Protective effect of Yi-Qi-Huo-Xue Decoction against ischemic heart disease by regulating cardiac lipid metabolism

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Available online 20 Oct., 2020

[ABSTRACT] Yi-Qi-Huo-Xue Decoction (YQHX) is the recombination of Dang-Gui-Bu-Xue Decoction (DBD), which is one of the well-known traditional Chinese Medicine (TCM) prescription, and has long been shown to have significant protective effects against myocardial ischemic injury. In previous studies, we found that YQHX could regulate lipid and glucose metabolism, promote angiogenesis, attenuate inflammatory response, and ameliorate left ventricular function in myocardial ischemia rat models. However, the underlying mechanism of how YQHX involves in lipid metabolism remains unclear so far. In this study, the underlying mechanism of YQHX in lipid metabolism disorders was elucidated in a myocardial ischemia rat model and a hypoxia-induced H9c2 cell injury model. YQHX (8.2 g·kg−1) and positive-control drug trimetazidine (10 mg·kg−1) were administered daily on the second day after left anterior descending (LAD) operation. At 7 days and 28 days after surgery, changes of cardiac morphology, structure, and function were evaluated by H&E staining and echocardiography, respectively. The plasma lipid levels and mitochondrial ATP content were also evaluated. Western blot and RT-PCR were used to determine the protein and mRNA expressions of AMPK, PGC-1α, CPT-1α, and PPARα. YQHX improved cardiac function and ameliorated lipid metabolism disorders. Furthermore, YQHX increased the expression of p-AMPK, PGC-1α, and CPT-1α without changing PPARα in ischemic rat myocardium. In vitro, YQHX activated the protein and mRNA expression of PGC-1α, CPT-1α, and PPARα in hypoxia-induced H9c2 cells injury, whereas AMPK inhibitor Compound c blocked the effects of YQHX. Taken together, the results suggest that YQHX reduces lipid metabolism disorders in myocardial ischemia via the AMPK-dependent signaling pathway.

[KEY WORDS] Yi-Qi-Huo-Xue Decoction; Lipid metabolism; Ischemic heart disease; AMPK-dependent pathway; Fatty acid oxidation.

[CLC Number] R965
[Document code] A
[Article ID] 2095-6975(2020)10-0779-14

Introduction

In recent years, according to the lifestyle changes, dietary habits, and an aging population, the incidence of ischemic heart disease has gradually become a leading cause of death, which affected more than 17 million people worldwide in 2008. Furthermore, the number is estimated to rise by 23.6 million in 2030 [1,2]. Despite a series of significant therapeutic developments that share the aim to rapidly restore coronary artery blood flood, poor prognosis of IHD remains a significant clinical problem, which urgently requires novel therapeutic strategies. The 2016 Heart Outcomes Prevention Evaluation-3 (HOPE-3) trial supports that using rosuvastatin, at a dose of 10 mg per day to lower cholesterol, decreases the risk of cardiovascular events among 12 705 participants in 21 countries without cardiovascular diseases [3]. Given that fatty acids provide more energy production than glucose, recently people have gradually realized that lipid metabolic dysfunction plays an important role in the pathogenesis of cardiovascular diseases [4-7]. It suggests that reverting cardiac metabolism back to utilizing fatty acid metabolism may be a plausible therapeutic choice for treating myocardial ischemia [8,9]. As a “metabolic master switch”, AMP-activated protein kinase (AMPK) maintains energy homeostasis and regulates lipid and glucose metabolism for adaptation to stresses response, especially during and following ischemia [10-12]. Ischemia causes a significant increase in the myocardial...
levels of AMPK phosphorylation and activity. AMPK activates or inactivates a series of metabolic pathways, including peroxisome proliferator activated receptor gamma co-activator 1α (PGC-1α), carnitine palmitoyltransferase-1α (CPT-1α), and peroxisome proliferator activated receptor α (PPARα), which is involved in fatty acid and cholesterol synthesis. However, the mechanism by which myocardial AMPK phosphorylation is activated in myocardial ischemia remains unclear so far.

Yi-Qi-Huo-Xue decoction (YQHX) is a compound Chinese medicine, which has been widely applied in the treatment of ischemic heart disease with efficacy and safety. Results of high-performance-liquid-chromatography (HPLC) and HPLC-linear ion trap-Orbitrap mass spectrometry analyses indicated that the active substances of YQHX include Astragaloside IV, Calycosin, Ferulic acid, ginsenoside Rg1 and ginsenoside Rb1. Our previous studies reported that YQHX could exert cardioprotective effects in ischemic rat myocardium by inhibiting oxidative stress and inflammation response, promoting angiogenesis and protecting mitochondrial function. Results of nuclear magnetic resonance metabolomics also showed YQHX could effectively regulate plasma and urine levels of lipid, amino acid and carbohydrate metabolism in rat models of IHD. Recently, we reported that YQHX could further activate AMPK phosphorylation in hypoxia-induced cardiomyocytes injury. However, as of right now, the mechanisms of YQHX on energy metabolism especially the activation of AMPK phosphorylation have not been completely evaluated. To address these unanswered questions, we use a rat myocardial infarction (MI) model, which is similar to the IHD patients affected by myocardial infarction to evaluate the change of lipid metabolism between the subacute (7 days) and chronic (28 days) stages after myocardial infarction. Then we use the hypoxia-induced H9c2 cell injury model with YQHX or/and AMPK inhibitor (Compound c) to assess the underlying lipid mechanisms of YQHX.

Materials and Methods

Preparation of YQHX

The herbs of YQHX were purchased from Dongzhimen Hospital of Beijing University of Chinese Medicine. YQHX is composed of five herbs (Table 1), including Astragalus membranaceus (Huang Qi, HQ), Angelica sinensis (Dang Gui, DG), Panax ginseng (Ren Shen, RS), Ligusticum wallichii (Chuan Xiong, CX), and Panax notoginseng (San Qi, SQ). The YQHX preparations were extracted by refluxing with boiling distilled water (1:10, g mL⁻¹) three times, respectively. After filtration, the aqueous extracts of YQHX were concentrated into a constant volume for use in animal experiments, and were prepared in powder form by freeze-drying in vacuo, respectively. YQHX freeze-drying powders (25 mg) were dissolved in 50 mL of DMEM in vitro. Subsequently, YQHX extracts were filtered through a 0.45 μm millipore filter prior to use. Then, a part of each filtrate was 4-fold diluted with distilled water and was further subjected to HPLC and UHPLC-LTQ-Orbitrap-MS, respectively.

Animal model and grouping

A total of 120 male Sprague-Dawley (SD) rats were purchased from Vital River Laboratory Animal Technology to be used for myocardial infarction surgery (Beijing, China, license number: SCXK2016-0006). Animals were housed at Dongzhimen Hospital Science cage under consistent conditions (12 h light or dark cycles). All experiments and protocols were performed in accordance with the Animal Care Committee and Use Committee of Dongzhimen Hospital Affiliated to Beijing University of Chinese Medicine (2017-11). The myocardial infarction rat model was established by a left anterior descending (LAD) coronary artery occlusion. Briefly, SD rats were anesthetized with 1% pentobarbital sodium at the dosage of 40 mg kg⁻¹ by intraperitoneal injection. Then, the thorax was opened to expose the heart, and the LAD was ligated with a 5–0 polypropylene suture except the Sham group. Finally, the chest was closed, and we used the sodium penicillin for 3 days after surgery for the purpose of anti-inflammation of the wound. From the second day after MI, rats in the YQHX group were treated with YQHX aqueous extract at the daily dosage of 8.2 g kg⁻¹. Rats in the positive control drug Trimetazidine (Servier, Tianjin, China, Series: H20055465) group were treated with Trimetazidine aqueous solution at the daily dosage of 10 mg kg⁻¹. Rats were acclimated for 1 week and randomly divided into four groups during the subacute (7 days) and chronic (28 days) stages, including Sham group (Sham), myocardial infarction model group (MI), YQHX group (YQHX) and positive-control drug Trimetazidine group (TMZ). All rats were anesthetized by pentobarbital sodium at the end of the study, and the cardiac samples were collected to analyze mRNA and protein expression levels.

Cell culture and grouping

Rat H9c2 cells were seeded 5 × 10⁴ cells/mL in 6-well
plates (Corning, New York, USA). H9c2 cells were maintained in high-glucose DMEM (Gibco, California, USA) supplemented with 10% fetal bovine serum FBS (BI, Israel), 1% of penicillin/streptomycin in a cell incubator with 5% CO₂ and 95% air at 37 °C. After 24–30 h culture, the culture medium was completely replaced with serum-free DMEM. After synchronization culture for 6 h, H9c2 cells were exposed to a hypoxic atmosphere with 1% O₂, 5% CO₂ and 94% N₂ for 12 h. The treatment of H9c2 cells with YQHX at the dosage of 200 μg mL⁻¹ (Dongzhimen Hospital, China) or/and AMPK inhibitor Compound c (5 μmol L⁻¹) (Selleck, Shanghai, China) were used before the initiation of hypoxia [25, 26]. H9c2 cells were randomly divided into four groups, including (1) Control group (C), (2) Ischemia/Hypoxia group (IH), (3) YQHX at the concentrations of 200 μg mL⁻¹ during hypoxia group (Y2), and (4) 200 μg mL⁻¹ YQHX supplemented with 5 μmol L⁻¹ Compound c during hypoxia group (Y2cc).

**Histopathology examination**

Hematoxylin and eosin (HE) staining was used to visualize cardiomyocyte architectures. Heart tissue samples were extracted from sacrificed rats, and immediately fixed in 4% paraformaldehyde solution. Then the cardiac samples were embedded in paraffin and were cut into 5 μm slices, which were stained with HE and observed under a light microscope (Olympus, Tokyo, Japan).

**Plasma lipid analysis**

At 7 and 28 days after MI, blood samples were collected from sacrificed rats, and centrifuged at 3000 r min⁻¹ at 4 °C for 20 min to obtain serum. Plasma levels of free fatty acids (FFA), total cholesterol (TC), triglyceride (TG), high-density lipoprotein (HDL), and low-density lipoprotein (LDL) were measured using a TBA-120 automatic biochemical analyzer (Olympus, Tokyo, Japan).

**Transmission electron microscopy**

Fresh samples of the left ventricle in the margin area were cut into thick slices, and rapidly fixed them in 2% paraformaldehyde and 2.5% glutaraldehyde at 4 °C for 2 h. After washing with (phosphate buffered saline) PBS three times, the samples were fixed with 1% osmium tetroxide for 2 h, and then dehydrated using acetone solutions. Next, the specimens were embedded with epoxy resin and heated polymerization at 60 °C. Finally, the embedding blocks were sliced into ultrathin sections. Images of different stained areas were recorded using a Hitachi-H7650 transmission electron microscopy (Tokyo, Japan).

**ATP content**

The fresh myocardial samples were dissected and homogenized in a cold homogenate buffer for 30 min. Then these were centrifuged at 12 000 g for 5 min at 4 °C to collect the supernatant. The myocardial mitochondrial ATP content were measured by a corresponding ATP Assay Kit (Beyotime, Shanghai, China).

**Western blot analysis**

Samples of myocardial tissues from the infarcted myocardial border, as well as H9c2 cells were prepared for protein analysis. Firstly, the heart tissues were homogenized and H9c2 cells were collected in an ice-cold RIPA lysis buffer. The protein extracts (40 μg) were separated by 12% SDS-PAGE blots (Bio-Rad, California, USA) and then transferred to the nitrocellulose membranes (Millipore, MA, USA). The nitrocellulose membranes were blocked with 5% fat-free dry milk in Tris-buffered saline for 2 h, washed, and then incubated with the following primary antibodies, including p-AMPK and AMPK (1: 1000, Cell Signaling Technology, Boston, USA), PGC-1α (1 : 1000, Abcam, MA, USA), CPT-1α (1 : 500, Proteintech, Rosemont, USA), and PPARα (1 : 1000, Proteintech, Rosemont, USA). The primary antibodies were incubated at 4 °C overnight, washed, and then incubated with the secondary antibodies for 2 h. After three washed, the blots were incubated with ECL and then detected by using the Gene Genius Bio Imaging System.

**Real-time quantitative PCR analysis**

The mRNA expressions of AMPK, PGC-1α, CPT-1α, and PPARα were determined by reverse transcriptase polymerase chain reaction (RT-PCR). Total RNA was extracted from cardiac tissues and H9c2 cells using Trizol reagent (Invitrogen, California, USA) and subjected to reverse transcription. Thereafter, the first-strand cDNA was synthesized using a Revert Aid TM First Strand cDNA Synthesis kit (Invitrogen, California, USA). Next, real-time quantitative PCR was performed in triplicate using SYBR® Premix Ex Taq kit on an ABI PRISM 7500 PCR instrument (Applied Biosystems, FosterCity, USA). The cDNA was denatured by 35 PCR cycles (2 min at 94 °C, 30 s at 94 °C, 30 s at 61 °C, 30 s at 72 °C). GAPDH was used for the invariant control, and the relative level of mRNA was calculated using the 2⁻ΔΔCt method. The forward and reverse sequences for the primers are listed in Table 2.

**Statistical analysis**

All data are expressed as mean ± SD, and the statistically significant differences between Sham group, MI group, YQHX group and other differences groups were calculated via one-way ANOVA and Duncan’s multiple range test. P values less than 0.05 were considered as statistically significant. All statistical analyses were conducted using SPSS 17.0 software and performed by GraphPad 6.0 Prism software.

**Results**

**Effects of YQHX on cardiac function in MI rats**

In order to assess whether YQHX improves cardiac function after myocardial infarction, we initially examined the cardiac function in different groups after MI or Sham opera-
Effects of YQHX on cardiac histopathology changes in MI

7 days after MI (Fig. 2A), rats in the MI group had myocardial edema and necrosis, and reduced inflammatory cell infiltration. 28 days after MI (Fig. 2B), the MI group myocardial fibers were arranged irregularly and disorderly as an interstitial substance with edema and cardiac muscle membrane damage. A considerable number of cardiomyocytes were replaced by increased fibroblasts and collagen. YQHX and TMZ treatment significantly improved myocardial edema and necrosis, and reduced inflammatory cell infiltration.

Effects of YQHX on plasma lipid levels in MI rats

To determine the effects of YQHX on lipid metabolism in MI rats, we next detected plasma lipid levels in different groups after MI or sham operation. 7 days after MI, the levels of TC in the MI group were significantly increased compared with the Sham group (P < 0.05). FFA, TG, and LDL levels in the MI group also were increased, but there were no statistically significant differences (P > 0.05). Compared with the MI group, YQHX group significantly decreased serum FFA and TG levels.

Table 2  Primers used in this study

<table>
<thead>
<tr>
<th>Gene</th>
<th>Species</th>
<th>Forward (5′−3′)</th>
<th>Reverse (5′−3′)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AMPK</td>
<td>Rat</td>
<td>GTTAAATCCCACTACCACAAC</td>
<td>GAGGACTCGGATCAATA</td>
</tr>
<tr>
<td>PGC-1α</td>
<td>Rat</td>
<td>GACCGTTCCAAGGATTCA</td>
<td>GGTCTTGGTCCAGGGCTC</td>
</tr>
<tr>
<td>CPT-1α</td>
<td>Rat</td>
<td>GGCTCTGGTGCGCGTACAT</td>
<td>CCGTGTTCTGCAAAACTCCA</td>
</tr>
<tr>
<td>PPARα</td>
<td>Rat</td>
<td>TCCACAAAGTGCGTGTCGTCGT</td>
<td>CTTTCCTGCGATGATGACCC</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Rat</td>
<td>GGCAAGTTCACGCGACAG</td>
<td>CGCCAGTAGACTCCACGAC</td>
</tr>
</tbody>
</table>

Table 3  Indicators of cardiac function in different groups 7 days after myocardial infarction (mean ± SD, n = 6)

<table>
<thead>
<tr>
<th>Group</th>
<th>EF% (mean ± SD)</th>
<th>FS% (mean ± SD)</th>
<th>LVIDs (mm) (mean ± SD)</th>
<th>LVIDd (mm) (mean ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham</td>
<td>85.68 ± 3.67</td>
<td>55.78 ± 4.24</td>
<td>2.43 ± 0.33</td>
<td>5.48 ± 0.39</td>
</tr>
<tr>
<td>MI</td>
<td>34.99 ± 2.76***</td>
<td>17.45 ± 1.54***</td>
<td>6.62 ± 0.49***</td>
<td>8.02 ± 0.57***</td>
</tr>
<tr>
<td>YQHX</td>
<td>50.62 ± 10.66***</td>
<td>27.03 ± 6.55***</td>
<td>5.09 ± 0.96</td>
<td>7.39 ± 1.10</td>
</tr>
<tr>
<td>TMZ</td>
<td>59.82 ± 7.33***</td>
<td>33.24 ± 5.01***</td>
<td>5.33 ± 0.91**</td>
<td>7.94 ± 0.95</td>
</tr>
</tbody>
</table>

Table 4  Indicators of cardiac function in different groups 28 days after myocardial infarction (mean ± SD, n = 8)

<table>
<thead>
<tr>
<th>Group</th>
<th>EF% (mean ± SD)</th>
<th>FS% (mean ± SD)</th>
<th>LVIDs (mm) (mean ± SD)</th>
<th>LVIDd (mm) (mean ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham</td>
<td>80.24 ± 7.64</td>
<td>50.81 ± 7.90</td>
<td>2.98 ± 1.05</td>
<td>6.40 ± 1.06</td>
</tr>
<tr>
<td>MI</td>
<td>31.11 ± 3.82***</td>
<td>16.32 ± 2.90***</td>
<td>7.86 ± 1.03***</td>
<td>9.49 ± 1.31***</td>
</tr>
<tr>
<td>YQHX</td>
<td>48.03 ± 5.42***</td>
<td>25.36 ± 3.27***</td>
<td>6.02 ± 0.83***</td>
<td>8.27 ± 0.73***</td>
</tr>
<tr>
<td>TMZ</td>
<td>42.84 ± 5.59***</td>
<td>22.08 ± 3.28***</td>
<td>6.38 ± 0.83***</td>
<td>8.29 ± 0.97***</td>
</tr>
</tbody>
</table>

***P < 0.001 vs Sham group; **P < 0.01, *P < 0.05, #P < 0.01, **P < 0.001 vs MI group
Fig. 1  YQHX and TMZ improved cardiac function including LVEF, LVFS, and LVIDs/d in MI rats. (A) Effect of YQHX (8.2 g·kg\(^{-1}\)·d\(^{-1}\)) and TMZ (10 mg·kg\(^{-1}\)·d\(^{-1}\)) on cardiac function 7 days after myocardial infarction (B) Effect of YQHX and TMZ on cardiac function 28 days after myocardial infarction. Data are expressed as mean ± SD, n = 6–8. *P < 0.05, **P < 0.01, ***P < 0.001 vs Sham group; †P < 0.05, ‡P < 0.01, §§P < 0.001 vs MI group

levels \((P < 0.05)\). However, TMZ group had no significant change, suggesting that metabolic pathways may be different between YQHX group and TMZ group (Table 5). 28 days after MI (Table 6), the levels of TG were significantly higher in the MI group than in the Sham group \((P < 0.001)\). However, YQHX treatment significantly decreased TC and TG levels compared with the MI group \((P < 0.01)\). The effects of TMZ treatment on TC and TG levels were similar with those of YQHX \((P < 0.05)\). But no significant differences were found between YQHX group and TMZ group. The above results showed that TG levels were lesser than TC levels in the YQHX group 7 days or 28 days after MI, suggesting YQHX treatment has better efficacy on TG than TC, especially at the subacute stage. Furthermore, we found that MI-induced HDL levels all had an increasing trend at two points time, indicating that it may be a compensatory response after myocardial infarction.

**Effects of YQHX on p-AMPK and AMPK expression in MI rats**

As a central regulator of cardiomyocyte energy homeostasis, AMPK controls fatty acid oxidation, glucose transport and intracellular signaling pathways in response to low fuel supplies \([27, 28]\). Hence, Western Blot analysis confirmed whether YQHX in regulated AMPK phosphorylation at threonine 172 protein expression 7 days and 28 days after acute myocardial infarction. As shown in Fig. 3A, the phosphorylation level of AMPK was significantly activated in the MI group \((P < 0.05)\) compared with the Sham group 7 days after MI. After treatment with YQHX and TMZ, AMPK phosphorylation was further up-regulated \((P < 0.05)\). Similar increase was observed by Western Blot at 28 days after MI (Fig. 3B). Compared with the Sham group, AMPK phos-

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**Table 5** Effects of YQHX and TMZ on plasma lipid levels in MI rats at 7 d (mean ± SD, \(n = 5–9\))

<table>
<thead>
<tr>
<th>Group</th>
<th>FFA/(mmol·L(^{-1}))</th>
<th>TC/(mmol·L(^{-1}))</th>
<th>TG/(mmol·L(^{-1}))</th>
<th>HDL/(mmol·L(^{-1}))</th>
<th>LDL/(mmol·L(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham</td>
<td>0.90 ± 0.24</td>
<td>1.26 ± 0.15</td>
<td>1.07 ± 0.36</td>
<td>0.48 ± 0.07</td>
<td>0.56 ± 0.05</td>
</tr>
<tr>
<td>MI</td>
<td>0.94 ± 0.47</td>
<td>1.48 ± 0.22*</td>
<td>1.09 ± 0.41</td>
<td>0.59 ± 0.10</td>
<td>0.75 ± 0.18</td>
</tr>
<tr>
<td>YQHX</td>
<td>0.53 ± 0.26*</td>
<td>1.43 ± 0.10</td>
<td>0.45 ± 0.15**</td>
<td>0.64 ± 0.10</td>
<td>0.74 ± 0.05</td>
</tr>
<tr>
<td>TMZ</td>
<td>1.02 ± 0.26*</td>
<td>1.36 ± 0.21</td>
<td>1.37 ± 0.28</td>
<td>0.54 ± 0.11</td>
<td>0.55 ± 0.12</td>
</tr>
</tbody>
</table>

\(^*P < 0.05\) vs Sham group; \(^*P < 0.05, **P < 0.001\) vs MI group; \(^*P < 0.05\) vs YQHX group

**Table 6** Effects of YQHX and TMZ on plasma lipid levels in MI rats at 28 d (mean ± SD, \(n = 5–8\))

<table>
<thead>
<tr>
<th>Group</th>
<th>FFA/(mmol·L(^{-1}))</th>
<th>TC/(mmol·L(^{-1}))</th>
<th>TG/(mmol·L(^{-1}))</th>
<th>HDL/(mmol·L(^{-1}))</th>
<th>LDL/(mmol·L(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham</td>
<td>1.08 ± 0.32</td>
<td>1.64 ± 0.17</td>
<td>0.94 ± 0.21</td>
<td>0.56 ± 0.03</td>
<td>0.98 ± 0.18</td>
</tr>
<tr>
<td>MI</td>
<td>1.14 ± 0.59</td>
<td>1.96 ± 0.19</td>
<td>1.70 ± 0.55***</td>
<td>0.68 ± 0.08</td>
<td>0.80 ± 0.14</td>
</tr>
<tr>
<td>YQHX</td>
<td>0.97 ± 0.35</td>
<td>1.27 ± 0.42***</td>
<td>0.64 ± 0.22***</td>
<td>0.51 ± 0.10</td>
<td>0.62 ± 0.24</td>
</tr>
<tr>
<td>TMZ</td>
<td>0.88 ± 0.44</td>
<td>1.43 ± 0.43*</td>
<td>0.96 ± 0.31**</td>
<td>0.47 ± 0.10</td>
<td>0.77 ± 0.36</td>
</tr>
</tbody>
</table>

\(^***P < 0.001\) vs Sham group; \(^*P < 0.05, **P < 0.01, ***P < 0.001\) vs MI group
phorylation at threonine 172 protein expression was significantly increased in the MI group ($P < 0.05$). YQHX treatment further markedly increased AMPK phosphorylation at threonine 172 protein expression compared with MI group ($P < 0.001$). TMZ alone treatment did slightly increase after MI 28 days, but there was no statistical difference ($P > 0.05$). These data indicate that YQHX and TMZ-induced the balance of glucose or lipid metabolism may be attributable to further activating the phosphorylation level of AMPK. We next quantified AMPK mRNA expression using quantitative real-time PCR. Compared with the Sham group, the AMPK mRNA levels had an increase trend in the MI group (Fig. 3C), while those in the YQHX group were higher than the MI group, especially at 7 days after MI ($P < 0.01$).

**Effects of YQHX on lipid metabolism-related gene and protein expression in MI rats**

To determine the molecular mechanisms responsible for regulating lipid metabolism by YQHX treatment, we next examined the lipid metabolism-related gene and protein expression by RT-PCR and Western Blot. As AMPK can directly stimulate PGC-1α activity, which subsequently increases mitochondrial biogenesis and regulates of fatty acid oxidation $^{[15, 29]}$. We investigated changes of PGC-1α gene and protein expression at 7 days and 28 days after MI. The results revealed that the PGC-1α mRNA and protein in the MI group at 7 days after MI were significantly down-regulated compared with the Sham group ($P < 0.05$, Figs. 4A and 4C). YQHX treatment caused a significant increase in PGC-1α expression at mRNA and protein level ($P < 0.05$). Then we observed a similar decrease in the MI group at 28 days ($P < 0.05$). YQHX was shown to markedly increase PGC-1α mRNA and protein expression ($P < 0.05$). However, TMZ significantly decreased PGC-1α protein expression at 28 days after MI ($P < 0.01$, Fig. 4B). TMZ alone treatment did slightly decrease in PGC-1α mRNA level at 28 days after MI (Fig. 4C), but there was no statistical difference ($P > 0.05$). Compared with YQHX group, PGC-1α mRNA and protein levels were significantly decreased in the TMZ group ($P < 0.05$), indicating that TMZ could affect energy metabolism by inhibiting fatty acid oxidation.

As well as regulating the activity of PGC-1α, AMPK also regulates lipid metabolism through direct phosphorylation of acetyl-CoA carboxylase (ACC), promoting fatty acid oxidation via indirect relieving the suppression of CPT1. CPT-1, a key enzyme of fatty acid oxidation, catalyzes the rate-limiting step for mitochondrial fatty acid uptake. The CPT-1 activity is strongly inhibited by the cytosolic malonyl-CoA, which is controlled by the balance of two enzymes, ACC and malonyl-CoA decarboxylase (MCD) $^{[6, 7]}$. CPT-1α is the one of subtypes of CPT-1. Studies showed that TMZ as a weak CPT-1 inhibitor blocks fatty acid oxidation. We then investigated changes of CPT-1α expression at 7 days and 28 days after MI. RT-PCR and Western Blot revealed that CPT-1α expressions in the MI group after MI 7 days were down-regulated compared with the Sham group, respectively ($P < 0.05$, Figs. 4A and 4D). After treatment with YQHX, CPT-1α
mRNA and protein levels were up-regulated \( (P < 0.05) \). However, TMZ treatment was shown to further inhibit the activation of CPT-1\(\alpha\) expression compared with the MI group, but there was no statistical difference. Then we observed a similar significant decrease in the MI group at 28 days \( (P < 0.05) \), whereas YQHX alone treatment did slightly increase at 28 days after MI \( (P > 0.05) \), Fig. 4B. Interestingly, compared with MI group, TMZ alone treatment did slightly decrease after MI 28 days, but there was no statistical difference between MI group and TMZ group \( (P > 0.05) \). This agreed with the TMZ results of 7 days, indicating that YQHX and TMZ could exert differences in their metabolic patterns, and the lipid metabolism effects of YQHX had more advantages in 7 days after MI than 28 days after MI. PPAR\(\alpha\), one of three isoforms of PPARs, plays a key role in fatty acid \( \beta \)-oxidation \( ^7 \). PPAR\(\alpha\) activity is controlled at multiple levels, including its downstream lipid oxidation gene CPT-1 and interaction with the PPAR\(\gamma\) coactivator-1\(\alpha\) PGC-1\(\alpha\) \( ^7, 30 \). However, the effect of PPAR\(\alpha\) on ischemia is still controversial. Some studies showed that PPAR\(\alpha\) was chronically activated after ischemia that is harmful to cardiac recovery \( ^31 \). As shown in Figs. 4A and 4E, we observed that the expression of PPAR\(\alpha\) was significantly decreased in the MI group \( (P < 0.05) \), whereas YQHX treatment did slightly increase and TMZ slightly decrease 7 days after MI. However, different groups showed no effect on PPAR\(\alpha\) \( (P > 0.05) \), except for TMZ significantly decreased PPAR\(\alpha\) protein expression 28 days after MI \( (P < 0.05) \) (Fig. 4A). The above data indicated that the metabolic pattern of YQHX on lipid metabolism was different with those of TMZ.

**Effects of YQHX on mitochondrial structures and functions in MI rats**

The mitochondria are recognized as the powerhouse of cardiomyocytes, which generate about 90% of the ATP to
maintain cardiac function (systolic and diastolic) and energy metabolism (lipid and glucose) \cite{32}. We investigated the effects of YQHX on mitochondrial ultrastructure and function in rat MI models. As shown in Fig. 5A, the changes in mitochondrial ultrastructure were observed by transmission electron microscopy. 7 days after MI, there were irregular and disorderly ultrastructure of the myocardium, along with massive mitochondria destruction such as swelling, rupture and fracture of cristae in the MI group compared with the Sham group. After YQHX treatment, the destruction of myocardium and mitochondria were significantly improved. Specifically, YQHX caused massive accumulation of mitochondria in the left ventricle marginal areas. 28 days after MI, the MI group showed that myocardial and mitochondrial ultrastructure further were damaged, including irregular and disorderly myocardial ultrastructure, mitochondria swelling, loss of cristae, rupture and even vacuoles. However, YQHX treatment improved those events, especially increased mitochondrial proliferation, accumulation and content, suggesting that mitochondrial aggregation the phenomenon of mitochondrial aggregation may be an intrinsic characteristic of YQHX in protecting cardiac function. In addition, we also measured myocardial mitochondrial ATP levels in a rat MI model between 7 days and 28 days (Figs. 5B and 5C). Compared with the Sham group, MI group significantly decreased ATP levels ($P < 0.001$). However, YQHX group showed a significant increase in ATP content ($P < 0.001$). The data indicated that YQHX improved mitochondrial energy metabolism.

Effects of YQHX on PGC-1α, CPT-1α, and PPARα expression hypoxia-induced myocardial injury in H9c2 cells

To confirm the mechanism of YQHX regulated lipid metabolism, the lipid metabolism-related mRNA and protein expression were further examined in vitro (Fig. 6). Compared with the Sham group, the levels of PGC-1α, CPT-1α, and PPARα mRNA and protein were down-regulated at 12 h of hypoxia ($P < 0.05$). After treatment with YQHX, levels of

Fig. 5 YQHX improves cardiac mitochondrial ultrastructure and function in rat MI model. The mitochondrial ultrastructure and ATP content were measured by transmission electron microscope and ATP Assay Kits. (A) YQHX improves cardiac mitochondrial structures in rat MI model for 7 days and 28 days (cardiac mitochondrial structures under transmission electron microscope). (B) Effect of YQHX on the concentration of ATP in rat MI model for 7 days. (C) Effect of YQHX on ATP content in rat MI model for 28 days. Data are expressed as mean ± SD, $n = 5$. ***$P < 0.001$ vs Sham group; ****$P < 0.001$ vs MI group.
Fig. 6  YQHX increased PGC-1α, CPT-1α, and PPARα protein and mRNA expression in H9c2 cells subject to normoxia or hypoxia. (A) PGC-1α protein expression. (B) CPT-1α protein expression. (C) PPARα protein expression. (D) PGC-1α mRNA expression. (E) CPT-1α mRNA expression. (F) PPARα mRNA expression. Data are expressed as mean ± SD, n = 3–6. *P < 0.05, **P < 0.01 vs control group; *P < 0.05, ***P < 0.001 vs YQHX group

PGC-1α, CPT-1α, and PPARα mRNA and protein were significantly up-regulated, compared with those in the ischemia/hypoxia group (P < 0.05). However, treatment with compound c, an AMPK inhibitor, completely blocked the YQHX-induced up-regulated effects (P < 0.05), suggesting that YQHX regulated PGC-1α, CPT-1α and PPARα protein via AMPK pathway.

Discussion

Traditional Chinese medicine (TCM) has been widely applied in the treatment of ischemic heart disease for thousands of years. YQHX is re-developed based on the theory of supplementing “Qi” and nourishing “blood” in the well-known TCM formulas Danggui Buxue Decoction (DBD). There have been accumulating studies showing the significant protective effects of YQHX on myocardial ischemic injury [13]. And accumulating evidence further supports that the multiple activities of YQHX may involve multiple molecular targets and pathways [16,17]. However, the underlying metabolic mechanisms and particular pathways have remained unclear so far. The main findings (Fig. 7) of this study are that YQHX can regulate lipid metabolism by activating p-AMPK, PGC-1α, CPT-1α, and PPARα expression, whereas these effects were blocked with AMPK inhibitor Compound c. These results suggest that YQHX reduces lipid metabolism disorders in myocardial ischemia via the AMPK-dependent signaling pathway.

Lipid metabolism disorder is not only one of the major mechanisms of ischemic heart disease induced by coronary atherosclerosis according to the lipid infiltration theory of atherosclerosis, but also considered as a part of cardiac energy substrate selection according to energy depletion hypothesis [8, 9, 20, 22]. Wang Y et al. [8] found that pure ischemic myocardial infarction could cause plasma lipid disorder, with manifestation of up-regulation of triglyceride (TG), low density lipoprotein (LDL), Apolipoprotein B (Apo-B), and 3-hydroxy-3-methyl glutaryl coenzyme A reductase (HMGCR). The FATP-CPT1 lipid transport pathway was down-regulated in ischemic model group. Danqi Pill could improve lipid metabolism disorder induced by myocardial infarction through up-regulating FATP-CPT1 lipid transport pathway. Wang Q et al. [9] further reported that Lipoprotein lipase (LPL) and key membrane proteins involved in lipid transport and uptake, such as CD36, FABP4, and CPT-1α was down-regulated in ischemic heart tissues...
compared with the Sham group. Furthermore, PPARα, PPARγ, and PGC-1α relevant transcriptional factors, encoding enzymes involved in fatty acid and glucose utilization, were all down-regulated in ischemic model group. Treatment with Danqi Pill regulated lipid metabolism disorder through PPARs-PGC1α pathway. However, up to now, there are few reports on whether YQHX treatment can regulate lipid metabolism in pure myocardial ischemia at two time points. We further aim to explore the metabolic mechanism by which YQHX regulates lipid metabolism in myocardial ischemic injury. TMZ, as a well-known metabolic anti-ischemic agent, inhibits fatty acid metabolism and indirectly increases the pyruvate oxidation, hence it is used as a positive control drug [24, 35]. It is reported that plasma FFA, cholesterol, triglycerides, HDL, and LDL are primary components of plasma lipids, which are high risk factors for Coronary Heart Disease (CHD) [36]. At the subacute stage (Table 5), our study observed that the levels of plasma TC significantly were increased, and TG, LDL, and FFA levels had an increased trend in pure myocardial ischemia. Treatment with YQHX could markedly decrease the FFA and TG levels. However, TMZ group did not show significant change. At the chronic stage (Table 6), the plasma TG levels were significantly increased in the MI group, but the changes of FFA, TC, HDL, and LDL in serum were not obvious. Treatment with YQHX and TMZ could markedly decrease the TG and TC levels. These data indicate that the metabolic mechanism of YQHX and TMZ exists differently at the early stage of myocardial infarction. To our knowledge, energy substrate preference of myocardial ischemia has a complex pathophysiology. The role of alterations in myocardial energetics has been controversial for decades. Here, we also observed the activity of glucose (GLU) and lactic acid (LA) in serum (Supplementary Table 1). As shown in Supplementary Table 1, there was no significant change in GLU and LA content in 7 days after MI, but GLU and LA content were significantly increased in 28 days after MI. These data indicate that the cardiac metabolic profile reverts back to the “fetal phase” at the early stage of cardiac remodeling, which may be a compensatory response. However, glycolysis and lactic acid oxidation exist metabolic decompensation at the chronic stage, leading to lactic acid accumulation. TMZ treatment merely reduced glucose 28 days after MI, while failed to reduce LA levels in serum. However, YQHX treatment significantly reduced the levels
of glucose and LA in serum, especially 28 days after MI. These results further indicate that YQHX treatment can regulate glucose and lipid metabolism at 7 days and 28 days after MI. However, the metabolisms of YQHX on the cardiac energy substrate selection especially lipid metabolism in the ischemic heart remain to be answered.

AMPK is a critical regulator of energy metabolism, which plays a critical role in cardiovascular diseases [37]. Under normal conditions, AMPK catalyzes the substrates of downstream metabolic enzymes such as CPT-1α and PPARα, and increases catabolism whilst it decreases anabolism, thereby relatively increasing myocardial ATP level [37]. During myocardial ischemia, cellular depletion of ATP and the increased AMP/ATP ratio induced AMPK phosphorylation at threonine 172. Activated AMPK is involved in multiple cellular processes, including autophagy, the change of cardiac fatty acid and glucose metabolism [11, 38], especially importing myocardial cytosolic fatty acid into the mitochondria. Furthermore, activated AMPK accelerates production of lactate, which forms a vicious cycle, further threatening ischemic myocardium [38]. In the present study, we investigated the effect of YQHX on the expression of AMPK phosphorylation in model rats with myocardial ischemia (Fig. 3). The results of Western Blot and RT-PCR showed that YQHX and TMZ further activated AMPK phosphorylation at two time points in the ischemic myocardium. Although there was no statistical difference between YQHX and TMZ group, TMZ was a higher AMPK phosphorylation than YQHX at 7 days after MI. Interestingly, at 28 days after MI, we found that AMPK phosphorylation in myocardium of YQHX was higher than that seen in TMZ. These findings suggest that YQHX and TMZ may have a different way to optimize energy substrate metabolism such as lipid and glucose metabolism further improving cardiac function at different stages, which were via the activation of AMPK-dependent signaling pathway. To compare the differences between YQHX and TMZ, we study the mechanisms of YQHX on lipid metabolism, and further explore the different mechanisms between YQHX and TMZ.

AMPK is involved in multiple signaling pathways. Studies have demonstrated that AMPK can directly stimulate PGC-1α activity, which further regulates mitochondrial biogenesis and electron transport chain via co-activating other transcription factors, including nuclear respiratory factor 1/2 (NRF-1/2), the synthesis of transcription factor A mitochondrial (Tfam) and estrogen-related receptors (ERRs) [39]. PGC-1α also regulates fatty acid oxidation together with PPARα [15, 38]. PPARα, as one of the predominant subtypes of PPARs, is involved in the regulation of fatty acid oxidation (FAO) enzyme genes such as CPT-1α to reduce lipid accumulation and maintain the balance of lipid metabolism [30, 40-42]. However, the effect of PPARα on ischemia remains controversial. Some studies showed that PPARα was chronic activated after ischemia, which is harmful to cardiac recovery [11]. Meanwhile, as a key enzyme of fatty acid β-oxidation, CPT-1α can reduce free fatty acid esterification and allow fatty acid entry into mitochondria for fatty acid oxidation [43]. TMZ is considered as a weak CPT-1 inhibitor that prevents fatty acid oxidation. Our study showed that YQHX treatment significantly up-regulated the expression of p-AMPK, PGC-1α, CPT-1α in model rats with myocardial ischemia at the subacute stages, whereas at the chronic stages YQHX merely significantly increased p-AMPK and PGC-1α expression, and attenuated the plasma glucose levels such as glucose and lactic acid, indicating that YQHX might have a better effect on lipid metabolism at the subacute stages. However, after treatment with positive drug TMZ, our study showed that TMZ increased the expression of p-AMPK, whereas attenuated the expression of CPT-1α and PPARα, indicating TMZ could protect myocardial metabolism by inhibiting fatty acid entry into mitochondria for oxidation. Therefore, these data also indicate that the metabolic way of YQHX and TMZ may exist differently. Furthermore, we also observed that YQHX significantly restored mitochondrial ultrastructure and mitochondrial ATP production after the MI different time points. These above data further indicate that YQHX may have a better effect on lipid metabolism at the subacute stages, whereas at the chronic stages YQHX seemed to be more effective in glucose oxidation.

To confirm the mechanism that YQHX regulated lipid metabolism, Western blotting was analyzed by hypoxia-induced myocardial injury in H9c2 cells after treatment of YQHX or/and AMPK inhibitor Compound c. In this study, we found that PGC-1α, CPT-1α, and PPARα mRNA and protein expression were significantly reduced in H9c2 cells during hypoxia (Fig. 6). Moreover, treatment with YQHX significantly increased the expressions of PGC-1α, CPT-1α and PPARα protein, whereas AMPK inhibitor compound c blocked the expressions of these molecules. The data indicate that YQHX can activate PGC-1α, CPT-1α, and PPARα expression to maintain the balance of lipid metabolism that may be related to AMPK-dependent signaling pathway.

Conclusions

The present findings provided evidence that YQHX could regulate lipid metabolism by activating p-AMPK, PGC-1α, CPT-1α, and PPARα expression, whereas the regulation of PGC-1α, CPT-1α, and PPARα expression were blocked with AMPK inhibitor compound c. YQHX may be a potent drug for prevention of ischemic heart disease.

Supplementary Materials

All the supporting information of this paper can be requested by sending E-mails to the corresponding authors.

References


