Berberine promoted myocardial protection of postoperative patients through regulating myocardial autophagy

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ABSTRACT

Background: Berberine has been verified to protect the heart from ischemia/reperfusion injury through animal experiments. However, the cardioprotective properties of berberine have not been established fully. This study was aimed at investigating whether berberine is cardioprotective in vivo and in vitro.

Methods: In the cardiomyoblast cells, the autophagosomes were observed by immunostaining. The apoptosis was detected by a flow cytometry. Beclin-1, LC3-II/I, adenosine monophosphate-activated protein kinase (AMPK), and mTOR in cardiomyocytes were detected by Western blot. Next, one hundred patients, who were undergoing percutaneous coronary intervention (PCI), were randomly assigned to the berberine group (n = 52) or control group (n = 48). Berberine was administered on them postoperatively. Their plasma was then analyzed for CRP, TNF-α and IL-6.

Results: In the cardiomyoblast cells, berberine reduced the autophagy and apoptosis induced by NaH2PO4. At the same time, berberine increased the activation of p-AMPK and inhibited the activation of p-mTOR induced by NaH2PO4 in vivo, berberine significantly reduced the levels of CRP, TNF-α and IL-6 in the patients’ plasma.

Conclusion: It was concluded that berberine therapy reduced myocardial injury partly by reducing myocardial autophagy and apoptosis through the AMPK/mTOR pathway.

1. Introduction

Patients with acute myocardial infarction (AMI) usually have high levels of inflammation. In the last few years, many studies were carried out to explore the inflammatory mechanism including oxidized LDL (ox-LDL), inflammation markers, and inflammatory cytokine, platelet activation to improve downstream coronary flow as well as myocardial function recovery [1–3].

Autophagy is an evolutionarily conserved catabolic process by which damaged and disproportionate cellular components are transported to lysosomes where they are degraded. Autophagy occurs in response to various stimuli, such as nutrient inadequacy and chemical, physiological, and pathological stress [4]. Autophagy and apoptosis have been reported to have some association with AMI [5]. As an important part of the regulation of energy metabolism, adenosine monophosphate-activated protein kinase (AMPK) signaling has always been a key biomarker in autophagy research. It is not only present in all metabolic organs, but it can also be activated by various stimuli, including changes in intracellular pressure, movement, hormonal imbalance, and other substances that can affect cell metabolism [6,7]. AMPK is also a stress-response enzyme, which can be activated by energy deficiency in the body or stress, resulting in two acute effects. First, it reduces energy consumption, inhibits anabolism, causes adenosine triphosphate (ATP) synthesis to maintain cell homeostasis, and induces autophagy by digesting its own structural substances to produce energy and reduce the cell's dependence on external nutrition. Second, it stimulates fatty acid oxidation to produce more ATP, supplementing cell metabolism [8]. On the contrary, mTOR is activated when energy is sufficient, and is inhibited when energy is lacking, which plays an inhibitory effect on AMPK. Activated mTOR can directly or interactively participate in protein phosphorylation and inhibits autophagy and apoptosis [9].

Berberine is a common isoquinoline alkaloid found in the four families and ten genera of Berberis, and its molecular formula is C20H18NO4. In recent years, it has been found that Berberine may be used as a drug for the treatment of cardiovascular and cerebrovascular diseases, hyperuricemia, hyperuricuria, diabetes and many chronic inflammatory diseases. As a result, berberine has attracted much attention in the cardiovascular and cerebrovascular field. However, berberine studies are mostly focused on acute cerebrovascular diseases and neurodegenerative diseases. The protective mechanism of berberine on AMI remains unclear.

In our study, we observed the postoperative administration of

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berberine in patients diagnosed with AMI undergoing primary PCI on cardiac function and inflammatory markers. We also explored the protection mechanism and effects of Berberine on cardiomyoblast cells.

2. Materials and methods

2.1. Myocardial cell culture

Cardiomyoblast cells (H9C2 cells, Mbio, Shanghai, China) were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine Serum (Sigma, USA) in 5% CO2 and 85% humidity incubator at 37 °C. For treatment, H9C2 cells were incubated with the study. After admission, all patients were immediately treated by protocol was designed and approved in 2015 by the ethics committee of and coagulation abnormalities were excluded from the experiment. This protocol was purchased. GAPDH (Santa Cruz, TX, USA) was used as loading control

2.2. Western blotting

Total proteins of H9C2 cells were isolated using a RIPA lysis buffer (Dingguo, Beijing, China). The protein concentration in the supernatant was determined by a BCA kit (Bio-Rad, Hercules, CA, USA). 50 μg of protein were separated by 10% SDS polyacrylamide gel electrophoresis with a constant voltage of 75 V for 120 min. The proteins were then transferred to nylon membranes (Dingguo, Beijing, China) at 60 V for 2 h. The membranes were incubated in 3% bovine serum albumin to eliminate nonspecific binding. To investigate autophagy and apoptosis, primary antibodies p-mTOR (Cell Signaling, MA, USA), p-AMPK (Cell Signaling, MA, USA), beclin-1 (Cell Signaling, MA, USA), LC3B I/II (Cell Signaling, MA, USA), and their respective secondary antibodies, were purchased. GAPDH (Santa Cruz, TX, USA) was used as loading control

2.3. Immuno-fluorescence staining

After treatment, cells were washed with 1 × PBS three times and fixed with 4% formaldehyde for 15 min at room temperature. Then, the cells were washed with PBS and treated with 0.1% Triton X-100 for 2 min. After washing in PBS, cells were blocked using 5% goat serum for 1 h. The LC3B I/II antibody (1:500) was added to the cells which were then incubated for 24 h at 4 °C. After PBS washes, a secondary fluorescent antibody was added for 1 h. After that, the cells were washed with PBS, DAPI was added and incubated with the cells for about 15 min. Cells were then visualized under a fluorescence microscope.

2.4. FITC-Annexin V staining for apoptosis

After 24 h of NaH2PO4 treatment, cells were collected and washed with cold PBS, 1 × 10^6 cells were resuspended in 100 mL 1 × binding buffer. Subsequently, 100 μL of cell suspension was transferred to a 5 mL culture tube, and 5 μL of annexin V and 5 μL of propidium iodide were added according to the instructions given on the Apoptosis Detection kit (BD Biosciences, USA). Then, cells were gently vortexed and incubated for 15 min at room temperature in the dark. After that, the cells were analyzed by flow cytometry

2.5. Participants

One hundred patients diagnosed with ST-elevated AMI were randomly divided into experiment group (n = 52) and control group (n = 48). Patients with severe liver disease, cardiogenic shock, chronic or severe renal failure, severe infections, chronic inflammatory disease, and coagulation abnormalities were excluded from the experiment. This protocol was designed and approved in 2015 by the ethics committee of Xuanwu Hospital of the Capital Medical University. It is important to also note that we received written consent from each participant before the study. After admission, all patients were immediately treated by chewing 300 mg of enteric coated-aspirin and 300 mg of clopidogrel, followed by continued use of aspirin (100 mg/day) combined with clopidogrel (75 mg/day, antiagulant). After undergoing direct PCI, all patients were treated with aspirin, clopidogrel, statin, angiotensin-converting enzyme inhibitor, angiotensin II receptor antagonist and low-molecular weight heparin. The berberine group was given additional berberine tablets (Shanxi Taiyuan Pharmaceutical Co., Taiyuan, China), 0.3 g/time, and taken three times a day.

2.6. Follow-up examinations

Follow-up examinations dealt with the main clinical cardiovascular adverse events (death, recurrent myocardial infarction, and stroke), as well as recurrent angina pectoris during the hospital stay. In addition, 2D echocardiography was conducted to determine the left ventricular ejection fraction (EF), whereas the six-minute walk test was performed to evaluate the different cardiac functions. Moreover, we obtained the patients’ blood samples 15 days after the operation. All test tubes were subjected to centrifugation at 3500 rpm for 20 min and at 4 °C to obtain plasma. The plasma samples were then stored at −80 °C. Detection of hs-CRP, TNFα and IL6 levels were measured with an enzyme-linked immunosorbent-assay kit (R&D Systems, MN, USA). The follow-up examinations were completed by two specialist physicians without knowing the patient’s clinical information.

2.7. Statistical analysis

Nonparametric variables within groups were compared using χ2 tests, while parametric variables were compared using Student’s t-tests. For correlation analysis, linear regression and logistic regression analysis were used to identify multivariable correlations. In all cases, a p value < 0.05 was considered as statistically significant.

3. Results

3.1. Berberine inhibits autophagy and apoptosis induced by NaH2PO4 in H9C2 cells

Total proteins extracted from H9C2 cells treated with NaH2PO4 for 24 h were analyzed to understand the effects of berberine on autophagy and apoptosis. The activation of autophagy-related proteins such as beclin-1 and LC3 II was increased significantly after treatment with NaH2PO4, whereas the expression of beclin-1 and LC3 II was reduced after treatment with berberine in a concentration-dependent manner (Fig. 1A).

Immunofluorescent staining also confirmed berberine inhibited the autophagy induced by NaH2PO4 in a concentration-dependent manner (Fig. 1B). FITC-annexin V was used to quantify the apoptotic cells after the NaH2PO4 treatment and berberine pretreatment. It was also observed that the number of apoptotic cells increased after NaH2PO4 treatment. However, berberine-treated cells showed a significant reduction in apoptosis (Fig. 1C). This shows that berberine can reverse the autophagy and apoptosis induced by NaH2PO4.

Further, we detected the activation of AMPK and mTOR signaling pathway and the results showed that the expression of p-AMPK was increased (2.5-fold) when treated with 10 mm/L of NaH2PO4, whereas the expression of p-mTOR was decreased (2-fold). Berberine inhibited the activation of p-AMPK and promoted the activation of p-mTOR in a concentration-dependent manner.

3.2. Follow-up results

The average age of 24 males and 24 females in the control group was 56.45 ± 11.68, while the average age of 28 males and 24 females in Treatment group was 57.37 ± 10.53. No statistical significance of basic information was observed between the treatment and the control.
No occurrence of death and stroke was observed in the two groups at 15 days after they underwent PCI (p > 0.05). EF and six-minute walk test distance at 15 days after the PCI were not significantly different between the two groups (p > 0.05). However, Hs-CRP, IL-6 and TNFα decreased significantly at 15 days after the PCI (p < 0.05, Table 1).

4. Discussion

Berberine has several preventive effects on cardiovascular diseases. For example, it promotes ischemia-induced angiogenesis [12], improved cardiac function in rat coronary microembolization model via a mechanism involving antiplatelet and anti-inflammatory effects [13],

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**Table 1**

<table>
<thead>
<tr>
<th>Groups</th>
<th>Treatment group (n = 52)</th>
<th>Control group (n = 48)</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>57.37 ± 10.53</td>
<td>56.45 ± 11.68</td>
<td>NS</td>
</tr>
<tr>
<td>Male/Female (n/n)</td>
<td>28/24</td>
<td>24/24</td>
<td>NS</td>
</tr>
<tr>
<td>Hypertension (n)</td>
<td>30</td>
<td>28</td>
<td>NS</td>
</tr>
<tr>
<td>Diabetes mellitus (n)</td>
<td>28</td>
<td>28</td>
<td>NS</td>
</tr>
<tr>
<td>EF (%)</td>
<td>55.3 ± 8.4</td>
<td>51.4 ± 9.5</td>
<td>NS</td>
</tr>
<tr>
<td>6-minute walk (m)</td>
<td>489 ± 86</td>
<td>466 ± 73</td>
<td>NS</td>
</tr>
<tr>
<td>Hs-CRP (mg/dL)</td>
<td>0.22 ± 0.31</td>
<td>1.88 ± 1.51</td>
<td>p &lt; 0.05</td>
</tr>
<tr>
<td>IL-6 (pg/ml)</td>
<td>37.43 ± 8.21</td>
<td>45.66 ± 6.68</td>
<td>p &lt; 0.05</td>
</tr>
<tr>
<td>TNFα (pg/ml)</td>
<td>10.77 ± 4.19</td>
<td>17.85 ± 5.76</td>
<td>p &lt; 0.05</td>
</tr>
</tbody>
</table>
attenuated myocardial ischemia/reperfusion injury by reducing oxidative stress and inflammation response [14], and down-regulated mitochondrial dysfunction and apoptosis [15]. However, these studies are superficial, and the mechanism of myocardial protection induced by berberine remains unclear. Excessive level of autophagy is one of main mechanism to stimulate pathological cardiac hypertrophy, which leads to heart failure [16]. In the present study, we analyzed the effects of berberine on autophagy and apoptosis by establishing a NaH$_2$PO$_4$-induced cell model. NaH$_2$PO$_4$ is a common reagent used to construct hypoxic/reoxygenation injury model of isolated cardiomyocytes [17]. Previous report showed that the expression of LC3 II is an important mediator of autophagosome formation. We found that the expression of beclin-1 and LC3 II increased when the cells were treated with high concentration of NaH$_2$PO$_4$ (10 mm/L). However, berberine significantly inhibited autophagy induced by NaH$_2$PO$_4$. Moreover, in this study, apoptosis was induced in NaH$_2$PO$_4$-treated cells and berberine inhibited the apoptosis in H9C2 cells induced by NaH$_2$PO$_4$, indicating berberine had an inhibitory effect on the excessive level of autophagy and apoptosis.

AMPK is one of the central regulators of cellular and organism metabolism and regulates several transcriptional factors. Moreover, autophagy is promoted by AMPK to maintain energy homeostasis. In this study, treatment with NaH$_2$PO$_4$ has shown to upregulate p-AMPK, whereas p-mTOR has shown to be down-regulated. Berberine inhibited the activation of p-AMPK and promoted the activation of mTOR induced by NaH$_2$PO$_4$, which are consistent with earlier findings where inactivation of mTOR by rapamycin has been reported to stimulate autophagy in the presence of nutrients, suggesting that mTOR down-regulates autophagy [18].

Next, this study evaluated the effects of berberine on inflammatory markers in patients with AMI and undergoing primary PCI. CRP is an acute-phase reactant that plays an active role in the progression of atherosclerosis through its direct proatherogenic effects on the vasculature. This protein is produced chiefly by the liver in response to pro-inflammatory cytokines and is secreted into systemic circulation Serum CRP is a strong independent predictor of the size of an infarct area [19]. IL-6 is a major inducer of CRP production in the liver, and its elevation is associated with short- and long-term prognosis in patients with unstable angina [20]. TNFα is a multifunctional circulating cytokine derived from endothelial and smooth-muscle cells, and macrophages associated with coronary atheroma [21]. Persistent overexpression of TNFα after ischemia leads to adverse coronary outcomes. In our study, although there were no difference in cardiac function such as EF (%) and 6-minute walk distance (m), berberine reduced all inflammatory biomarkers 15 days after operation when compared with control, indicating that berberine provides cardiac protection against cardiac injury.

Together, our findings elucidate that in vitro, Berberine led to a reduction in autophagy and apoptosis through the AMPK/mTOR pathway. Berberine protects patients from cardiac injury partly via reduction in autophagy and apoptosis.

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Ethics approval
All experiments and procedures were approved by the Institutional Ethics Committees of Capital Medical University (CMU-201500605).

Competing interests
None.

References