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Curcumin alleviates isoproterenol-induced cardiac hypertrophy and fibrosis through inhibition of autophagy and activation of mTOR

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Abstract. – OBJECTIVE: Curcumin has been reported to possess cardioprotective effects. However, the potential molecular mechanism of curcumin is still not clear. The aim of the present study was to investigate the role of curcumin in regulating autophagy and mammalian target of rapamycin (mTOR) signaling in isoproterenol-induced cardiac hypertrophy and fibrosis in the rat.

MATERIALS AND METHODS: Rats model of cardiac hypertrophy and fibrosis was induced by isoprenaline (5 mg/kg/day, subcutaneous injection), which were treated with or without curcumin (200 mg/kg/day, intragastric administration). Masson's trichrome staining was performed to investigate the effect of curcumin on fibrosis of cardiac hypertrophy rat. The expression of hypertrophic and fibrosis markers was determined by RT-qPCR. The protein expression of autophagic markers, mTOR, and phosphorylated-mTOR (p-mTOR) was performed by Western blotting.

RESULTS: Isoprenaline treatment significantly up-regulated the mRNA expression of hypertrophic (ANP and MYH7) and fibrotic (procollagen I and III) markers in the hearts from rats. All of these markers were reversed by curcumin treatment in isoproterenol-treated rats. Histological analysis showed that curcumin attenuated the interstitial fibrosis of heart triggered by isoproterenol. Moreover, isoproterenol significantly reduced the mRNA levels of mTOR and the protein expression of p-mTOR. However, isoprenaline caused a significant induction of the mRNA levels of LC3 and Beclin-1 and the protein expression of LC3-II and Beclin-1, as well as LC3-II/I ratio. Curcumin abolished these isoprenaline-mediated changes in mTOR/autophagy signaling pathway.

CONCLUSIONS: Our data demonstrated that curcumin targeted mTOR/autophagy axis could attenuate cardiac hypertrophy and fibrosis in a rat model.

Key Words:
Curcumin, Cardiac hypertrophy, Fibrosis, Autophagy, mTOR.

Introduction

Cardiac hypertrophy is usually induced by an increase in biomechanical stress and characterized by the growth of heart mass and individual cardiomyocytes[1]. There is increasing evidence that cardiac hypertrophy is one of the main reasons for leading to heart failure, and the prevention of cardiac hypertrophy may represent a new management strategy for improving survival in patients with cardiovascular diseases (CVDs)[2,3]. Recently, autophagy has been investigated as one of the possible therapeutic approaches to treatment of cardiac hypertrophy[4,5]. Interestingly, autophagy appears to play dual roles in myocardial hypertrophy. Many reports[6,7] have suggested that the high level of autophagy plays a beneficial role in cardiac hypertrophy, while other studies[8,9] show the contrasting functions of autophagy in myocardial hypertrophy. Therefore, autophagy may play adaptive or maladaptive roles in the pathologic process of cardiac hypertrophy depending on the conditions and the extent of stimulation.

Curcumin is a polyphenolic compound and is derived from turmeric (*Curcuma longa*) that has been used for the treatment of inflammatory diseases[10,11]. Recent works also show that curcumin plays a protective role in CVDs, including cardiac hypertrophy[12]. Multiple signaling pathways implicated in the hypertrophic response can be regulated by curcumin[13], similar to enalapril, an angiotensin-converting enzyme inhibitor (ACEI), which is well known for improving cardiac hypertrophy[14]. A review published by Hashemzaei et al[15] declares that curcumin can trigger autophagy via Beclin-1-dependent or Beclin-1-independent pathways. In human vascular endothelial cells, curcumin protects against oxidative stress-induced cell damage

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by activating autophagy[16]. Curcumin protects cardiomyocytes from ischemic damage through activating adenosine monophosphate-activated protein kinase (AMPK) pathway and inhibiting mTOR signaling promoting autophagy[17]. In contrast to these studies[16,17], the protective effect of curcumin against hypoxia/reoxygenation-induced H9c2 cardiomyocyte injury attributes to the inhibition of autophagy. All of these results demonstrate that the effects of curcumin on autophagy during cardiovascular dysfunction are controversies. Therefore, the regulatory mechanism of curcumin on autophagy in the process of cardiac hypertrophy is necessary for further investigation.

In the present work, we have undertaken to determine whether curcumin protects against isoproterenol-induced cardiac hypertrophy and fibrosis by regulating autophagy.

Materials and Methods

Animal Treatment

Male Sprague-Dawley rats (ten-week-old; body weight: 200-250 g; n = 24) were purchased from Vital River Laboratories Co., Ltd (Beijing, China) and allowed to acclimate to the environment for 1 week. The rats were randomly divided into four groups. Control group (Con, n = 6) received normal saline by subcutaneous injection. The model group received isoprenaline (Iso, 5 mg/kg/day) treatment by subcutaneous injection for 7 days. Control group plus curcumin (Cur, n = 6) group was treated with normal saline by subcutaneous injection combined with curcumin (200 mg/kg/day) by intragastric administration. Curcumin plus isoprenaline group (Cur + Iso, n = 6) was treated with isoprenaline (5 mg/kg/day) by subcutaneous injection for 7 days combined with curcumin (200 mg/kg/day) by intragastric administration for 4 weeks. All of the rats were killed by an overdose of sodium pentobarbital (2%; 200 mg/kg; Sigma-Aldrich, St. Louis, MO, USA) 24 h after their last treatment. Hearts were collected and immediately frozen in liquid nitrogen and 4% formalin at room temperature for gene and protein analysis and paraffin-embedded, respectively. The experiment was approved by the Ethics Committee of Beijing Luhe Hospital, Capital Medical University (Beijing, China) and was performed in accordance with its guidelines.

Histomorphology

Hearts were collected immediately following sacrifice and fixed with 4% formalin at room temperature for 24 h. Tissues were embedded with paraffin and were cut into 3 μm-thick sections, which were stained with Masson's trichrome (cat. no. SBJ-0290; Nanjing SenBeiJia Biological Technology Co., Ltd., Nanjing, China) as previously described[18]. Interstitial fibrosis was visualized under a microscope (magnification, ×50; Leica DM 2500; Leica Microsystems GmbH, Wetzlar, Germany). Fibrotic measurements were performed with an automated image analysis system (Image-Pro Plus 5.0, Media Cybernetics Inc., Rockville, MD, USA).

RNA Extraction and Reverse Transcription-Quantitative Polymerase Chain Reaction (RT-qPCR)

Total RNA was extracted using TRIzol® (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA), according to the manufacturer's protocol. The RNA was quantified by measuring the absorbance at 260 nm using Nanodrop 2000 (Thermo Fisher Scientific, Inc., Waltham, MA, USA). cDNA was synthesized by reverse transcription reactions with 2 μg total RNA using moloney murine leukemia virus reverse transcriptase (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA). cDNAs served as the template for the PCR, which was performed using the TaqMan Universal PCR Master Mix (Thermo Fisher Scientific, Inc.) with a DNA Engine (ABI 7300; Thermo Fisher Scientific, Inc., Waltham, MA, USA). The reaction conditions were carried out according to the manufacturer's protocol. The PCR primers were shown in Table I. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) levels were utilized to normalize the expression of the target genes. The relative expression levels of genes were calculated using the $2^{-\Delta\Delta Ct}$ method[19].

Western Blotting

Protein was extracted using RIPA Lysis Buffer (Beyotime Institute of Biotechnology, Haimen, China). The concentration was determined using the Bicinchoninic Acid Kit for Protein Determination (Sigma-Aldrich; St. Louis, MO, USA). Samples containing 50 μg of protein were separated by 10% SDS-PAGE gel and transferred to nitrocellulose membranes (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Primary antibodies mTOR (cat.no: sc-293089, dilution, 1:1,000), p-mTOR (cat.no: sc-293132, dilution, 1:500) and

Table I. Primers were used to RT-qPCR.

Gene	Forward primer (5′-3′)	Reverse primer (5′-3′)
ANP	GGAGCCTGCGAAGGTCAA	TATCTTCGGTACCGGAAGCTGT
BNP	CAGAAGCTGCTGGAGCTGATAAG	TGTAGGGCCTTGGTCCTTTG
MYH6	GCCCTTTGACATCCGCACAGAGT	TCTGCTGCATCACCTGGTCCTCC
MYH7	GCGGACATTGCCGAGTCCCAG	GCTCCAGGTCTCAGGGCTTCACA
MYH7B	CCCGATTCTCAACACCAACACCTCT	CATCAGGCACCCAGACCCGT
Procollagen I	TATGCTTGATCTGTATCTGCCACAAT	TCGCCCTCCCGTTTTTG
Procollagen III	CAGCTGGCCTTCCTCAGACT	TGCTGTTTTTGCAGTGGTATGTAA
mTOR	TGCTGGTGTCCTTTGTGAAG	TTGTGCTCTGGATTGAGGTG
LC3	ATCATCGAGCGCTACAAGGGTGA	GGATGATCTTGACCAACTCGCTCAT
Belin-1	TTCAAGATCCTGGACCGAGTGAC	AGACACCATCCTGGCGAGTTTC
GAPDH	GCACCGTCAAGCTGAGAAC	TGGTGAAGACGCCAGTGGA

Beclin-1 (cat.no: sc-48341, dilution, 1: 1,000) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA), LC3I/II (cat.no: 4108, dilution, 1:1,000) from Cell Signaling Technology (Danvers, MA, USA). And then, the membranes were incubated with the appropriate horseradish peroxidase-conjugated secondary antibody (cat.no: sc-516102; dilution: 1:10,000; Santa Cruz Biotechnology, Santa Cruz, CA, USA), following visualized using chemiluminescence (Thermo Fisher Scientific, Inc. Waltham, MA, USA). GAPDH primary antibody (cat. no: 2118; dilution: 1:2,000; Cell Signaling Technology, Inc.) was used to as the control antibody. Signals were analyzed with Quantity One® software version 4.5 (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

Statistical Analysis

Data were presented as mean ± SEM. Statistical analysis was performed using GraphPad Prism Version 7.0 (GraphPad Software, Inc., La Jolla, CA, USA). Inter-group differences were analyzed by one-way analysis of variance, followed by Tukey's post-hoc analysis (pairwise comparison). The *p*-value less than 0.05 was considered to indicate a statistically significant difference.

Results

Curcumin Attenuated Isoproterenol-Induced Cardiac Hypertrophy in Rats

To investigate the beneficial effect of curcumin on isoproterenol-induced cardiac hypertrophy *in vivo*, we first established a cardiac hypertrophy model in the rats. We measured the ratio of heart weight to body weight, isoprenaline caused a significant induction in the ratio of heart weight to body weight by 27.7%. On the other hand, curcumin treatment significantly decreased the ratio of heart weight to body weight by 13.1% (Figure 1A). In addition, the hypertrophic markers, atrial natriuretic peptide (ANP), and brain natriuretic peptide (BNP), α-myosin heavy chain (α-MHC; also known as MYH6), β-MHC (also known as MYH7) and MYH7B, were evaluated in the hearts from rats. RT-qPCR analysis showed that the expression of ANP was up-regulated more than 6-fold in the hearts of isoprenaline-treated rats, but it was significantly down-regulated in the combined isoprenaline+curcumin group (Figure 1B). Of note, BNP was not altered by isoprenaline, curcumin or their combination (Figure 1C). MYH7 and the ratio of MYH7 to MYH6 are considered as the molecular markers of myocardial hypertrophy[20]. Consistent with previous reports[21,22], isoproterenol treatment markedly decreased MYH6 mRNA expression level and elevated the mRNA expression level of MYH7 (Figure 1D and 1E). Curcumin treatment significantly reversed isoproterenol-induced the up-regulation of MYH7 and the down-regulation of MYH6. MYH7B gene shows a transcriptional response similar to MYH6 in response to cardiac hypertrophy[23]. MYH7B was decreased by isoproterenol treatment, and the expression of MYH7B had a similar expression of MYH6 in the combined isoprenaline+curcumin group (Figure 1F).

Curcumin Improved Isoproterenol-Induced Cardiac Fibrosis in Rats

To investigate the effect of curcumin on the fibrosis associated with isoprenaline-induced cardiac hypertrophy, Masson's trichrome staining revealed that the area of interstitial fibrosis (2.90 ± 0.60%) was significantly increased in isoprenaline-treated group compared with control group

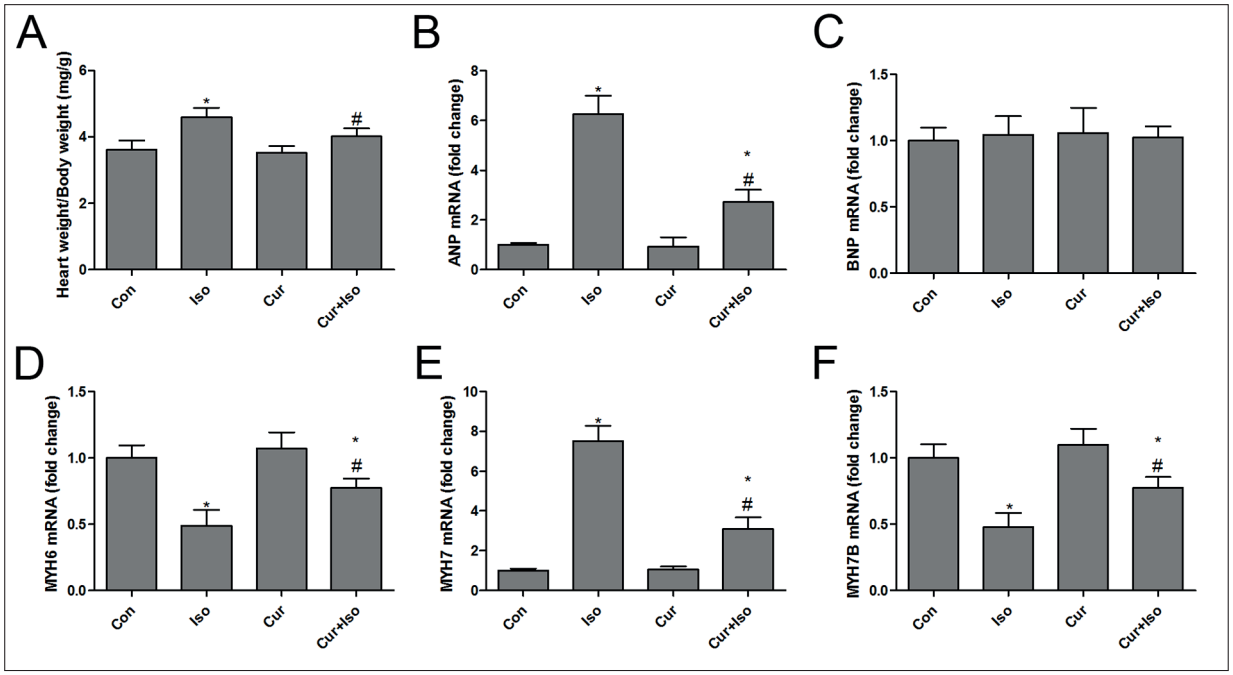

Figure 1. Canonical markers of cardiac hypertrophy in response to curcumin and isoproterenol treatment. The ratio of heart weight/body weight (mg/g) was changed by curcumin and isoproterenol treatment **(A)**. The changes in expression of atrial natriuretic peptide (ANP) **(B)**, brain natriuretic peptide (BNP) **(C)**, α-myosin heavy chain (α-MHC; also known as MYH6) **(D)**, β-MHC (also known as MYH7) **(E)** and MYH7B **(F)**. Values represent the mean ± SEM, n = 6 in each group. $^{*}p < 0.05$ compared with control group; $^{\#}p < 0.05$ compared with isoproterenol-treated group.

(0.55 ± 0.17%). However, curcumin did limit the increased area of interstitial fibrosis caused by isoproterenol (Figure 2A and 2B). In addition, we measured the cardiac gene expression of the fibrotic markers, procollagen I, and procollagen III. Isoprenaline treatment caused a significant induction in the fibrotic markers, procollagen I, and procollagen III by 429% and 314%, respec-

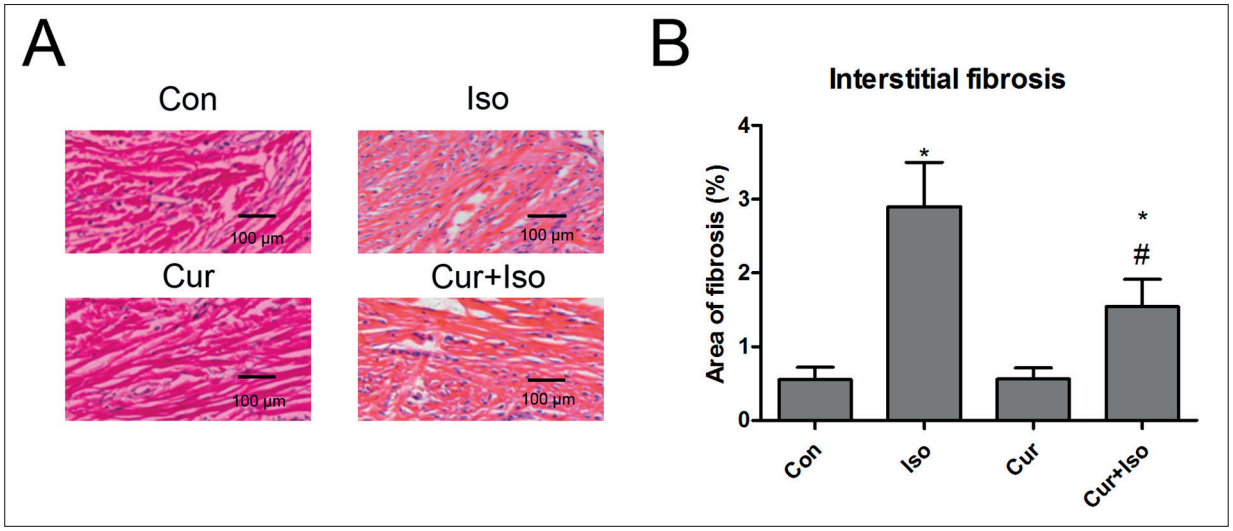

Figure 2. Curcumin improved isoproterenol-induced cardiac fibrosis in rats. Masson's trichrome staining of hearts in the control, isoproterenol, curcumin and isoproterenol combined with curcumin treatment group **(A)**. Bar diagrams represent the area of interstitial fibrosis (%) **(B)**. Values represent the mean ± SEM, n = 6 in each group. $^{*}p < 0.05$ compared with control group; $^{\#}p < 0.05$ compared with isoproterenol-treated group.

tively. However, curcumin treatment significantly decreased the isoprenaline-mediated induction of procollagen I and procollagen III by 57.8% and 51.7%, respectively (Figure 3A and 3B). Furthermore, no significant difference was observed between the control group and the curcumin treatment alone (Figure 3A and 3B).

Curcumin Inhibited Autophagy and Activated mTOR in Isoproterenol-Induced Cardiac Hypertrophy and Fibrosis in Rats

To further explore the potential molecular mechanisms by which curcumin protects against isoproterenol-induced cardiac hypertrophy and fibrosis, the autophagy and mTOR signaling were supervised by RT-qPCR and Western blotting analysis in the hearts from rats. Previous studies[24,25] suggest that mTOR signalling possesses a cardioprotection through the inactivation of autophagy *in vivo* and *in vitro*. The mRNA levels of mTOR were significantly inhibited in the hearts from isoprenaline-treated rats as compared to those of the control group. However, curcumin combined with isoprenaline treatment resulted in the up-regulation of mTOR mRNA expression (Figure 4A). In contrast to that the mRNA expression of the autophagy markers, LC3 and Belin-1, was significantly increased in the hearts from isoprenaline-treated rat, which was blocked by curcumin treatment (Figure 4B and 4C). Western blotting results showed that the expression of phosphorylated mTOR (p-mTOR) was markedly suppressed by isoprenaline, which had no effect on mTOR protein expression (Figure 5A). The ratio of p-mTOR to mTOR was significantly decreased by isoprenaline, but the combined treatment could significantly reverse isoprenaline-induced the decrease of the ratio of p-mTOR to mTOR (Figure 5A and 5B). Figure 5C and 5D showed the effect of curcumin treatment on isoprenaline-induced autophagy. Isoprenaline treatment caused a significant increase in autophagy in the hearts from rat. However, curcumin treatment could significantly inhibit isoprenaline-induced autophagy in the hearts from rat. These findings suggest that curcumin protects against isoproterenol-induced cardiac hypertrophy and fibrosis, at least partially, through inhibition of autophagy and activation of mTOR.

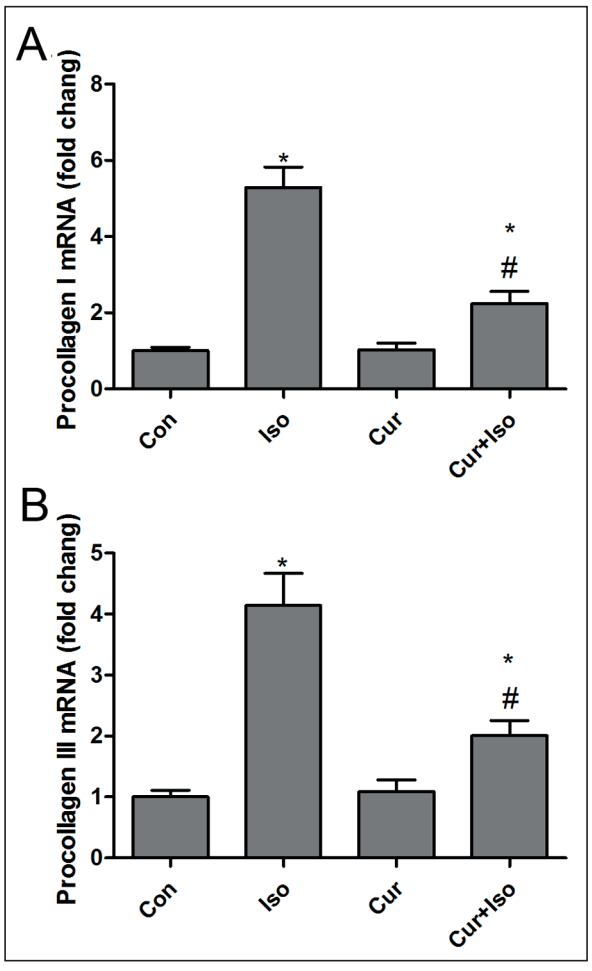

Figure 3. Canonical markers of cardiac fibrosis in response to curcumin and isoproterenol treatment. The mRNA expression of procollagen I **(A)** and procollagen III **(B)** was measured by RT-qPCR in the hearts from rats. Values represent the mean ± SEM, n = 6 in each group. $^{*}p < 0.05$ compared with control group; $^{\#}p < 0.05$ compared with isoproterenol-treated group.

Discussion

In the current work, we investigated the cardioprotective effect of curcumin treatment on isoprenaline-induced cardiac hypertrophy and fibrosis. Our results demonstrated that curcumin inhibited isoprenaline-induced cardiac hypertrophy and fibrosis in our murine model. We also found that isoprenaline-induced the activation of autophagy and the inhibition of mTOR were reversed by following curcumin treatment. We provided novel evidence that curcumin might reduce autophagy by activating mTOR signaling during isoprenaline-induced cardiac hypertrophy and fibrosis.

Previous scholars[4,26] report that some mechanical stresses, transverse aortic constriction (TAC), pulmonary arterial hypertension (PAH) and angiotensin II (Ang II) promote cardiac hypertro-

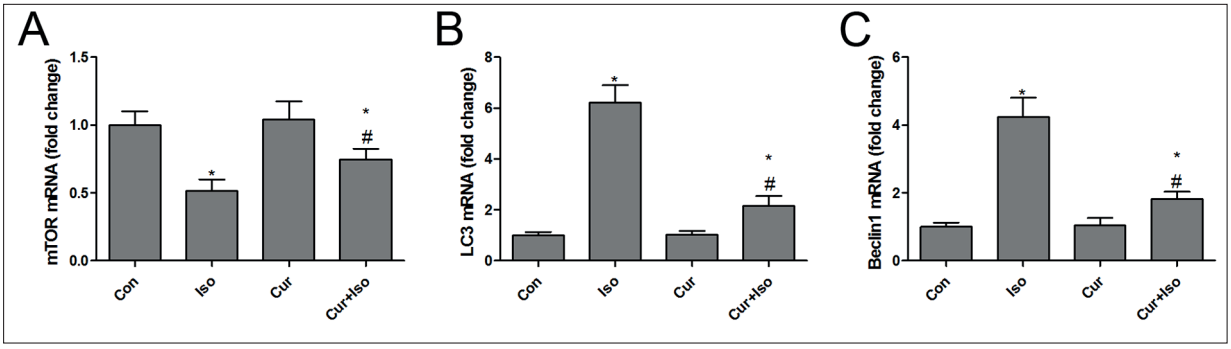

Figure 4. Curcumin inhibited autophagy and activated mTOR in isoproterenol-treated rats. The mRNA expression of mTOR **(A)**, LC3 **(B)** and Beclin-1 **(C)** was measured by RT-qPCR in the hearts from rats. Values represent the mean ± SEM, n = 6 in each group. $^{*}p < 0.05$ compared with control group; $^{\#}p < 0.05$ compared with isoproterenol-treated group.

phy accompanied by high levels of autophagic responses in the heart, inhibition of autophagy reverses cardiac hypertrophy. On the contrary, a high-fat diet induces cardiac hypertrophy accompanied by the inhibition of autophagy, rapamycin has been found to ameliorate cardiac hypertrophy by up-regulating autophagy in this process[27]. Lu et al[28] showed that isoproterenol induces cardiac hypertrophy through reduction of autophagy by decreasing the expression of autophagy-related

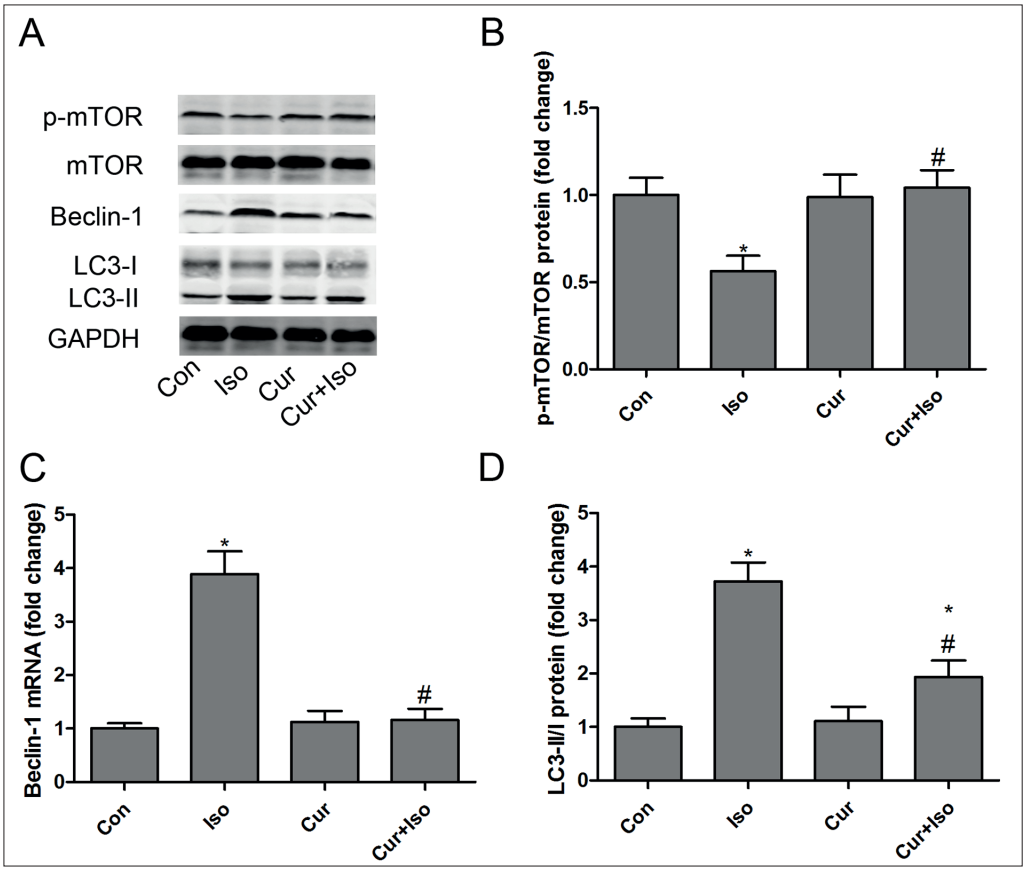

Figure 5. Curcumin inhibited autophagy and activated mTOR in isoproterenol-treated rats. The protein expression of p-mTOR and mTOR (**A and B**), Beclin-1 (**A and C**) and LC3-I/II (**A and D**) was measured by Western blotting in the hearts from rats. Values represent the mean ± SEM, n = 6 in each group. $^{*}p < 0.05$ compared with control group; $^{\#}p < 0.05$ compared with isoproterenol-treated group.

gene 5 (Atg5) and LC3-II. Zhang et al[29] suggested that isoproterenol induces excessive autophagy in cardiomyocyte hypertrophy models *in vitro* and *in vivo* by increasing the expression of Beclin1, Atg7 and LC3-II. In agreement with previous results[29], isoproterenol markedly increased the mRNA and protein levels of beclin-1 and LC3-II as well as the LC3-II/LC3-I ratio in our murine model, suggesting that autophagy was activated in isoproterenol-induced cardiac hypertrophy rats.

Curcumin has been found to play a cardioprotective effect in isoproterenol-[30], ischemia-reperfusion (I/R)-[31] or doxorubicin-induced cardiotoxicity[32], which appears to be due to their ability to anti-oxidized, anti-inflammatory, anti-thrombotic and anti-apoptotic properties[30-33]. The present investigation was designed to elaborate the effect of curcumin on attenuation of isoproterenol-induced cardiac hypertrophy and fibrosis in rat. Our findings showed that curcumin (200 mg/kg/day) effectively reduced hypertrophic and fibrotic biomarkers in the hearts from isoproterenol-treated rats. Moreover, alternative signaling molecules are involved in the hypertrophic and fibrotic responses in isoproterenol-treated rats. These included abnormally expressed Beclin-1, LC3-I, LC3-II, and p-mTOR. In recent years, the autophagy pathway mechanism is involved in curcumin ameliorated heart failure[15]. Curcumin alleviates hypoxia/reoxygenation (H/R)-induced H9c2 cardiomyocytes dysfunction through inhibition of autophagy by reducing Beclin-1 and LC3-II expression[34]. To our knowledge, this is the first study to report that curcumin attenuates isoproterenol-induced cardiac hypertrophy and fibrosis by inhibiting autophagy.

On the other hand, AMPK and mTOR signaling as the directly regulators are also implied in curcumin-induced autophagy[15]. Curcumin possesses a protective effect on cardiomyocytes through activation of AMPK and inhibition of mTOR signaling in myocardial I/R model, meanwhile the autophagy is induced in this process[17]. Kim and Guan[35] indicated that genetic or pharmacologic inhibition of mTOR signaling can induce autophagy. The inhibition of mTOR results in increased autophagy-initiating UNC-5 like autophagy activating kinase (ULK) 1/2 kinase activity[35]. In mammalian cells, mTOR promotes the phosphorylation of ULK1, preventing the interaction and phosphorylation of ULK1 by AMPK, which is essential for ULK1 activation and autophagy[36]. One study showed that isoprenaline-induced the activation of autophagy was accompanied with the inhibition of mTOR in the hearts from rats. However, curcumin supplementation could be beneficial for the heart in the presence of isoproterenol, suggesting that mTOR activation underlied the mechanism of curcumin inhibition of isoproterenol-induced cardiac hypertrophy and fibrosis.

Conclusions

We indicated that inhibition of autophagy and activation of mTOR might be a key mechanism for the anti-cardiac hypertrophy and fibrosis activity of curcumin in an experimental animal model.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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