Effect of Allopurinol on Myocardial Energy Metabolism in Chronic Heart Failure Rats After Myocardial Infarct

Zhenzhen Wang, MD, Juan Ding, MD, Xiang Luo, MD, Siliang Zhang, MD, Gang Yang, MD, Que Zhu, MD, and Dichuan Liu, MD

SUMMARY

To determine the effect of the xanthine oxidase (XO) inhibitor allopurinol on myocardial energy metabolism in a chronic heart failure rat model after myocardial infarct.

An AMI model was established in 6-week-old rats via the ligation of the anterior descending coronary artery. Thirty-five rats were randomly divided into the following 3 groups: an ALLO group, an AMI group, and a Sham group. Heart failure was successfully diagnosed via echocardiography and blood tests. Xanthine oxidase (XO), malondialdehyde (MDA), PGC-1α, CPT-1, and GLUT4 were monitored in the myocardium.

The TEM results demonstrated that myofilament lysis and mitochondrial swelling were alleviated in the ALLO group compared with the AMI group (without ALLO). The results also demonstrated that cardiac function was significantly improved in the ALLO group compared with the AMI group. Compared with the AMI group, the ALLO group exhibited increased respiratory-chain enzyme activity, as well as increased PGC-1α and CPT-1 mRNA and protein expression, decreased MDA content, and decreased XO and GLUT4 mRNA and protein expression.

ALLO improves myocardial energy metabolism in rats with chronic heart failure, which may result from the regulation of PGC-1α in the setting of glycolipid metabolism, enhancing the production of ATP. (Int Heart J 2016; 57: 000-000)

Key words: Reactive oxygen species (ROS), Mitochondria

Chronic heart failure (CHF) is the terminal stage of heart disease and is the leading cause of death in affected patients. The incidence of CHF in adults is nearly 1-2% in developed countries and is above 10% among patients older than 70 years of age. Synthetic treatments and assist devices have been introduced to improve myocardial remodeling, strengthen myocardial contraction, and alleviate cardiac overload.

From the Department of Cardiology, Second Affiliated Hospital, Chongqing Medical University, Chongqing, PR China.

The study was support by the National Natural Science Foundation of China (grant No. 30670870) and the Chongqing Municipal Science and Technology Commission of the Natural Science Foundation of China (cstc2012jgA10085).

Address for correspondence: Dichuan Liu, MD, Department of Cardiology, Second Affiliated Hospital, Chongqing Medical University, Linjiang Road No. 76, Chongqing, 400010, PR China. E-mail: LDC670220@163.com

Received for publication April 4, 2016. Revised and accepted May 20, 2016.

Released in advance online on J-STAGE November 4, 2016. (This is “Advance Publication”.)

All rights reserved by the International Heart Journal Association.
function among patients with CHF warrant further research. In this study, we examined the changes in cardiac function, oxidative stress, and myocardial energy metabolism that occur in rats with CHF following ALLO administration. Our findings also provided clinicians with a theoretical basis for the treatment of heart failure using ALLO.

**METHODS**

**Animal model:** Male Sprague-Dawley (SD) rats (6-weeks old) were provided by the Experimental Animal Center at Chongqing Medical University. They were raised in 12:12 hour light-dark cycles and fed a standard diet (10% fat, 20% protein and 70% carbohydrates) and purified water. All animal experimental protocols were approved by the Animal Care and Use Committee of Chongqing Medical University and complied with its laboratory animal management and use regulations. Thirty-five rats were randomly divided into an ALLO group (n = 12), an AMI group (n = 13), and a Sham group (n = 10). Male SD rats were anesthetized with sodium pentobarbital (50 mg/kg, IP) prior to surgery. The ALLO group and the AMI group were treated via coronary artery ligation to establish an AMI model as previously described. The Sham group was subjected only to thoracotomy without ligation of the coronary arteries. The left chest was closed in 3 layers (rails, muscles, and skin) 15 minutes after occlusion, and the rats were allowed to recover from anesthesia on their own. Following surgery, penicillin was injected at a dose of 40,000 U/day. Four weeks following surgery, the rats in the ALLO group were treated with ALLO by gavage at a dose of 50 mg/kg·day, whereas the rats in the AMI and Sham groups received 1 mL of NS per day as a control; these treatments were administered for 8 weeks. The dose of ALLO was chosen based on a study by Xiao, et al. All rat experiments were approved by the local institutional animal research committee. Eight SD rats died. In the control group, one rat died when establishing models. One died within one day after operation. One death occurred on the fifth day after operation and one death occurred on the eighth day after operation. In the treatment group, one death occurred when establishing models. One died within one day after operation. One died on the seventeenth day after operation. In the sham-operated group, one rat died on the sixth day after operation. The cause of death may have been acute heart failure, infection, malignant arrhythmia, or some other cause. The remaining 27 rats were raised until the endpoint.

**Cardiac functional assessment via echocardiography:** Transthoracic Doppler echocardiographic measurements were performed using a commercially available echocardiographic system (GE vivid7, Fairfield, CT, USA) following 4 and 12 weeks after surgery. The long axis of the left ventricle was displayed using two-dimensional echocardiography. Parameters related to cardiac function such as left ventricular internal diastolic dimension (LVIDd), left ventricular internal systolic dimension (LVIDs), and ejection fraction (EF%) were measured via M-mode ultrasound. Myocardial structural evaluation via fluorescent microscopy: Specimen collection: Following the echocardiographic measurements, rats were sacrificed by cervical dislocation. Non-infected myocardial samples of the left ventricle were subsequently obtained for additional examinations, including MDA content, BNP content, mitochondrial respiratory-chain enzyme activity, paraffin sectioning, and ultrastructural examination via electron microscopy; the residual myocardial specimens were preserved in lipid-nitrogen. Myocardial structural evaluation via transmission electron microscopy: Specimen collection: Following the echocardiographic measurements, myocardial tissue samples were fixed in 4% paraformaldehyde for 24 hours before being embedded in paraffin and cut into pieces (5-micrometer thick pieces), stained with hematoxylin-eosin, and observed under a fluorescent microscope (OLYMPUS-BX51, Japan). Myocardial ultrastructure via transmission electron microscope: Specimen collection: Blocks of tissue with thicknesses of 1 mm were taken from anesthetized animals and immersed in 4% glutaraldehyde, fixed for 2 hours, cleaned via 3 rinses of PBS, fixed in 1% osmium tetroxide for 2 hours, dehydrated by ethanol and acetone, soaked in epoxy resin, embedded and polymerized, and cut into sections with thicknesses of 0.5 μm. Ultrathin sections with thicknesses of 60 nm were fixed and prepared under a fluorescent microscope and stained with uranium acetate and lead citrate. Myocardial ultrastructure was observed and photographed using a transmission electron microscope (TEM, H-7500 type, Hitachi, Japan) equipped with a digital CCD (Gatan-780, Gatan Inc., USA).

**Malonaldehyde content:** The oxidation of lipid is indicative of the oxidative stress level in the body. Peroxidation products, particularly malonaldehyde (MDA) content, were examined using a Lipo Peroxidation MDA Assay Kit (Beyotime Institute of Biotechnology, Beijing, China). Approximately 100 mg of myocardial tissue was placed in 0.1 mL cold PBS and ground for 20 seconds; 10% TCA precipitation protein was subsequently added to the samples, which were centrifuged at 1,600 g for 10 minutes at 4°C. The liquid supernatants were extracted and detected using a Lipid Peroxidation MDA Assay Kit. MDA content was measured using Multi-Mode Microplate Readers (SpectraMax M5) at 532 nm.

**Respiratory-chain enzyme activity (I~IV):** Respiratory-chain enzyme activity (I~IV) was measured using a Tissue Mitochondria Separation Kit (Beyotime Institute of Biotechnology). Fresh heart tissue (100 mg) was washed, cut and ice bathed in PBS, and centrifuged at 600 g for 10 seconds. The supernatant was discarded, and the samples were digested using pancreatic enzymes and centrifuged at 11,000 g for 5 minutes. The supernatant was discarded, leaving only purified mitochondria. The activity of the mitochondrial respiratory chain complexes in the samples was detected via calorimetry (Zhanchen Biotechnology, Guangzhou, China).

**mRNA expression of XO, PGC-1α, CPT-1, and GLUT4 in the myocardium via quantitative RT-PCR:** Quantitative real-time PCR (qRT-PCR) was used to measure the mRNA expression of XO, PGC-1α, CPT-1, and GLUT4 in 35 mg of myocardial tissue. Total RNA was extracted using an RNA extraction kit (OMEGA Biotech, Norcross, GA). Both the concentration and purity of the extracted RNA were subsequently determined. The RNA was then reverse transcribed into cDNA using an RNA PCR Kit (TaKaRa, Dalian, China) according to the manufacturer’s protocol. A 20 μL reaction system contains approximately 500 ng of RNA. The reaction conditions were maintained at 42°C for 2 minutes, 37°C for 15 minutes, and 85°C for 5 seconds. The volume of the fluorescence quantitative PCR reaction system was 20 μL and contained 10 μL of SY-BREx TaqTM II, 0.8 μL of upstream and downstream primers,
2 μL (100 ng) of cDNA template, and 6.4 μL of ddH2O. The reaction conditions were as follows: denaturation at 95°C for 50 seconds and 15 seconds, annealing at 55°C for 15 seconds, and polymerization at 72°C for 30 seconds, for 39 cycles. The results were calculated using the 2−ΔΔCt method. To obtain the relative quantitative values for gene expression, β-actin was used as an endogenous control. The sequences of the primers used for RT-PCR are listed in Table I.

**Protein expression of XO, PGC-1α, CPT-1, and GLUT4 via Western blotting:** The tissue samples were cracked for 30 minutes and disintegrated using a protein extraction kit (Beyotime Institute of Biotechnology). The total GE gel was subsequently prepared. Equal amounts of protein (20 μg) were loaded onto each lane of the SDS-PAGE gel. Electrophoresis was performed at a constant voltage of 80 V (stacking gel)/120 V (separation gel). The protein samples were blotted onto polyvinylidene difluoride membranes (PVDF) with currents of 250 mA. The membranes were blocked using 5% skimmed milk powder for 1 hour. The primary antibodies [XO: (1:600), sc-22006, Santa Cruz Biotechnology, Santa Cruz, CA, USA; GLUT4: (1:1000), sc-53566, Santa Cruz Biotechnology; CPT-1: (1:5000), ab128568, Abcam, UK; PGC-1α: (1:5000), ab54481, Abcam; and β-actin: (1:200), Boster Biotechnology Co., Ltd, Wuhan, China] were incubated with the samples overnight at 4°C. The membrane was washed 3 times using TBST for 10 minutes. The secondary HRP antibodies were incubated with the membranes for 1 hour at room temperature and visualized via enhanced chemiluminescence (ECL). The results were acquired using Quantity One 4.6.2 image analysis software; the protein and β-actin (as an internal control) densities ratio were calculated as relative expression quantities.

**Statistical analysis:** Data were analyzed using SPSS 17.0 software (Inc., Chicago, IL). The data with a normal distribution are expressed as the mean ± standard deviation. Comparisons between groups with a normal data distribution were analyzed using one-way analysis of variance. Otherwise, the rank sum test was utilized. A P value less than 0.05 was considered statistically significant.

**Results**

**Echocardiographic measurements:** After 4 weeks, 4 rats in the AMI group, 3 rats in the ALLO group, and 1 rat in the sham group died. Both the AMI and ALLO rats demonstrated diminished cardiac function at 4 weeks characterized by a decreased LVEF and increased LVIDd and LVIDs compared with the Sham rats (P < 0.05). After 12 weeks, LVIDd and LVIDs of the ALLO group decreased by 27.40% and 27.75%, respectively, and LVEF increased by 27.75% compared with the AMI group (P < 0.05), which indicated that the administration of ALLO preserved the cardiac function of rats suffering from heart failure (Figure 1 and Table II).

**HE staining and transmission electron microscopy (TEM):** The HE staining and TEM results demonstrated well-aligned myocardial fibers, rich and homogeneous cytoplasm, and complete nuclei in the sham group (as depicted in Figures 2A and 2D). However, in the AMI group, both fractured and disorganized myocardial fibers were observed, as well as myocardial tissue edema, absent nuclei, and large amounts of fibrous tissue.

**Table I. Primer Sequences Used for the qRT-PCR Analysis**

<table>
<thead>
<tr>
<th>Primer</th>
<th>Target transcript</th>
<th>Primer Sequence (5′-3′)</th>
<th>Product size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>XO</td>
<td>XO-F</td>
<td>AATGCCGAACCTGAAACAAC TTTTCTATGCTTCCACGTT</td>
<td>243</td>
</tr>
<tr>
<td></td>
<td>XO-R</td>
<td>TTTCCTATGCCTTCCACGTT</td>
<td></td>
</tr>
<tr>
<td>PGC1α</td>
<td>PGC1α-F</td>
<td>AGCCTCTTTGGCCAGATCTT GCAATCCGTCTCCATCCACC</td>
<td>259</td>
</tr>
<tr>
<td></td>
<td>PGC1α-R</td>
<td>GCAATCCGTCTCCATCCACC</td>
<td></td>
</tr>
<tr>
<td>CPT1</td>
<td>CPT1-F</td>
<td>GAGTCTCGAATCTTTTGCGA AAGGTGATGCTGACTGCAAGA</td>
<td>187</td>
</tr>
<tr>
<td></td>
<td>CPT1-R</td>
<td>AAGGTGATGCTGACTGCAAGA</td>
<td></td>
</tr>
<tr>
<td>Glut4</td>
<td>Glut4-F</td>
<td>GCCATCGTCATTGCCATCTCTGCATT</td>
<td>222</td>
</tr>
<tr>
<td></td>
<td>Glut4-R</td>
<td>CGTTTACCTGTCATTGCCGTC</td>
<td></td>
</tr>
<tr>
<td>β-actin</td>
<td>β-actin-F</td>
<td>CCTGACTGAGCGTGCGCTATT GGCTTCGGGAGGAACAAAA</td>
<td>429</td>
</tr>
<tr>
<td></td>
<td>β-actin-R</td>
<td>GGCTTGTCGGAGGAGCAATTA</td>
<td></td>
</tr>
</tbody>
</table>

Figure 1. Echocardiographic measurements of the rats in the different groups (12 weeks). A: Normal cardiac function in the sham group. B: Diminished cardiac function in the AMI group. C: Improved cardiac function in the ALLO group compared with the AMI group (n = 9 per group).
sue surrounding the infarcted area (as depicted in Figures 2B and 2E). The ALLO group exhibited improved myocardial tissue edema, aligned myocardial fibers, and complete cell nuclei (as depicted in Figures 2C and 2F), which suggests that ALLO attenuates the progression of heart failure following myocardial infarction.

Myocardial MDA and BNP contents: The results of the TBARS (thiobarbituric acid reactive substance) measurements indicated that myocardial MDA content was 10.64 ± 0.96 nmol/mg·prot in the AMI group, which was significantly different from the levels observed in both the sham group (4.36 ± 0.70 nmol/mg·prot) and the ALLO group (5.89 ± 0.46 nmol/mg·prot) (*P < 0.05). These results serve as convincing evidence that ALLO decreases MDA production, a toxic by-product of heart failure following myocardial infarction, and minimizes mitochondrial damage (*n = 4 per group).

The levels of BNP were detected. The results show that myocardial MDA content was 10.64 ± 0.96 nmol/mg·prot in the AMI group, which was significantly different from the levels observed in both the sham group (4.36 ± 0.70 nmol/mg·prot) and the ALLO group (5.89 ± 0.46 nmol/mg·prot) (*P < 0.05). These results serve as convincing evidence that ALLO decreases BNP content and improves cardiac function (*n = 4 per group).

Myocardial mitochondrial respiratory chain enzyme complex activity: The activity of the myocardial mitochondrial respiratory chain enzyme complexes was examined and is depicted in Figure 3. The activity of the enzymes was higher in the sham group than in the AMI and ALLO groups (*P < 0.05). The activity of the enzymes was higher in the ALLO group than in the AMI group (*P < 0.05). There were no significant differences in the activity of the enzymes of complex II among the groups (*P < 0.05). Therefore, among the rats with heart failure following AMI, the activity of the enzymes of complexes I, III,
and IV were significantly decreased, which resulted in decreased myocardial ATP production. ALLO appears to attenuate myocardial mitochondrial damage by inhibiting oxidative stress, and thus mitigating the damage to respiratory enzyme complexes I, III, and IV.

**mRNA expression of XO, PGC-1α, CPT-1, and GLUT4:** We attributed the protective effects of ALLO to the regulation of key enzymes involved in both glucose and lipid metabolism. PGC-1α is an essential regulator of mitochondrial energy metabolism and biogenesis. CPT-1 is involved in fatty acid oxidation and GLUT4 plays an important role in the oxidation of glucose. To determine the potential mechanisms underlying the protective effects of ALLO, the mRNA expression of molecular markers (XO, PGC-1α, CPT-1, and GLUT4) was subjected to qRT-PCR analysis in the sham, AMI and ALLO groups. As depicted in Figure 4 (compared with the sham group, the mRNA expression of XO increased by 152.56% and 377.86% in the ALLO and AMI groups (P < 0.05); the mRNA expression of GLUT4 increased by 87.94% and 195.25% (P < 0.05); the mRNA expression of PGC-1α decreased by 50.31% and 84.42% (P < 0.05); and the mRNA expression of CPT-1 decreased by 24.76% and 54.45% (P < 0.05)). Compared with the AMI group, the mRNA expression levels of XO increased by 27.37% and 39.76% (P < 0.05), and the protein expression levels of PGC-1α and CPT-1 increased by 218.85% and 65.14% (P < 0.05).

**Protein expression of XO, PGC-1α, CPT-1, and GLUT4:** The expression levels of XO, PGC-1α, CPT-1, and GLUT4 proteins were analyzed via Western blotting; the bands and statistical results are depicted in Figures 5 and 6. Compared with the sham group, the protein expression levels of XO increased by 91.65% and 163.87% in the ALLO and AMI groups (P < 0.05); the protein expression level of GLUT4 increased by 16.08% and 92.69% (P < 0.05), and the protein expression level of PGC-1α decreased by 19.94% and 39.14% (P < 0.05); the protein expression level of CPT-1 decreased by 13.11% and 71.58% (P < 0.05). Compared with the AMI group, the protein expression levels of XO and GLUT4 decreased by 27.37% and 39.76% (P < 0.05), and the protein expression levels of PGC-1α and CPT-1 increased by 31.56% and 205.71%, respectively (P < 0.05).

To enter cardiac myocytes, fatty acids must cross the cell membrane via transporters such as fatty acid translocase (FAT/CD36), plasma membrane fatty acid binding protein (FABP₄),...
and tissue specific fatty acid transport proteins (FATP). By adding a coenzyme A moiety to fatty acids via fatty-acyl-CoA synthetase, a long-chain acylcarnitine is synthesized by carnitine palmitoyl transferase I (CPT-1) and is translocated across the IMM into the matrix before being taken up into the mitochondria and converted into acetyl-CoA to enter the Krebs cycle. CPT-1 is the rate-limiting step in fatty acid oxidation (FAO). Glucose is taken up into cardiomyocytes via GLUT4. Inside the cell, glucose is phosphorylated to glucose-6-phosphate, which subsequently enters glycolysis via phosphofructokinase I. Pyruvate, the end product of glycolysis, is taken up into the mitochondria and converted into acetyl-CoA by pyruvate dehydrogenase (PDH) to enter the Krebs cycle. GLUT4 is the key enzyme of glucose oxidation; acetyl-CoA is the common product of glycolysis and FAO; the Krebs cycle produces NADH and FADH, which feed their electrons into the ETC and drive oxidative phosphorylation (as depicted in Figure 7).

**DISCUSSION**

ALLO attenuates cardiomyocyte apoptosis, stabilizes endothelial cells, and increases adenosine triphosphate energy delivery. The most recent clinical research points to its anti-oxidant properties and its facilitation of myofibrillar ATP delivery as a means of enhancing the energetic profile of the failing human heart. The goal of this study was to determine if ALLO improves cardiac function in a rat model of HF. Our findings demonstrated that cardiac function was significantly diminished at 4 weeks following MI; ALLO treatment improved cardiac function, reduced cardiomyocyte lipid oxidation, promoted respiratory-chain enzyme (I, III and IV) activity, and improved glycolipid metabolism.

The accumulation of a large number of ROS in CHF induced cardiomyocyte lipid peroxidation, which promoted the production of MDA. XO is a prominent source of ROS in the setting of CHF. Therefore, we hypothesized that ALLO, an XO inhibitor, attenuated lipid oxidation by mitigating cardiomyocyte injury. Therefore, the MDA content of the myocardium was measured to examine the protective effects of ALLO in CHF. As ROS injure mitochondria, we studied the influence of ALLO on the mitochondria of cardiomyocytes in CHF. The activity and microstructure of the respiratory-chain enzymes were also examined in this study. The results demonstrated that the activity of the respiratory-chain enzyme complexes (I, III and IV) was increased at 8 weeks in the ALLO group; however, the activity of respiratory-chain enzyme II exhibited no significant changes. It is possible that specific subunits of the respiratory-chain enzyme complexes (I, III and IV) are easily influenced by mitochondrial DNA coding. Additionally, we observed that mitochondrial swelling was effectively attenuated following ALLO administration, demonstrating that ALLO enhanced mitochondrial function in the myocardium in the setting of CHF. Energy derived from fatty acid metabolism accounts for 70% of the myocardium’s ATP supply. The oxidation of one fatty acid molecule (such as palmitate) generates 104 molecules of ATP, whereas glucose metabolism generates only 31 molecules of ATP. It is therefore possible that the myocardium compensates for the effects of heart failure by transitioning from fatty acid metabolism to glucose metabolism. However, the amount of energy that may be derived from glucose is much less than the amount that may be derived from fatty acids. Therefore, cardiac function is determined by the availability of the primary energy provider of the myocardium. Ventura-Clapier et al demonstrated that PGC-1α is an important regulatory factor of mitochondrial energy metabolism and biogenesis. The expression level of PGC-1α is decreased in CHF. We measured the changes in PGC-1α expression, as well as the expression levels of its downstream genes. Our results demonstrated that the expression of PGC-1α was increased following 8 weeks of ALLO administration, as was the expression of CPT-1. However, the expression of GLUT4 was also decreased, which suggests that ALLO improves glycolipid metabolism in CHF.

In this study, an animal model of CHF was successfully established. The results of the echocardiographic examinations demonstrated that ALLO administration decreased LVIDd and LVIDs and increased EF, which indicates that ALLO improves cardiac function. MDA content was also decreased, which indicated that ALLO attenuates oxidation-induced injury. Additionally, ALLO increased the activity of respiratory-chain enzyme complexes I, III and IV in the mitochondria via the inhibition of XO. Our results also demonstrated that ALLO up-regulated the mRNA and protein expression of PGC-1α, which induced the up-regulation of CPT-1 and the down-regulation of GLUT4. However, the underlying mechanism of this phenomenon remains unknown. We did not detect the contents of ATP. This research may play an important role in the evaluation of ALLO as a therapeutic agent; however, additional clinical trials are necessary to more closely examine its potential as a therapeutic agent.
Conclusion: This study demonstrated that ALLO improves cardiac function in the setting of CHF. The inhibition of XO by ALLO attenuated the injury caused by ROS and facilitated in-cardiac function in the setting of CHF. The inhibition of XO by metabolism.

Conflicts of interest: The authors declare there are no conflicts of interest.

REFERENCES