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# MiR-30a regulates cardiomyocyte autophagy induced by ischemia-reperfusion through Beclin-1

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**Abstract:** Autophagy, referred as autophagy for short, is lysosome-dependent degradation pathway existing in eukaryotic cells, which runs through the whole process of normal cell growth and development and pathophysiology, and is an important way for biological degradation of intracellular proteins, to complete organelle transformation and to maintain homeostasis. Autophagy plays a significant role in myocardial ischemia-reperfusion (IR) injury. So it is important to inhibit autophagy to protect cardiomyocytes besides anti-apoptosis. MiRNA has been demonstrated to protect cardiomyocytes against apoptosis during IR, while whether it has anti-autophagy effect has not been known. The aim of this study was to investigate whether miR-30a regulated autophagy by regulating Beclin-1 protein, which is the marker of autophagosome during myocardial IR injury. In this study, we found that IR induced cardiomyocytes autophagy, together with down-regulation of miR-30a and upregulation of Beclin-1 protein. And, we have found that Beclin-1 protein was regulated by miR-30a, using the method of transferring miR-30a mimic or AMO-204 into the cardiomyocytes, before. These studies provided evidence that miR-30a played an important role in regulating autophagy through Beclin-1 protein during IR.
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【Key words】Autophagy; Beclin-1; cardiomyocytes

Autophagy plays a critical role in the growth and development, cell differentiation and the environmental stress response of organism, and its physiological function is involved in normal growth and development process of cells and maintaining metabolic balance; to promote the production of amino acids by self-degradation when nutritional deficiencies occurs[1-3]; under certain pressure conditions, such as in the presence of injury or nutritional deficiencies, to remove unwanted or injured cellular organ cells. Autophagy is a type of programmed cell death. It has been suggested to be essential for cell homeostasis. It can determine the cell survival together with apoptosis and necrosis [4-5]. Autophagy level is very low in physiological conditions, and is upregulated in many pathophysiological processes[6-7]. Because cardiomyocytes are terminally differentiated cells which can not divide again, suitable autophagy is essential for the maintenance of cardiomyocytes homeostasis. So, autophagocytic deficiencies or excess is associated with many cardiac pathologies, such as ischemia, IR, and heart failure [8-9]. It has been found that autophagy increased after IR [10], but it is still unclear whether autophagy protects the heart against IR injury or contributes to cell death. It seems that modest levels of autophagy appear to be protective. While high levels of autophagy may cause self-digestion and promote cell death [11].Autophagy is regulated by many autophagy related genes (Atgs) which are involved in autophagosome formation [12-13]. Beclin-1 is an important marker of autophagy [14]. So it is possible to control the process of autophagy by up-regulating or down-regulating LC3, and the molecular mechanism for this effect has yet to be elucidated. As we know, microRNAs (miRNAs or miRs), which negatively regulate protein expression in diverse biological and pathological processes, have been demonstrated to play an important role in myocardial injury [15-17]. It has been observed that many miRNAs regulate cell apoptosis,such as miR-1, miR-133, miR-199, miR-208, miR-320,miR-21, and miR-30a, etc [18-21]. However, it is well known that when apoptosis is blocked, the cells, which preferentially die by apoptosis, may die by autophagy

[22]. So it will be beneficial for cell survival if autophagy is inhibited together with apoptosis. We found that miR-30a,which has anti-apoptosis effect, may also regulate Beclin-1 expresion through the 9 complementary bases, according the bioinformatics of Targetscan.So the present study was undertaken to see whether miR-30a was dysregulated by ischemia-reperfusion (IR), and if it may inhibit autophagy during hypoxiareoxygenation by regulating Beclin-1.

## Materials and methods

### Animal care

All animal experiments were approved by the Animal Research Ethics Committee of Guangzhou Medical University, China. The investigation conformed with the guide for the care and use of laboratory animals published by the US National Institutes of Health.

### IR model and experimental protocols

SD rats (250-300 g) were anesthetized with 10% chloral hydrate (300 mg/kg, i.p.) before endotracheal intubation.IR was induced by ligating the left anterior descending artery (LAD) for 30 min, followed by loosening the ligature for 120 min. Successful ligation of LAD was evidenced by immediate regional cyanosis in the anterior ventricular wall and the apex of the heart with color change greater than 40% of the left ventricle (LV) and confirmed by electrocardiography(ECG).

### Experimental protocols

Twenty rats were equally randomly assigned into two groups: Control group (Con group, n = 10), where the rats underwent thoracotomy without ligation; IR group (n = 10), where the rats were treated with ischemia for 30 min and reperfusion for 120 min.

### Infarct size measurement

Infarct size of the myocardium was measured as previously described. Infarct area (INF) and area at risk (AAR) were determined by computerized planimetry.The percentage of INF/AAR was calculated.

### LDH assay

Blood serum was collected after 180min reperfusion for determination of lactate dehydrogenase (LDH).

### Quantitative real-time RT-PCR of miR-30a

Total RNA of cells was isolated by using TRIzol reagent,and reverse transcribed according to the manufacturer's instructions (Fermentas, in CA). The annealing temperature of miRNA-204 was set at 60°C. The comparative Ct (threshold cycle) method with arithmetic formulae (2-ΔΔCt) was used to determine relative quantitation ofgene expression of both target and housekeeping genes (b-actin). The primers of miR-30a used in the study are shown in Table 1.

Table 1 Primers used for quantitative real-time RT-PCR

| Type | Name | Sequence |
|---|---|---|
| PCR-primer | miR-30a-F | 5-GACGGTACCTGGTGGAGAACA ACTTCG-3 |
| | miR-30a-R | 5-CAGAAGCTTCATCAAACCTTCAATCCC-3 |
| | β-actin-R | 5'-ATGGTGGGTATGGGTCAGAAGG-3' |
| | β-actin-F | 5'-TGGCTGGGGTGTTGAAGGTC-3' |

**Western-blotting**

Protein concentration was determined with BCA protein assay kit according the manufacturer's protocol. Equal amounts of protein (60ug) from the cardiomyocytes were subjected to Western-blotting analysis to evaluate Beclin-1 expression with ECL detection kit (Amersham Biosciences, Piscataway, NJ). Beclin-1 immunoreactivity was detected using a rabbit antiserum specific for rat Beclin1 protein (Sigma, USA) as primary antibodies. Detection of antigen-antibody complex formation was performed with horseradish peroxidase (HRP)-conjugated goat anti-rabbit secondary antibody. The Beclin-1 concentrations were normalized with b-actin.

**Statistical analysis**

Quantitative data are presented as mean ± standard error. Statistical significance was determined using T test or one-way ANOVA. $P < 0.05$ was considered statistically significant.

**Results**

**Myocardium injury was induced by IR**

The extent of myocardial infarction was evaluated after reperfusion. Representative photographs of midventricular cross sections of evans blue and TTC-stained hearts were taken from Control and IR groups. INF/AAR and LDH were shown in Figure 1.

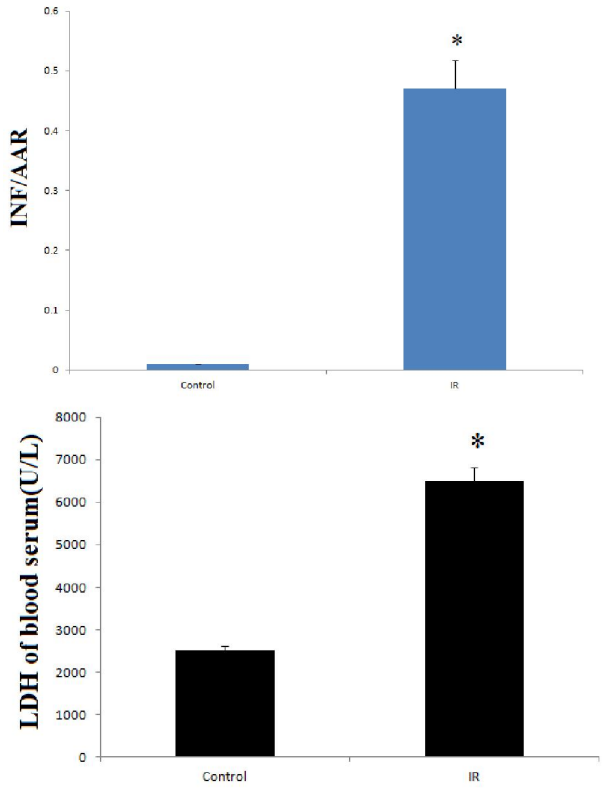



Figure 1 The heart injury induced by IR.

Note; The ratio of INF/AAR. It was found that IR increased the relative INF size compared with Con group. (n = 10, *P = 0.000 <0.05, compared with Con group). LDH assay of blood serum. The activitie of LDH was increased by IR compared with Con group. (n= 10, *P = 0.000 < 0.05, compared with Con group).

**IR decreased the expression of miR-30a**

To demonstrate the effect of IR on miR-30a, we compared the miR-30a between the control group and IR group (n=10). It was found that IR significantly decreased miR-30a with the method of Real-time PCR.(Figure 2)

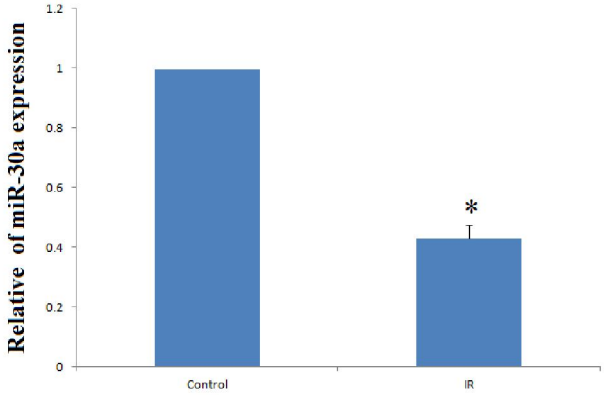
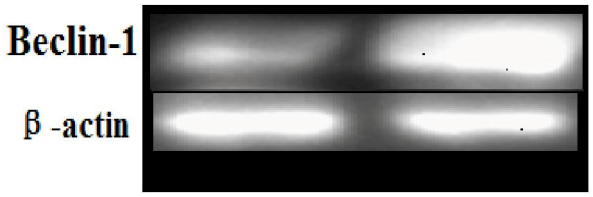



Figure 2 Results of miR-30a expression with RT-PCR after IR injury. It was found that miR-30a was down-regulated by IR. (n =10, P = 0.026 < 0.05).

**IR up-regulated the protein level of BECLIN-1**

As a marker of autophagosome, the protein level of Beclin-1 represents the amount of autophagosome. So we compared the ratio of Beclin-1/β-actin between the control collaborators. It has been reported that autophagy contributed to cell death when apoptosis is inhibited,and sometimes the early stages of autophagy were required for apoptosis. Beclin-1 was the marker of autophagosome. So the ratio of Beclin-1/β-actin could stand for the level of autophagy. In our study, we found that IR up-regulated the protein expression of Beclin-1 together with increasing the ratio of IR group (n=10), and found that it was enhanced by IR. (Figure 3)



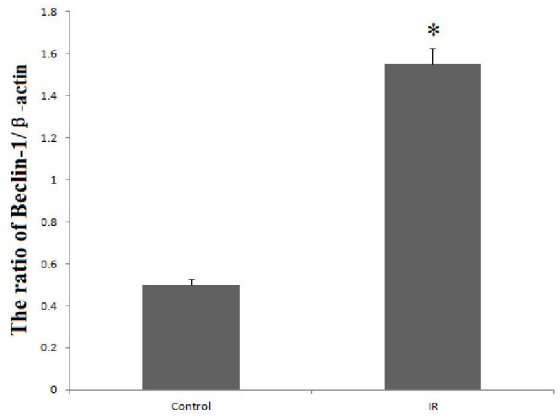



Figure 3 Results of BECLIN-1 protein expression with western blot after IR injury.
Note: The ratio of BECLIN-1/LC3-I in different groups. It was found that BECLIN-1 was up-regulated by IR. (n = 10, *P = 0.000 < 0.05).

**Discussion**

Autophagy is a lysosom-dependent degradation pathway widely existing within eukaryotic cells as a self-protection mechanism in response to the pessimal stimulation, and the function of autophagy are not only involved in surrounding and isolating the damaged organelles, but more important in transporting the surrounded materials to lysosomes for degradation, and producing amino acids, ATP and other substances to maintain the energy metabolism cycle of the body, autophagy has attracted great interest because it was involved in many physiological processes. If autophagy destroys the cytosol and organelles beyond a certain threshold, autophagic cell death will occur. Autophagy was detrimental during reperfusion although it protected the cardiomyocytes during ischemia, which has been demonstrated by Matsui and his autophagy cell. So it would be beneficial for the revascularized hearts, to find a method of regulating Beclin-1 expression. MiRNA is a group of small, non-coding RNAs which regulates gene expression in a sequence-dependent manner. They are endogenous regulators of gene expression, and have been demonstrated to be involved in cardiac IR injury. According to the bioinformatics of Targetscan, miR-30a, which has anti-apoptosis effect, may regulate the expression of Beclin-1.In our study, it was found that IR could down-regulate miR-30a together with up-regulated Beclin-1 protein.When miR-30a mimic was transferred into cardiomyocytes,Beclin-1 protein was attenuated, and Beclin-1 protein was up-regulated by AMO-204 which was concentration dependence as other miRNAs [23]. But LC3-I was not regulated by miR-30a, as the previous study [24]. These results demonstrated that miR-30a may regulate cardiomyocytes autophagy through Beclin-1 during IR injury.

Our results demonstrated that miR-30a played an important role by regulating Beclin-1 protein during IR. So it became possible to control the autophagy under a beneficial threshold by regulating miR-30a expression, for protecting the cardiomyocyte against IR injury.

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