Puerarin Decreases Collagen Secretion in AngII-Induced Atrial Fibroblasts Through Inhibiting Autophagy Via the JNK–Akt–mTOR Signaling Pathway

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Abstract: Puerarin is used to treat cardiovascular diseases due to its anti-inflammatory and antifibrotic effects. However, its mechanism of action in atrial fibroblasts is unknown. In this study, we investigated the autophagy pathway and molecular changes in angiotensin II (AngII)-stimulated atrial fibroblasts in response to puerarin treatment. Atrial fibroblasts were cultured and then subjected to stimulation with AngII and puerarin or other chemical drugs (3-MA, CQ, and SP600125). Quantitative real-time polymerase chain reaction and Western blot experiments were used to quantify the expression levels of mRNA and protein. mCherry-GFP-LC3 adenovirus was applied to reflect the autophagic flux. The results showed aggravating levels of autophagy and collagen deposit in the presence of AngII. Puerarin inhibited autophagy and decreased collagen secretion in a dose-dependent manner in atrial fibroblasts. Furthermore, phosphorylation of JNK was down-regulated in response to puerarin, whereas phosphorylation of Akt and mammalian target of rapamycin (mTOR) was upregulated. Interestingly, reduced autophagy and collagen secretion were observed when the JNK signaling pathway was blocked using SP600125. We also observed upregulation of Akt and mTOR phosphorylation in the presence of SP600125. These results suggest that puerarin exerts its antifibrotic effect in atrial fibroblasts partly through the inhibition of autophagy. Furthermore, the mechanism of action of puerarin in fibroblast autophagy seems to be mediated partly through JNK–Akt–mTOR signaling.

Key Words: puerarin, atrial fibroblasts, autophagy, JNK signaling, atrial remodeling

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INTRODUCTION

Atrial fibrillation (AF) is one of the most common tachyarrhythmias, especially in the elderly. The mechanisms of AF are complicated. Previous studies found that atrial structural and electrical remodeling is one of the pathological mechanisms underlying AF.[1,2] Fibrosis is characterized by myofibroblast accumulation, collagen deposition, and extracellular matrix remodeling.[3–6] Fibrosis in atria results in atrial structural remodeling and influences the electrical conduction in the atria, providing a substrate for AF generation and maintenance.

Autophagy, an evolutionarily conserved metabolic process, ensures cellular homeostasis when cells encounter stress.[7,8] Under normal conditions, long-lived proteins are degraded by autophagy, and the degradation products are recycled. However, excessive autophagic activation leads to the digestion of essential molecules or organelles.[9,10] Three types of autophagy have been described[7]: chaperone-mediated autophagy, microautophagy, and macroautophagy (hereafter known simply as autophagy). Previous studies have shown that autophagy participates in various aspects of cardiovascular disease, including ischemia–reperfusion, heart failure, cardiac hypertrophy, and fibrosis.[11–13] Liu et al[14] indicate that activation of autophagy mitigates cardiac fibrosis induced by angiotensin II (AngII). By contrast, other studies show that autophagy inhibition has a protective effect in cardiac fibrosis.[15,16] Therefore, there is controversy concerning whether autophagy is beneficial or detrimental in cardiac fibrosis, especially in atrial fibrosis and AF.

Puerarin is an isoflavone extracted from the root of the traditional Chinese medicine pueraria.[17] It has been used as a traditional Chinese medicine for a long time in curing various diseases, including cardiovascular diseases, because of its anti-inflammatory and antifibrotic properties.[18–20] A recent study reported that puerarin is neuroprotective in cerebral ischemia by attenuating autophagy at the ischemic penumbra in neurons.[21] Liu et al[22] report that puerarin reduces cardiac hypertrophy induced by pressure overload through the activation of autophagy. However, Tang et al[23] consider enhanced autophagy as related to myocardial hypoxia/reoxygenation injury and demonstrate that puerarin alleviates myocardial hypoxia/reoxygenation injury via the Akt signaling pathway. Considering these contradictory findings about autophagy in cardiac diseases, we investigated the role of autophagy and the effect of puerarin in AngII-induced atrial

fibroblasts. In this study, we aimed to explore whether puerarin relieves atrial fibrosis induced by AngII and understand the potential mechanism.

MATERIALS AND METHODS

Reagents and Antibodies

The cell culture medium (dulbecco's modified eagle medium basic) was purchased from Gibco (Shanghai, China). AngII was purchased from Bachem (Bubendorf, Switzerland, No.4006473). Fetal bovine serum was from GEMINI (Woodland, CA). Puerarin was purchased from Selleck Chemicals (Houston, TX). mCherry-GFP-LC3 was purchased from ViGene (Jinan, China). Antibodies for collagen I (COL-I, ab138492, 1:500), collagen III (COL-III, ab7778, 1:1000), P62/SQSTM1 (ab109012, 1:2000), phospho-Akt (ab81283, 1:1000), and Akt (ab179463, 1:1000) were purchased from Abcam (Cambridge, United Kingdom). Phospho–mammalian target of rapamycin (mTOR) (2971, 1:1000), mTOR (2972, 1:1000), phospho-SAPK/JNK (4668, 1:1000), and SAPK/JNK (9252, 1:1000) antibodies were purchased from Cell Signaling Technology (Boston, MA). LC3 antibody (L7543, 1:500), dimethyl sulfoxide (DMSO), and chloroquine diphosphate salt (CQ) were purchased from Sigma (St. Louis, MO). 3-methyladenine (3-MA) and SP600125 were from MedChemExpress (Monmouth, NJ). GAPDH antibody (ab011-100, 1:5000), goat anti-rabbit (GAR007, 1:10,000), and goat anti-mouse (GAM007, 1:10,000) horseradish peroxidase (HRP)-IgG were obtained from Multi Sciences (Hangzhou, China).

Cell Culture

Atrial fibroblasts were isolated from mouse atria, as previously described.[24,25] The animal experiments were approved by the Institutional Animal Care and Use Committee of Sir Run Run Shaw Hospital of Zhejiang University and conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Briefly, mice (provided by the Experimental Animal Centre of Sir Run Run Shaw Hospital) were anesthetized by isoflurane, followed by cervical dislocation. The heart was exposed, and bilateral atria were cut off. Atria were washed with cold phosphate buffer saline and cut into 1-mm pieces that were placed into digestive fluid (0.1% trypsin and 0.1% collagenase type II). Cells were collected, and atrium fibroblasts were separated according to differential adherent velocity of fibroblasts and cardiomyocytes. Cells were cultured in the dulbecco's modified eagle medium containing 10% (vol/vol %) fetal bovine serum in a humidified atmosphere at 37°C with 5% CO_2. Cells at passages 2–3 were used in experiments. Cells were treated with AngII or varying concentrations of puerarin (10, 20, 30, and 40 μM) on reaching 70% confluence. For the inhibitor experiment, 3-MA (10 mM) and CQ (10 μM) were used to inhibit autophagy. Cells were

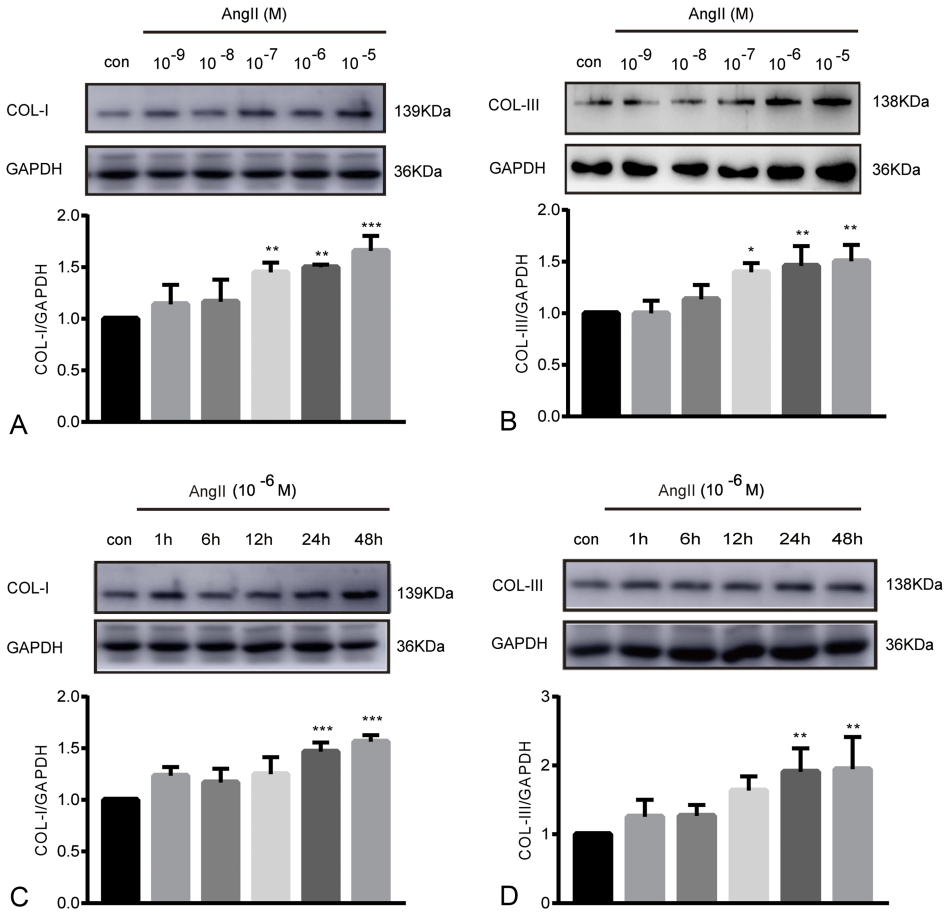

FIGURE 1. Expression of collagen I (COL-I) and collagen III (COL-III) in AngII-induced atrial fibroblasts. Cells were treated with AngII at different concentrations (10^{-9} M, 10^{-8} M, 10^{-7} M, 10^{-6} M, and 10^{-5} M) and times (1, 6, 12, 24, and 48 hours). Expression of COL-I and COL-III in response to different concentrations of AngII stimulation for 48 hours (A and B). Expression of COL-I and COL-III at different times of AngII stimulation at 10^{-6} M (C and D). Data are expressed as the mean ± SD of 3 independent experiments, and the most representative bands are shown. $^{*}P < 0.05$ versus the control group (con); $^{**}P < 0.01$ versus the control group; $^{***}P < 0.001$ versus the control group.

harvested for mRNA and protein analysis, as described below. In addition, AngII and CQ were dissolved in sterile water. Puerarin, 3-MA, and SP600125 were dissolved in DMSO, and the highest concentration of DMSO in the cell medium was 0.1% (v/v%). The vehicle control (DMSO) was performed in this study, and the results showed that DMSO (0.1%) did not affect the results (data were not shown).

Western Blotting Analysis

Fibroblasts were washed with the cold phosphate buffer saline buffer and lysed on ice in the RIPA lysis buffer (Cytoskeleton, Denver, CO) containing protein phosphatase inhibitor (Roche, Basel, Switzerland) and protease inhibitors (Beyotime, Shanghai, China) at a ratio of 100:10:1 (v/v/v) for 10 minutes. Cells were then scraped and solubilized for 30 minutes at 4°C in the lysis buffer, followed by centrifugation at 12,000 rpm for 15 minutes. Total protein concentration was determined by a bicinchoninic acid protein assay kit (Beyotime) and subsequently normalized. Protein samples (30 μg) were separated by 8%, 10%, or 12% sodium dodecyl sulfate electrophoresis, then transferred to polyvinylidene difluoride membranes (Bio-Rad, Hercules, CA), and blocked with 5% milk (BD, Franklin Lakes, NJ) in TBS-T for 90 minutes. Membranes were subsequently incubated with different primary antibodies overnight at 4°C. Next, membranes were washed 5 times in TBS-T and further incubated with HRP-labeled secondary antibodies (anti-rabbit IgG or anti-mouse IgG, as appropriate). To detect the protein bands, an ECL kit (FD, Hangzhou, China) was used. GAPDH was regarded as an internal control.

Quantitative Real-Time Polymerase Chain Reaction

Total cellular RNA was extracted using Trizol (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions, and cDNA was synthesized using a reverse transcription kit (Takara, Dalian, China) and used for subsequent qPCR. All samples were measured in triplicate. Differences in gene expression were calculated using the $2^{-(\triangle\triangle CT)}$ method. Gene primers are as follows: GAPDH (F-AGGTCGGTGTGAACGGATTTG, R-TGTAGACCATGTAGTTGAGGTCA); COL-I (F-ATCCTGCCGATGTCGCTAT, R-CCACAAGCGTGCTG-TAGGT); COL-III (F-CATGACTGTCCCACGTAAGCA, and R-ATTCGCCTTCATTTGATCCCA). GAPDH was used as an internal reference gene.

Ad-mCherry-GFP-LC3 Transfection

To observe autophagic flux, atrial fibroblasts were transfected with an mCherry-GFP-LC3 adenovirus according to the manufacturer's instructions. Briefly, cells were seeded in 20-mm diameter glass-bottomed dishes at a density of 30,000 cells/well for 24 hours. Then, an mCherry-GFP-LC3 adenovirus (multiplicity of infection = 100) was added to the

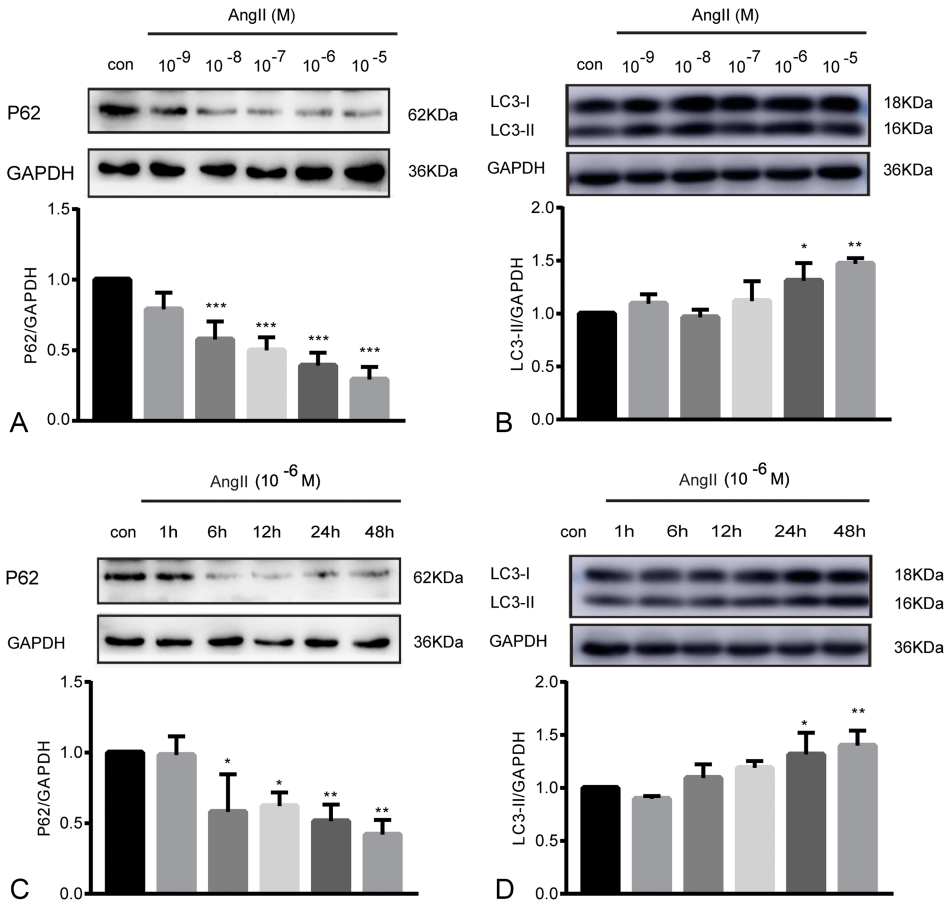

FIGURE 2. Expression of P62/SQSTM1 (P62) and LC3-II in AngII-induced atrial fibroblasts. Cells were treated with AngII at different concentrations (10^{-9} M, 10^{-8} M, 10^{-7} M, 10^{-6} M, and 10^{-5} M) and times (1, 6, 12, 24, and 48 hours). Expression of P62 and LC3-II in response to different concentrations of AngII stimulation for 48 hours (A and B). Expression of P62 and LC3-II at different times of AngII stimulation at 10^{-6} M (C and D). Data are expressed as the mean ± SD of 3 independent experiments, and the most representative bands are shown. *$P < 0.05$ versus the control group (con); **$P < 0.01$ versus the control group; ***$P < 0.001$ versus the control group.

medium, and the samples were incubated for 24 hours, at which time the medium was replaced with a complete medium. This was followed by incubation with puerarin or AngII for another 48 hours. At that time, the cells were fixed with 4% paraformaldehyde. Images were captured by confocal microscopy (Nikon, Japan). Numbers of red and yellow puncta (merged channel) were counted, which reflected autophagic flux.

Statistical Analysis

The results are expressed as the mean ± SD. The data analysis was conducted using GraphPad Prism 7.0. Differences among groups were analyzed using 1-way analysis of variance with Tukey's post hoc test. $P < 0.05$ was regarded as statistically significant.

RESULTS

AngII Upregulates COL-I/COL-III Expression and Activates Autophagy in Atrial Fibroblasts in a Dose- and Time-Dependent Manner

AngII is a major effector of the renin–angiotensin system (RAS), which plays an important role in regulating various types of cardiovascular disease, including hypertension, collagen deposition, and interstitial fibrosis.[26] In this

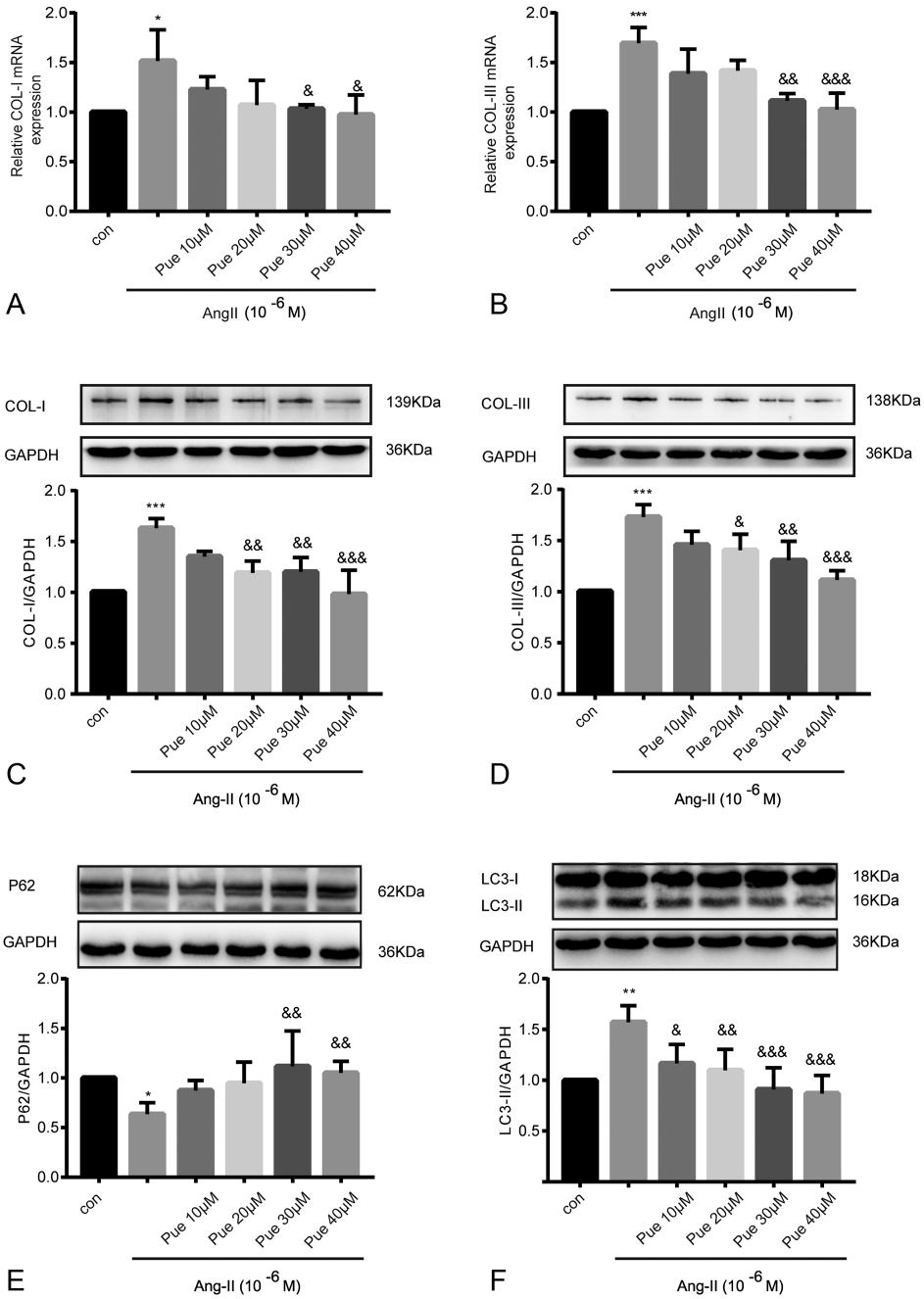

FIGURE 3. Effects of puerarin (pue) on collagen secretion and autophagy in AngII-induced atrial fibroblasts. Cells were pretreated with different concentrations of puerarin (10, 20, 30, and 40 μM) for 1 hour before stimulation with AngII (10^{-6} M) for 48 hours. mRNA expression of collagen I (COL-I) and collagen III (COL-III) (A and B). Protein expression of COL-I and COL-III (C and D). Protein expression of P62 and LC3-II (E and F). Data are expressed as the mean ± SD of 3 independent experiments, and the most representative bands are shown. *$P < 0.05$ versus the control group (con); **$P < 0.01$ versus the control group; ***$P < 0.001$ versus the control group; &$P < 0.05$ versus the AngII (10^{-6} M) group; &&$P < 0.01$ versus the AngII (10^{-6} M) group; &&&$P < 0.001$ versus the AngII (10^{-6} M) group.

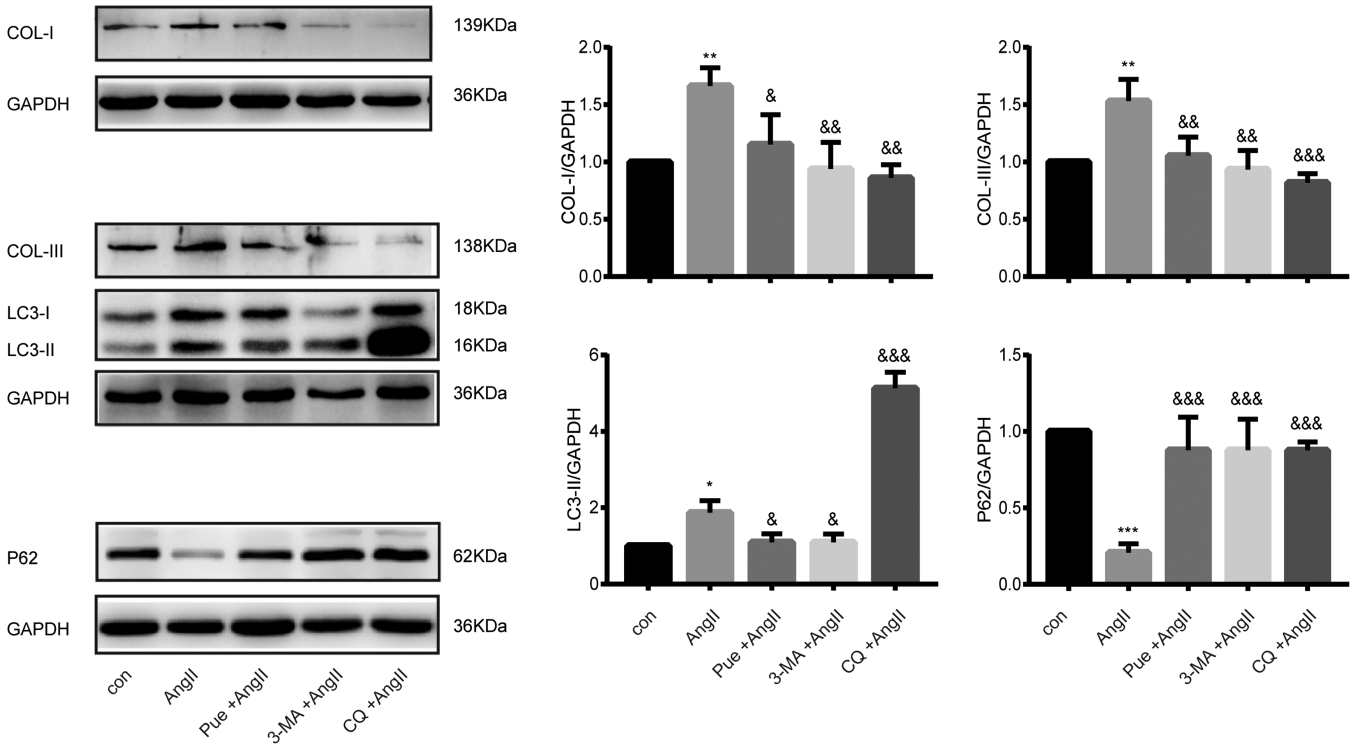

FIGURE 4. Effects of puerarin, 3-MA, and CQ on collagen secretion and autophagy in AngII-induced atrial fibroblasts. Cells were pretreated with puerarin (40 μM), 3-MA (10 mM), and CQ (10 μM) for 1 hour before stimulation with AngII (10^{-6} M) for 48 hours. The expression of collagen I (COL-I), collagen III (COL-III), P62, and LC3-II were detected by Western blot. The data are expressed as the mean ± SD of 3 independent experiments and the most representative bands are shown. $^{*}P < 0.05$ versus the control group (con); $^{**}P < 0.01$ versus the control group; $^{***}P < 0.001$ versus the control group; $\&P < 0.05$ versus the AngII group; $\&\&P < 0.01$ versus the AngII group; $\&\&\&P < 0.001$ versus the AngII group.

study, the secretion of both COL-I and COL-III by atrial fibroblasts was elevated in response to AngII stimulation, and this was shown in a dose- and time-dependent manner (Fig. 1). Meanwhile, compared with that of the control group, LC3-II expression was increased and P62 expression was decreased in response to AngII exposure (Fig. 2). These results suggest that AngII upregulates fibroblast autophagy and promotes COL-I and COL-III secretion.

Puerarin Reduces Atrial Fibroblast Collagen Secretion and Autophagy Induced by AngII

Cells were pretreated with different concentrations of puerarin for 1 hour before stimulation with AngII. Cells were then collected for mRNA or protein detection after 48 hours. As shown in Figure 3, puerarin inhibited COL-I and COL-III expression at both the mRNA (Figs. 3A, B) and protein levels (Figs. 3C, D). Treatment of atrial fibroblasts with AngII promoted LC3-II expression and inhibited P62 expression; however, pretreatment with puerarin reversed these changes (Figs. 3E, F). Furthermore, when we blocked autophagy with 3-MA, a PI3-kinase inhibitor, or CQ, a lysosome acidification inhibitor, the results showed that the process of autophagy was disturbed and that collagen secretion was reduced as well, which was similar to effects observed in response to puerarin treatment (Fig. 4). That is, puerarin inhibits collagen secretion in atrial fibroblasts induced by AngII, suggesting that the effect of puerarin is attributed to the inhibition of autophagy.

Puerarin and SP600125 Inhibit Autophagic Flux Induced by AngII

To further investigate the impacts of puerarin and the JNK signaling pathway on autophagy, a special marker, mCherry-GFP-LC3, was used as an adenovirus. Atrial fibroblasts were transfected with the adenovirus, and the cells were then stimulated with AngII or pretreated with puerarin or SP600125 1 hour before being stimulated with AngII. In the merged pictures, yellow puncta represent autophagosomes, and red puncta represent lysosomes (Fig. 5). Compared with the control group, the group with stimulation with AngII had markedly increased yellow puncta. However, yellow puncta were decreased by ~65% and ~63% in the puerarin and SP600125 pretreated groups, respectively. These results further demonstrate that puerarin and blocking the JNK signaling pathway both inhibit atrial fibroblast autophagy induced by AngII.

Puerarin Suppresses Phosphorylation of JNK, While Promoting Phosphorylation of Akt and mTOR

To investigate the molecular mechanisms wherein puerarin suppresses fibroblast autophagy and reduces collagen

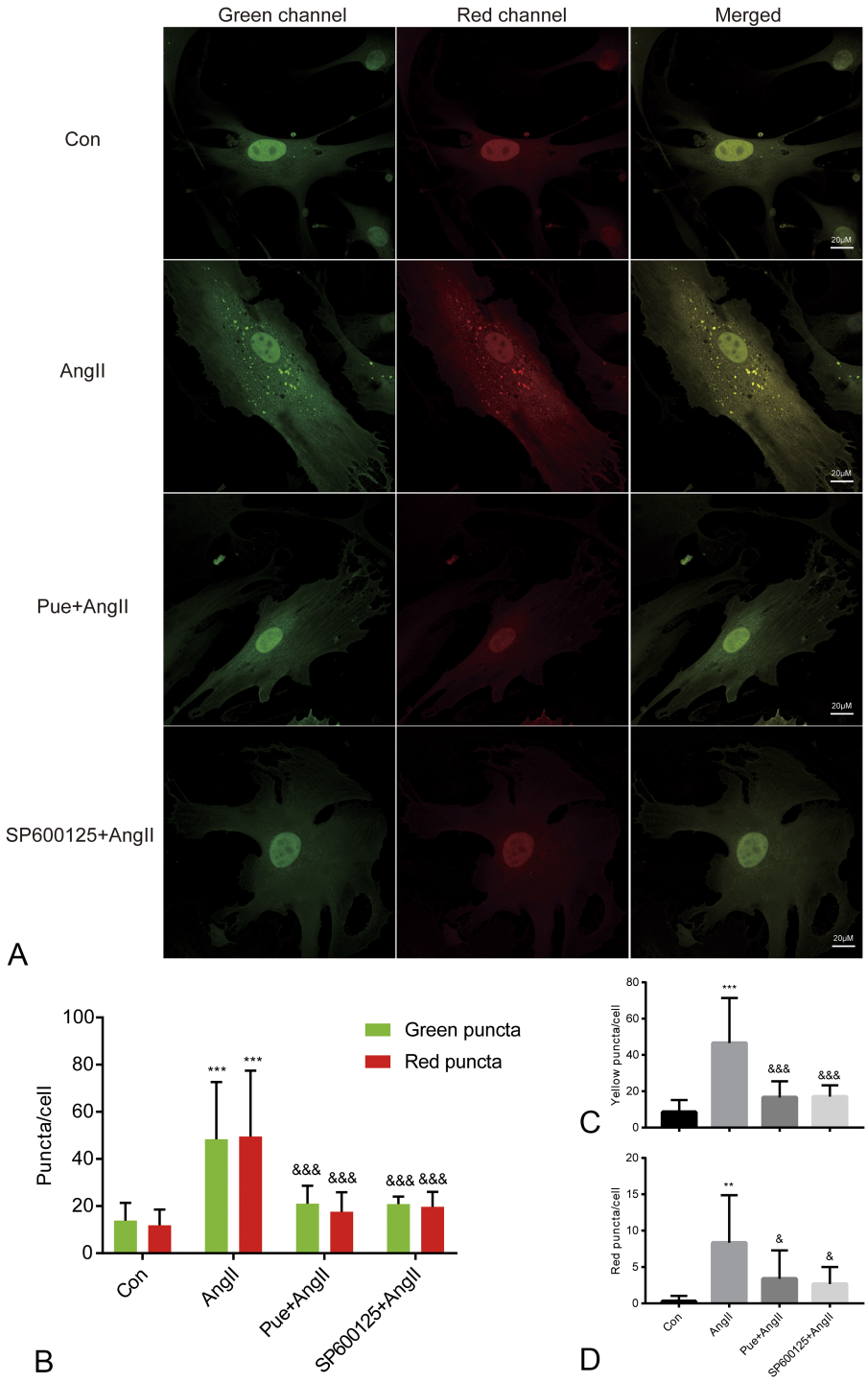

FIGURE 5. Puerarin and SP600125 suppress autophagosomes formation in AngII-induced atrial fibroblasts. Cells were transfected with an mCherry-GFP-LC3 adenovirus (MOI = 100). Then, pretreated with puerarin (40 μM) or SP600125(10 μM) for 1 hour before stimulation with AngII (10^{-6} M) for 48 hours. A, Representative confocal fluorescence microscopy images (600X) of mCherry-GFP-LC3 localization in AngII-induced atrial fibroblasts. B, Quantitative analysis of green puncta in green channel and red puncta in red channel. C and D, Quantitative analysis of autophagosomes (yellow puncta in merged channel) and autolysosomes (red puncta in merged channel). The data are expressed as the mean ± SD of 3 independent experiments, and the most representative pictures are shown. $^{**}P < 0.01$ versus the control group (con); $^{***}P < 0.001$ versus the control group (con); &$P < 0.05$ versus the AngII group; &&&$P < 0.001$ versus the AngII group.

secretion, the JNK and Akt–mTOR signaling pathways were examined. A previous study reported that JNK is activated by AngII and that it facilitates cardiac fibrosis.[27] In the current study, phosphorylation of JNK was elevated under stimuli by AngII, whereas phosphorylation of Akt and mTOR was inhibited. However, the changes observed in JNK, Akt, and mTOR were reversed on pretreatment with puerarin (Fig. 6).

Blocking the JNK Pathway Reduces COL-I and COL-III Expression, Restores Phosphorylation of Akt and mTOR, and Inhibits Autophagy Induced by AngII

To further investigate the relationship between the JNK signaling pathway and autophagy, SP600125, a broad-spectrum JNK inhibitor, was used to block the JNK pathway.

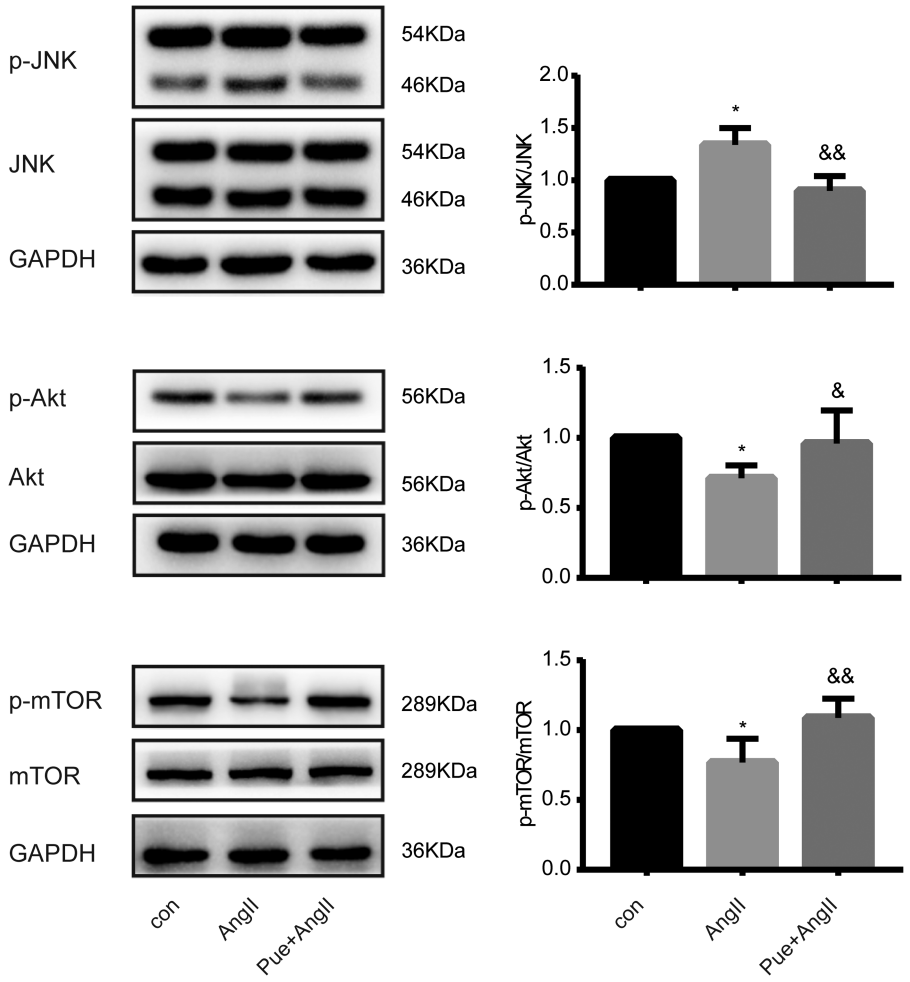

FIGURE 6. Puerarin suppresses phosphorylation of JNK and restores phosphorylation of Akt and mTOR in AngII-induced atrial fibroblasts. Cells were pretreated with puerarin (40 μM) for 1 hour before stimulation with AngII (10^{-6} M) for 20 minutes. Data are expressed as the mean ± SD of 3 independent experiments, and the most representative bands are shown. *$P < 0.05$ versus the control group (con); &$P < 0.05$ versus the AngII group; &&$P < 0.01$ versus the AngII group.

As shown in Figure 7, COL-I and COL-III expression were suppressed in response to treatment with SP600125. In addition, concurrent decreases in LC3-II expression and increases in P62 expression were observed, suggesting that autophagy induced by AngII is inhibited when the JNK pathway is blocked. Similarly, phosphorylation of Akt and mTOR was restored on inhibition of the JNK signaling pathway using SP600125. In summary, blocking JNK signaling contributes to relieving collagen secretion by atrial fibroblasts and inhibits autophagy activation induced by AngII through an Akt–mTOR–dependent mechanism.

DISCUSSION

For thousands of years, puerarin has been used as a traditional Chinese herb to treat many diseases, including cardiovascular diseases. Previous studies have demonstrated that puerarin exerts antifibrotic effects[18] and inhibits autophagy induced by myocardial hypoxia/reoxygenation injury.[23] In this study, we investigated the effects of puerarin on atrial fibrosis after activation of the RAS. We found that collagen secretion by atrial fibroblasts was markedly increased under AngII stimulation, while autophagy in fibroblasts was enhanced. However, when atrial fibroblasts were pretreated with puerarin, collagen secretion was decreased, while autophagy was inhibited. Puerarin suppressed the phosphorylation of JNK and facilitated the phosphorylation of Akt and mTOR. We speculate that the antifibrotic and antiautophagic effects of puerarin may be related JNK and Akt–mTOR signaling.

Interstitial fibrosis is a disease with an excessive deposition of the extracellular matrix, which exists in the end-stage of almost all cardiac diseases. The main components of the extracellular matrix are collagen and fibronectin.[28] Numerous studies have verified the relationship between atrial fibrosis and AF.[29,30] Atrial fibrosis not only causes atrial structural remodeling but also leads to electrophysiological changes in patients with AF.[29] It is also reported that this atrial remodeling occurred in a chronic canine AF model and that the electrical and structural remodeling increased vulnerability to AF induction.[31] The atrial effective refractory period was shortened when the dogs were treated with AngII. By contrast, the shortened atrial effective refractory period was recovered when the dogs were administered candesartan.[32] As a consequence, atrial fibrosis is a key point in improving atrial fibrosis in the treatment of AF.

RAS activation has extensive effects on cardiovascular homeostasis. Studies have demonstrated that a strong

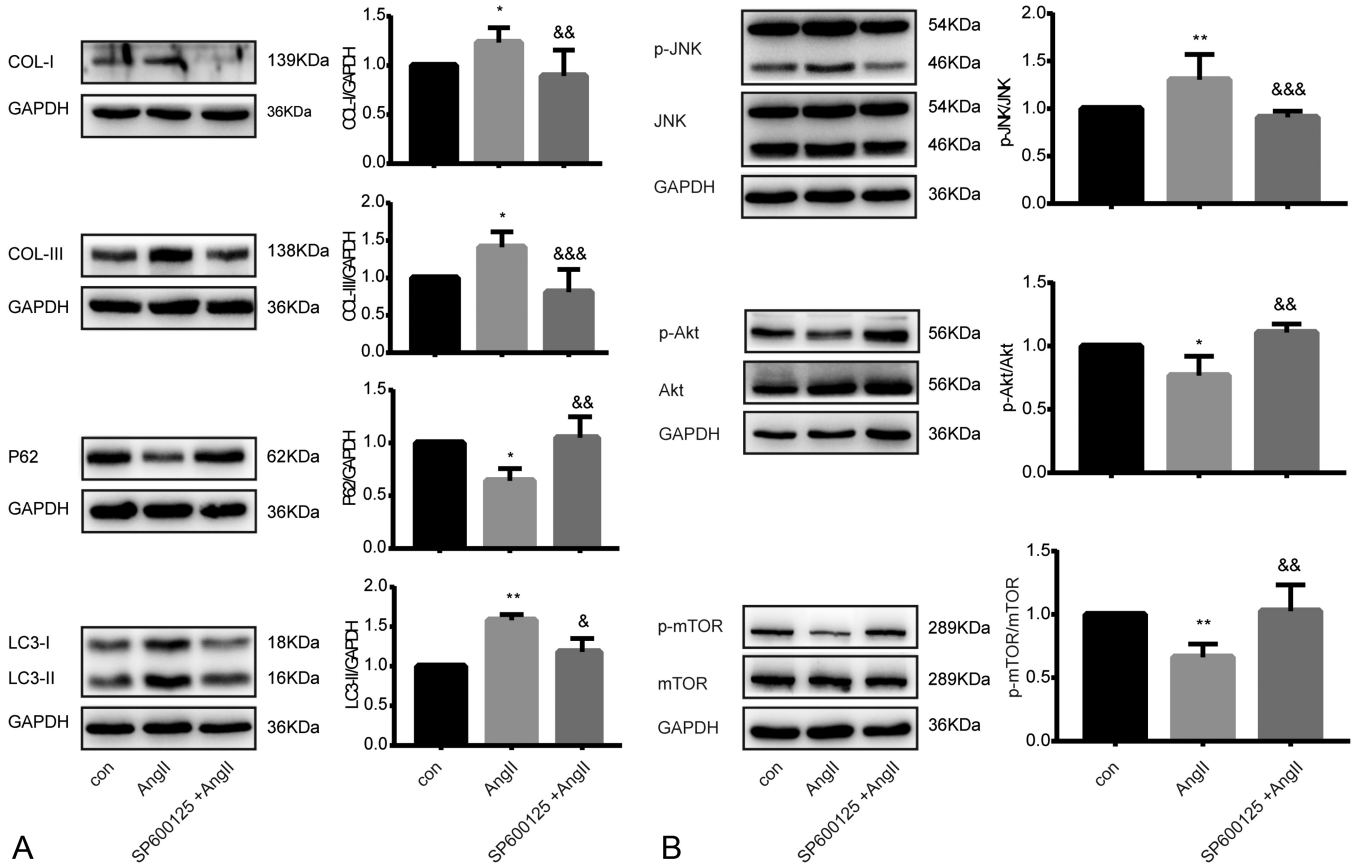

FIGURE 7. Effects of SP600125 in AngII-induced atrial fibroblasts. Cells were pretreated with SP600125 (10 μM), a JNK inhibitor, for 1 hour before stimulation with AngII (10^{-6} M) for 48 hours (for COL-I, COL-III, P62, and LC3-II detection) or 20 minutes (for phosphorylation of JNK, Akt, and mTOR detection). A, Protein expression of COL-I, COL-III, P62, and LC3-II. B, Protein expression of JNK, Akt, and mTOR. Data are expressed as the mean ± SD of 3 independent experiments, and the most representative bands are shown. *$P < 0.05$ versus the control group (con); **$P < 0.01$ versus the control group; &$P < 0.05$ versus the AngII group; &&$P < 0.01$ versus the AngII group. &&&$P < 0.001$ versus the AngII group.

relationship exists between the activation of RAS and hypertension, cardiac hypertrophy, heart failure, and AF.[33–35] AngII is the effector molecular of RAS, and, as is shown in this study, AngII increased collagen secretion by atrial fibroblasts in a concentration-, time-, and dose-dependent manner, further supporting the contention that the activation of RAS is one of the reasons collagen deposits in the atria. Furthermore, we also demonstrated that autophagy in atrial fibroblasts is activated by AngII (Fig. 1). Therefore, we speculate that increased collagen is related to activation of autophagy in atrial fibroblasts.

Puerarin is an old Chinese traditional medicine extract from Kudzu roots. It has been documented that puerarin possesses anti-inflammatory, antiarrhythmic, antioxidant, and antifibrotic effects.[18,36–38] Recent studies indicate that the cardiovascular protective effects of puerarin are related to autophagy.[22,23,39] In this study, we demonstrated that puerarin decreases collagen secretion induced by AngII in atrial fibroblasts. AngII increases protein expression of LC3-II and decreases that of P62 (Fig. 2), suggesting that autophagy is activated by AngII. However, observed changes in LC3-II and P62 were reversed in response to pretreatment with puerarin, and puerarin inhibited the production of COL-I and COL-III concomitantly (Fig. 3). Puerarin-mediated reduction of collagen secretion might be effective by inhibiting the activation of autophagy induced by AngII. To verify this, 3-MA was used to block autophagosome formation, and CQ was used to block lysosome acidification. The results illustrated that both 3-MA and CQ reduce collagen production similarly (Fig. 4), further confirming the effects of puerarin on AngII-induced fibroblasts to suppress autophagy. To further assess autophagic flux, an mCherry-GFP-LC3 adenovirus, which highlights autophagosomes and autolysosomes, was transfected into atrial fibroblasts. Yellow puncta, which indicate red and green light-spot fusion, are representative of autophagosomes in merged pictures. Owing to the acidic environment in lysosomes, GFP is degraded when autophagosomes and lysosomes fuse. Therefore, red spots are representative of lysosomes. As shown in Figure 5, there were only a few autophagosomes (yellow puncta) in the control group. Furthermore, numbers of autophagosomes were increased after stimulation with AngII; however, yellow puncta were decreased when fibroblasts were pretreated with puerarin. In summary, puerarin inhibited autophagosome formation in

AngII-induced atrial fibroblasts. These results suggest that puerarin reduces collagen production and relieves atrial fibrosis induced by AngII by inhibiting autophagy.

Autophagy has a complex regulatory mechanism involving many signaling molecules and pathways. Based on our results, puerarin restrains fibroblast autophagy and relieves collagen secretion. However, we do not know how puerarin regulates autophagy in atrial fibroblasts induced by AngII. mTOR is the most important target of autophagy regulation,[40] and Akt is an upstream target of mTOR. Studies have found that the activation of Akt upregulates the phosphorylation of mTOR and inhibits autophagy.[41–43] In this study, we found that AngII inhibits the phosphorylation of Akt and mTOR and activates autophagy in atrial fibroblasts (Fig. 6). However, phosphorylation of Akt and mTOR was restored on pretreatment with puerarin. In addition, autophagy in atrial fibroblasts was inhibited in response to puerarin administration. These findings suggest that puerarin regulates autophagy in AngII-induced atrial fibroblasts through the Akt–mTOR signaling pathway.

To further investigate regulation of the Akt–mTOR pathway, a JNK inhibitor, SP600125, was used. JNK is one of the mammalian MAPKs and is typically activated in response to inflammatory cytokines and cellular stress. Previous studies demonstrated that cardiac fibrosis and atrial fibrosis were alleviated when the JNK signaling pathway was inhibited by SP600125.[44,45] Yuan et al[20] reported that puerarin attenuated pressure overload–induced cardiac hypertrophy by suppressing the PI3K/Akt and JNK signaling pathways. We found that the autophagic flux induced by AngII was suppressed when the fibroblasts were pretreated with SP600125 (Fig. 5). As shown in Figure 7, SP600125 not only led to decreased collagen secretion but also led to restrained autophagy activation. Inhibition of Akt and mTOR phosphorylation was also restored when JNK was blocked. These results indicate that JNK signaling participates in the puerarin-mediated regulation of autophagy.

CONCLUSION

RAS activation influences many aspects of the cardiovascular system and causes a series of pathophysiological changes, including atrial remodeling. As a component of the heart, atrial fibroblasts not only provide structural support for the heart but are also involved in extracellular matrix homeostasis. Therefore, we cannot ignore the role of atrial fibroblasts in atrial remodeling. In this study, we demonstrated that AngII facilitates collagen secretion and activates autophagy in fibroblasts. However, puerarin suppressed autophagy levels through JNK–Akt–mTOR signaling and reduced COL-I and COL-III secretion. In this study, the absence of in vivo experiments is a limitation, which we plan to address. We have designed experiments to validate the effects of puerarin in vivo in subsequent studies. In summary, this study investigated the protective effects of puerarin on AngII-induced atrial fibroblasts and illuminated the mechanism whereby this occurs, providing findings to improve atrial remodeling after RAS activation.

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