, trimetazidine at concentrations 10–100 μM in the presence of 25–100 nM extramitochondrial Ca^{2+} could boost mitochondrial Ca^{2+} level, thus, leading to the enhancement of oxoglutarate dehydrogenase activity and then, ATP synthesis (32). Salducci et al. (33), using rat liver mitochondria in contact with Ca^{2+}, also demonstrated that trimetazidine restored ATP synthesis and calcium accumulation decreased by cyclosporine A in a dose-dependent manner. Trimetazidine prevented I/R injury to guinea pig retina via suppressing retinal lipid

peroxidation and histopathologic changes (34). Trimetazidine decreased the utilization of free fatty acids for energy production due to its inhibitory effect on mitochondrial 3-ketoacyl CoA thiolase in beta-oxidation (35).

Mitochondrial fission/fusion has been recognized as critical processes in the health of mitochondria and cells (36). Mitochondrial fission is required for daughter cell inheritance of mitochondria during cell division, while fusion is required for complementation of mitochondrial genomes (37). At the molecular level, mitochondrial fission/fusion is primarily regulated by Mfn1/2, Opa1, and Drp1, which together keep mitochondrial membrane intact. Mfn1/2, two-pass outer membrane proteins, mediate outer membrane fusion via heterotypic interactions between neighboring mitochondria (38). Conversely, OPA1 is an inner membrane protein, which simultaneously ensures matrix connectivity by regulating the melding of inner membranes (39). Drp1 is a cytosolic protein, which is recruited from the cytosol to prospective sites of fission on the mitochondrial outer membrane to catalyze mitochondrial fission (40). We demonstrated that trimetazidine maintained fission/fusion in rats with acute myocardial ischemia via upregulating the expression of Mfn1, Opa1, and Drp1.

Study limitations

Our study had several limitations such as the small number of cases, lack of long-term curative effects, and the limited dose of trimetazidine, which all might weaken the results. Therefore, we will further accumulate the cases, strength the design, and use different dose of trimetazidine to explore the effects of trimetazidine.

Conclusion

In conclusion, our study found that acute myocardial ischemia results in damage to mitochondrial functions, and treatment with trimetazidine may at least partly reverse myocardial ischemia by reducing IS in hearts, enhancing mitochondrial respiratory function, promoting mitochondrial biosynthesis, and maintaining mitochondrial fission/fusion. Therefore, trimetazidine may be a good choice in the prevention of myocardial ischemia in patients with heart disease.

Competing interests: The authors declare that they have no competing interests.

Conflict of interest: None declared.

Peer-review: Externally peer-reviewed.

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