**Original Article**

**Ang (1-7) improves cardiac function after resuscitation from cardiac arrest**

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**Abstract:** Objective: The aim of this study was to investigate the mechanistic action and therapeutic effects of Ang (1-7) on myocardial dysfunction after cardiopulmonary resuscitation from cardiac arrest. Methods: Sprague-Dawley rats were subjected to tracheotomies, arteriovenous punctures, invasive mechanical ventilation, and cardiopulmonary resuscitation with angiotensin-(1-7) Ang (1-7) or without the peptide. The sham group of rats received tracheotomies, arteriovenous punctures, and cardiopulmonary resuscitation with saline. Heart rate (HR), mean arterial pressure (MAP), left ventricular systolic pressure (LVSP), left ventricular end-diastolic pressure (LVEDP), and left ventricular maximal ascending and descending rates (± LVdp/dtmax) were measured before treatment (Baseline) and 6 hours after resuscitation. MicroRNA expression of Mas receptor (MasR), lncAng362, miR222, or phosphatase and tensin homolog (Pten) in myocardial tissues was analyzed by quantitative real-time PCR. Protein expression of MasR and Pten in myocardial tissues was analyzed by Western blotting. Results: Ang (1-7) treatment significantly enhanced its receptor MasR and downstream molecule Pten expression and inhibited lncAng362 and miR222 expression in rat cardiac tissues after resuscitation from cardiac arrest. Ang (1-7) treatment significantly improved rat cardiac function after resuscitation, with significant higher LVSP and LVDP, but lower LVEDP compared to control group. Conclusion: Ang (1-7) treatment significantly improved rat cardiac function after resuscitation from cardiac arrest by promoting MasR and Pten expression and suppressing lncAng362 and miR222 expression. Ang (1-7) could serve as a novel therapeutic agent for treatment of cardiac dysfunction after cardiac arrest.

**Keywords:** Cardiac insufficiency, cardiopulmonary resuscitation, Ang (1-7), lncAng362, Pten

**Introduction**

Cardiac arrest is a worldwide public health issue, with approximately 70-90% mortality and severe morbidity [1]. Patients surviving from cardiac arrest after cardiopulmonary resuscitation (CPR) mostly suffer from cardiac insufficiency, as well as other physical ailments [2]. Thus, attenuating cardiac dysfunction after resuscitation is an effective measure improving the survival rate of patients with cardiac arrest.

Renin-angiotensin signaling pathways play an important role in cardiac insufficiency after resuscitation [3]. Triggering AT1R signaling by angiotensin II (AngII) suppresses CPR and activation of Mas receptor (MasR) signaling affects cardiac function after cardiac arrest [4]. Angiotensin-(1-7) Ang (1-7) is a key molecule in renin-angiotensin signaling pathways, mainly produced through the digestion of AngII by ACE2 [5]. Ang (1-7) has the ability to bind to Mas receptors (MasR) and function as a key vascular protective peptide [6, 7], inducing beneficial cardiovascular responses. However, it is still unclear whether Ang (1-7) promotes cardiac function after cardiopulmonary resuscitation from cardiac arrest.

lncAng362 is a long-chain non-coding RNA (lncRNA) which does not encode proteins but regulates epigenetic [8], transcription [9], and post-transcriptional gene expression [10]. IncAng362 is found near the site of microRNA miR-222 [11]. IncAng362 and miR-222 co-regulate the proliferation of blood vessels after the activation of AngII [11, 12]. miR-222 interacts with its target protein phosphatase and tensin homolog (Pten) and mediates multiple signaling pathways linked to a variety of cardiovascular
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effects [13]. AngII can also generate Ang (1-7) under the action of angiotensin converting enzyme 2. However, the effects of Ang (1-7) on IncAng362 expression and consequent cardiac function are not fully understood. The present study investigated the function and mechanistic action of Ang (1-7) on myocardial dysfunction in a rat model of cardiopulmonary resuscitation after cardiac arrest.

Methods

Animals

Sprague-Dawley rats (8 weeks, 250-300 g) were randomly divided into three groups (n=10 per group): sham-operated group (Sham), cardiac arrest resuscitation group (Control), and resuscitation with Ang (1-7) treatment group (Ang (1-7)). The process of cardiac arrest resuscitation included CA, CPR, and return of spontaneous circulation (ROSC). Cardiac tissues were harvested from rats at 6 hours after ROSC. All rats used in the experiments were purchased from the Experimental Animal Center,Guiyang College of Traditional Chinese Medicine. Animal use was approved by the Animal Ethics Committee of Guizhou Provincial People’s Hospital in China.

Cardiac arrest resuscitation and cardiac function monitoring

Rats were fasted with water for 12 hours before the operation, then subjected to anesthesia with intra-peritoneal injections of chloral hydrate (300-380 mg/kg), of which 100 mg/kg was administered every hour to maintain the effects. Transesophageal pacing induced ventricular fibrillation rat cardiac arrest models were established, as described previously [14]. Briefly, mechanical ventilation was performed for 30 minutes to ensure hemodynamic stability in all the groups. The 5F pacing cardiac electrodes were inserted into the esophagus about 7 cm through the mouth. The proximal end of the pacemaker was connected to the output of the AC transformer. The 50 V AC 25 V stimuli was used to induce 90 s ventricular fibrillation. After stop stimulation, cardioversion changes were observed. When ventricular fibrillation automatically converted to sinus rhythm, the stimulation was immediately increased until the induction of ventricular fibrillation persisted [15] (Figure 1). Four minutes after ventricular fibrillation, electrical stimulation was stopped with immediate implementation of cardiopulmonary resuscitation. Chest compressions were performed with a modified Lanshike Pneumatic CPR machine (MCPRL00A, Guangzhou Lanswick Co., Ltd., China), with a chest compressional frequency of 200 beats/minute and a depth of compression of 1/3. CPR began after the femoral vein epinephrine 0.02 mg/kg (administration volume of 2 ml/kg), up to 3 times 2-4J two-way defibrillation. ROSC standard was followed, as described previously [16]. During the experiment, rectal temperature was maintained in the normal range (37.8-38.7°C). Ventilation was assisted with ALC-V9 animal ventilator (100% pure oxygen) (Shanghai Orte Biotech Co., Ltd., China), with the rate of 80 beats/min. The amount of gas was 6.5 mL/kg. BL-420S biological signal acquisition and analysis system (Chengdu Ao-sheng Technology Corporation, China) was used to measure cardiac function, such as heart rate (HR), mean arterial pressure (MAP), left
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ventricular systolic pressure (LVSP), left ventricular end-diastolic pressure (LVEDP), and left ventricular maximal ascending and descending rates (±LVdp/dtmax).

Administration of Ang (1-7)

Rats were intravenously administrated with Ang (1-7) (800 ng/kg/min) (MedChem Express Co., Ltd., Shanghai, China) when CPR began. Rats in the control group received intravenous injections of saline (2 ml/kg) saline.

Cardiac tissues

Rats were sacrificed by injections with a lethal dose of phenobarbital (250 mg/kg). Rat chests were opened and the hearts were isolated from the aortic root, rinsed with phosphate-buffered saline (PBS), and the left and right ventricles were separated. All cardiac tissues were immediately stored in a liquid nitrogen tank.

Quantitative real-time PCR (qRT-PCR)

Total RNA was extracted from cardiac tissues with TRIzol Reagent (Beijing Tiangen Biochemical Technology Co. Ltd., Beijing, China), cDNA was synthesized with PrimeScript RT reagent Kit (TaKaRa, Otsu, Japan). qRT-PCR was performed with SYBR Premix ExTaq II (TliRNaseH Plus), ROX plus (TaKaRa, Otsu, Japan), and primers under a PCR program: 95° for 30 seconds followed by 45 cycles of 95°C for 5 seconds and 60°C for 40 seconds on an ABI 7500 fluorescence quantitative PCR system. Primers for IncAng362: forward 5'-TATGCGTGTTCTGCTGT-3', reverse 5'-ATCAAGAAATTAGACATCG-3'. Primers for MasR: forward 5'-ACATTATCATGACTTGTCTGTGGA-3', reverse 5'-CGCAAGAAGAGAACCAG3'. Primers for miR222: forward 5'-GCTACATCTGGCTACTGGT-3', reverse 5'-GGCCAACCGCGAGAAGATG-3'. Primers for Pten: forward 5'-TGAAGACCATAACCCACCACAGC-3', reverse 5'-CATTACACCAGTTGCTCCCTTC-3'. Primers for GAPDH: forward 5'-GTCAACTGGCTACTGGGT-3', reverse 5'-GGCAACCGCGAGAAGATG-3'. Expression levels of MasR, Pten genes, IncAng362, and miR-222 were analyzed with 2^−ΔΔCT method. GAPDH gene served as the internal control.

Western blot

Proteins were extracted from myocardial tissue with RIPA lysis buffer, according to manufacturer instructions (Beyotime Institute of Biotechnology, Haimen, China). Protein concentrations were determined with the BCA assay (Sigma, St Louis, MO, USA). Proteins (50 μg/sample) were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and then transferred onto polyvinylidene fluoride membranes (Millipore corp., Billerica, MA, USA). The membranes were blocked with 5% non-fat milk in TBST (10 mM Tris, pH 7.4,
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Figure 3. Effects of Ang (1-7) stimulation on IncAng362 and miR222 expression in myocardial tissues. Total RNA was extracted from rat myocardial tissues in sham, Ang (1-7)-stimulated, or control group and subjected to qRT-PCR. IncAng362 (A) or miR222 (B) expression fold change in Ang (1-7)-treated or control group was calculated in comparison with the sham group. GAPDH gene served as internal control. Data are from three independent experiments, presented as mean ± SD, and analyzed by One-way ANOVA. **P<0.01, ***P<0.001.

IncAng362 and miR222 have been associated with AngII-induced vascular smooth muscle cell proliferation [11] and blood vessels. To test the effects of Ang (1-7) ligation on expression of IncAng362 and miR222, this study quantified transcripts of both RNAs in myocardial tissues in rats resuscitated from cardiac arrest by qRT-PCR. Analyses of gene mRNA in the tissues demonstrated that Ang (1-7) significantly reduced expression levels of both IncAng362 (Figure 3A) and miR222 (Figure 3B), compared with controls absent from Ang (1-7).

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Statistical analysis

Data are presented as mean ± mean ± SD (standard deviation) and were analyzed by One-way ANOVA, using SPSS 19.0 software (IBM, Armonk, NY, USA). Bivariate correlation was used in correlation analysis and Pearson’s correlation coefficient was used in two-tailed tests. P < 0.05 indicates statistical significance.

Results

Ang (1-7) ligation enhanced MasR expression in myocardial tissues

MasR plays a key role in cardiac remodeling and fibrosis in post-myocardial infarction [17]. To examine the effects of Ang (1-7) ligation on MasR expression after resuscitation from cardiac arrest, this study established rat ventricular fibrillation cardiac arrest models by using transesophageal pacing and treated the rats with Ang (1-7). Quantification of MasR mRNA expression in myocardial tissues by qRT-PCR demonstrated that Ang (1-7) significantly promoted MasR gene expression compared with the one without Ang (1-7) or sham treatment (Figure 2A). This study further profiled MasR protein expression in myocardial tissues of the three groups of rats. Western blotting showed that Ang (1-7) treatment markedly enhanced MasR protein production in the tissues, compared to sham or control treatment without Ang (1-7) (Figures 2B, S1), with a 4.9 fold increase on average (Figure 2C).

Ang (1-7) treatment suppressed IncAng362 and miR222 production in myocardial tissues

IncAng362 and miR222 have been associated with AngII-induced vascular smooth muscle cell proliferation [11] and blood vessels. To test the effects of Ang (1-7) ligation on expression of IncAng362 and miR222, this study quantified transcripts of both RNAs in myocardial tissues in rats resuscitated from cardiac arrest by qRT-PCR. Analyses of gene mRNA in the tissues demonstrated that Ang (1-7) significantly reduced expression levels of both IncAng362 (Figure 3A) and miR222 (Figure 3B), compared with controls absent from Ang (1-7).

Ang (1-7) treatment improved cardiac function after arrest resuscitation

To examine the effects of Ang (1-7) treatment on cardiac function after resuscitation from cardiac arrest, this study measured the param-
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Figure 4. Effects of Ang (1-7) treatment on Pten expression in myocardial tissues. Myocardial tissues were isolated from rats in sham, Ang (1-7)-treated, or control group. A. Total RNA was extracted from myocardial tissues of rats in the three groups and subjected to qRT-PCR. Pten mRNA expression fold increase was calculated compared to the sham group. GAPDH gene served as internal control. B. Pten protein expression in rat myocardial tissues was examined by Western blot with anti-Pten antibodies. β-actin served as internal control. C. Pten protein expression was quantified with the BandScan4.3 software. Pten protein expression fold increase in Ang (1-7)-treated or control group was calculated in comparison with the one in the sham group. Data are from three independent experiments, presented as mean ± SD, and analyzed by One-way ANOVA. *P<0.05, ***P<0.001.

Parameters of cardiac function. Parameters of cardiac performance, such as LVSP, ±LVdp/dtmax, LVEDP, MAP, and HR were observed in rats before and after treatment. No significant differences in baseline (before surgery) parameters such as total body weight, chloral hydrate, CPR, HR, MAP, LVSP, LVEDP, +LVdp/dtmax and -LVdp/dtmax were observed among the three groups of rats. Measurement of those parameters after the operation demonstrated that rats in the control group under resuscitation from cardiac arrest had lower LVSP (Figure 5A) and LVdp/dtmax (Figure 5C and 5D), but higher LVEDP (Figure 5B), compared to the sham group. In contrast, rats receiving Ang (1-7) treatment after resuscitation had significantly higher LVSP (Figure 5A), lower LVEDP (Figure 5B), and higher +LVdp/dtmax (Figure 5C) and -LVdp/dtmax (Figure 5D), compared to the control group without Ang (1-7) treatment (Figure 5A-D). No significant differences were observed in MAP or HR in rats between the Ang (1-7)-treated group and control group (Data not shown). This study further analyzed the relationship between expression of IncAng362 and cardiac function in myocardial tissue. Correlation analyses demonstrated that IncAng362 expression in myocardial tissues had significant negative correlation with LVSP (Figure 6A) and LVdp/dtmax (Figure 6B and 6C), but had significant positive correlation with LVEDP (Figure 6D).

Discussion

The present study demonstrated that Ang (1-7) ligation significantly enhances expression of its receptor MasR, inhibits expression of IncAng362 and miR222, increases Pten molecule production in rat myocardial tissues, and consequently improves rat cardiac function after resuscitation in vivo. To the best of our knowledge, this is the first report showing that Ang (1-7) has the ability to enhance cardiac performance after cardiopulmonary resuscitation from cardiac arrest.

Ang (1-7) has been shown to induce expression of vascular endothelial growth factor and matrix metalloproteinase-9 [19] and improve vascular endothelial function, delaying the development of heart failure after myocardial infarction [20]. Absence of Ang (1-7)/MasR ligation leads to heart dysfunction in vivo, decreasing left ventricular filling pressure, LVdp/dtmax, cardiac output, and cardiac index in Mas deficiency [21]. The present study found that Ang (1-7) treatment after resuscitation from cardiac arrest markedly enhanced expression of MasR in myocardium, suggesting a novel molecular mechanism by which Ang (1-7) exhibits protective effects on the cardiovascular system.

IncAng362 and miR222 are two types of non-coding RNAs linked with the Ang (1-7)/MasR signaling complex. It has been shown that Ang (1-7) downregulated the expression of IncAng362 and miR222. Moreover, specific knockdown of IncAng362 inhibited miR222 expression [11], indicating that IncAng362 collaborates with miR222 in response to upstream Ang (1-7) signaling. This study also showed that ligation of Ang (1-7) with MasR markedly
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Figure 5. Effects of Ang (1-7) treatment on cardiac function after cardiopulmonary resuscitation from cardiac arrest. Rats were divided into three groups: sham group, Ang (1-7), or saline (Con) intravenous administration group after cardiopulmonary resuscitation. Parameters of cardiac function LVSP (A), LVEDP (B), +LVdp/dt (C) or -LVdp/dt (D) were monitored with BL-420S biological signal acquisition and analysis system. Data are from three independent experiments, presented as mean ± SD, and analyzed by one-way ANOVA. *P<0.05, **P<0.01.

Figure 6. Correlation of IncAng362 and parameters of cardiac function. Bi-variate correlation analysis between IncAng362 and LVSP (A), +LVdp/dt (B), or -LVdp/dt (C), or LVEDP (D) was performed using One-way ANOVA. Pearson’s correlation coefficient was used in two-tailed tests. P<0.05 is considered to be statistically significant.

Reduced expression of both IncAng362 and miR222, suggesting that there is a positive correlation between IncAng362 and miR222 and a possible co-regulation relationship between IncAng362 and miR222 expression in myocardial tissues after cardiopulmonary resuscitation. However, different from previous reports based on vascular smooth muscle cell culture system in vitro [12], it was found that increased expression of IncAng362 in the control group of rats aggravated myocardial injuries after resuscitation from cardiac arrest in vivo.

Pten is widely expressed in the cardiovascular system and plays an important role in cardiovascular disease [22]. Numerous studies have found that overexpression of Pten negatively regulates cardiomyocyte hypertrophy induced by AngII through multiple pathways [23, 24]. MicroRNAs bind to the 3’ untranslated region of target messenger RNA, inhibiting their translation or directly degrading them and regulating the post-transcriptional level of genes, thereby regulating cell proliferation, differentiation, and apoptosis [25]. Pten is a direct target of miR222 that can regulate the expression of Pten at a post-transcriptional level [26]. It has been shown that Pten can inhibit the activation of AKT/eNOS signaling pathways and protect the heart [27]. In a cardiac fibrosis model, Pten overexpression can inhibit collagen metabolism and matrix metalloproteinases synthesis, thus inhibiting cardiac fibrosis [26]. The present study showed that Ang (1-7) significantly increased expression of Pten in rat myocardial tissues after cardiopulmonary resuscitation from cardiac arrest. It is possible that the mechanistic action...
of IncAng362 on cardiac function might be through the regulation of miR222 and Pten expression in myocardial tissues. Based on results, it is suggested that the mechanistic action of Ang (1-7) on cardiac function after cardiopulmonary resuscitation might be through the upregulation of MasR, downregulation of IncAng362 and miR222, and upregulation of Pten expression in myocardial tissues (Figure 7).

In conclusion, Ang (1-7) treatment attenuates cardiac insufficiency after cardiopulmonary resuscitation from cardiac arrest. The present study suggests that Ang (1-7) could serve as a potent therapeutic reagent for treatment of myocardial dysfunction after resuscitation.

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Disclosure of conflict of interest
None.

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**Figure S1.** Supplemental whole film for Figure 2B. Total proteins were extracted from myocardial tissues of the three groups of rats (Sham, control or Ang (1-7) treatment), and subjected to gel electrophoresis. MasR protein expression in the tissues was examined by Western blot with anti-MasR specific antibodies.

**Figure S2.** Supplemental whole film for Figure 4B. Total proteins were extracted from myocardial tissues of rats in Sham, control or Ang (1-7)-treated group. Pten protein expression in the tissues was examined by Western blot with anti-Pten specific antibodies.