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KLF15 protects against isoproterenol-induced cardiac hypertrophy via regulation of cell death and inhibition of Akt/mTOR signaling

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ABSTRACT

Increasing evidence indicate that the Krüppel-like factor KLF15, a member of Cys2/His2 zinc-finger DNA-binding proteins, attenuates cardiac hypertrophy. However, the role of KLF15 in cardiovascular system is largely unknown and the exact molecular mechanism of its protective function is not fully elucidated. In the present study, we established a mouse model of cardiac hypertrophy and found that KLF15 expression was down-regulated in hypertrophic hearts. To evaluate the roles of KLF15 in cardiac hypertrophy, we generated transgenic mice overexpressing KLF15 of KLF15 knockdown mice and subsequently induced cardiac hypertrophy. The results indicated that KLF15 overexpression protects mice from ISO-induced cardiac hypertrophy, with reduced ratios of heart weight (HW)/body weight (BW) and cross-sectional area. We also observed that KLF15 overexpression attenuated cardiac fibrosis, inhibited apoptosis and induced autophagy in cardiomyocytes compared with KLF15 knockdown mice. More importantly, we found that the KLF15 overexpression inhibited the Akt/mTOR signaling pathway. Taken together, our findings imply that KLF15 possesses potential anti-hypertrophic and anti-fibrotic functions, possibly via regulation of cell death pathways and the inhibition of Akt/mTOR axis. KLF15 may constitute an efficient candidate drug for the treatment of heart failure and other cardiovascular diseases.

1. Introduction

Cardiac hypertrophy, the thickening of the myocardium, is a pathological outcome derived from the reactions of cardiomyocytes to a variety of physiologic and pathological stimuli such as neurohumoral stimuli and biomechanical stress (pressure or volume overload) [1–3]. Indeed, mammalian cardiomyocytes are devoid of division ability soon after birth, and, therefore, have no choice but to undergo hypertrophy in case of sustained increase in workload requirement. However, cardiac hypertrophy, despite its role as a compensatory response that contributes to the preservation of cardiac function, generally results in heart failure and rapid death due to arrhythmias [2,3]. Currently, studies have listed heart failure as one of the foremost causes of deaths worldwide. It is well-known that cardiac hypertrophy is caused by the abnormal expression of genes such as microRNAs [4], transcription factors and co-regulators.

The Kruppel-like factor (KLF) transcription factors are largely recognized for their regulatory functions in a large number of cell biological processes such as differentiation, proliferation, survival and growth [5–14]. Relatively, KLF15 is the best-studied member of KLF family. Previous evidences indicate that KLF15 can control cell growth by sensing TGF-β. KLF15 is also known to be involved in type II diabetes and in the regulation of hepatic lipid metabolism. It was also established that KLF15 is a crucial regulator of myocardial lipid metabolism via interacting with p300 and by recruiting this critical co-activator to promoters [15]. More importantly, findings point toward an inhibitory role of KLF15 in cardiac hypertrophy through suppression of fundamental cardiac transcription factors such as MRTF-A and MRTF-B, GATA4, MEF2 and myocardin [16,17] and that overexpression of cardiac KLF15 levels protects from cardiac hypertrophy *in vivo* [17]. Equally, KLF15 is proven as a critical negative regulatory gene for cardiac remodeling during pressure overload [18] and as a negative regulator of the connective tissue growth factor (CTGF) expression and cardiac fibrosis [19]. However,

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the molecular mechanisms underlying the role of KLF15 in cardiovascular system, especially in the pathological processes involved in cardiac hypertrophy, are still not well understood.

Here, we aimed to investigate the role of KLF15 in pressure overload-induced cardiac hypertrophy and the underlying molecular mechanism. By using KLF15 transgenic mice, we demonstrated that KLF15 overexpression inhibited isoproterenol (ISO)-induced cardiac hypertrophy and its associated fibrosis and cell apoptosis but induced autophagy. The probable molecular mechanism of this protective effect of KLF15 may be associated with the inhibition of the Akt/mTOR signaling.

2. Materials and methods

2.1. Animals

This study employed transgenic and wild type male C57BL/6 mice weighing 23 ± 2.2 g. Transgenic mice with cardiac-restricted expression of KLF15 were generated as described previously [20]. KLF15−/− mouse was generated as previously described [21]. Mice in each group were placed in a group-specific cage in a room with a 12:12-h light-dark cycle. The mice were fed a standard chow diet and given ad libitum access to water. This study was conducted in accordance with the guidelines for the care and use of laboratory animals of Shandong University. All experimental protocols were reviewed and approved by the Ethics Committee Board of Shandong University.

2.2. Animal models of cardiac hypertrophy

To investigate the role of KLF15 on cardiac hypertrophy, we established a mouse model of isoproterenol-induced myocardial injury model. Briefly, after anesthesia of littermates, KLF15 transgenic mice (KLF15+/+) or knockout mice (KLF15−/−) with 1.5% isoflurane, an incision (approximately 1 cm) was posed on the posterior of each mouse in the region between shoulder blades. Following, ISO, previously dissolved in a physiological solution of 0.9% NaCl was delivered at 40 $mg.kg^{-1}.d^{-1}$ via the Alzet® microosmotic pump model 1007D inserted into the infrascapular subcutaneous tissue in the incision place. After the insertion of the pump, the incision was sutured. At the end of the experiments (5 weeks of ISO administration), mice were anesthetized to minimize pain, sacrificed by cervical dislocation, and dissected for heart collection. Hearts were kept at −80 °C until use.

2.3. Histological analysis

After dissection, hearts were rinsed using the saline solution and immersed in 10% formalin. To expose the ventricles, the hearts were transversely sectioned close to the apex. Following, 4–5 μm thickness heart sections were generated and stained with H&E for histopathology or picrosirius red for collagen deposition. Stained sections were then observed using a light microscope from Leica Microsystems. The cross-sectional area of myocytes was determined on sections stained with WGA (Invitrogen) using an image quantitative digital analysis system (NIH Image J, version 1.47).

2.4. Quantitative real-time PCR

For gene expression analysis, total RNA was isolated from mouse cardiac tissue homogenates using TRIZol (Invitrogen) and cDNA synthesis was done by means of the Maxima First Strand cDNA Synthesis Kit for RT-qPCR (Thermo Fisher Scientific). Thereafter, the real-time qPCR experiments were carried out on the CFX96 Touch™ Real-Time PCR Detection System (Biorad) according to the vendor's instruction manual. The PCR settings met the following conditions: 30 s at 95 °C, 45 cycles at 95 °C for 5 s and 58 °C for 34 s. The relative expression of a given gene was calculated based on the ΔΔCt method. GAPDH was used as internal control.

2.5. Western blotting

Heart tissue sections were lysed using RIPA lysis buffer in mixture with protease inhibitors. Total protein in the lysates was determined using the BCA protein quantification approach. Next, 40 μg of lysates were cell proteins were applied to 12% SDS–polyacrylamide gel. Subsequently, protein transfer onto PVDF membranes and blocked with 5% fat-free milk for 1 h before being incubated with primary antibody against KLF15 (Abcam, ab61207), LC3-II, caspase 3, cleaved caspase 3, cleaved PARP, Atg3, Beclin1, mTOR, p-mTOR, Akt, p-Akt or GAPDH at 4 °C overnight. Then, after washing, membranes were incubated with HRP-conjugated secondary antibody for 2 h prior to visualization with the Chemiluminescent ECL reagent (Beyotime). The Image J software was used for densitometric analysis and the relative protein levels were determined using the GAPDH as loading control.

2.6. Statistical analysis

All values are expressed as the mean ± SEM. Statistical differences among groups were determined using either Student's *t*-test (for two groups) or one-way ANOVA or two-way ANOVA (for more than two groups) using Graph-Pad Prism Software.

3. Results

3.1. Establishment of cardiac hypertrophy mouse models

Prior to the investigation of KFL15 effect, we aimed to establish a mouse model of hypertrophy. Isoproteronol (ISO) was used to induce cardiac hypertrophy in experimental animals. The results (Fig. 1A) indicated that the heart weight/body weight ratios was significantly ($p < 0.01$) increased for mice administered with ISO when compared with the normal and vehicle-treated mice. Likewise, after the analysis of cell cross-sectional area in H&E stained heart cryosections, we observed that the myocyte cross-sectional areas obtained from ISO-treated mice were markedly higher than those obtained from the vehicle and normal mice (Fig. 1B). As shown in Fig. 1C, the H&E staining revealed that cardiac cells in the ISO-treated mice were denser than those in the normal control or vehicle mouse hearts. We also assessed whether ISO was able to induce fibrosis in the mice heart. As a result, significant fibrosis was detected in hearts of ISO-treated mice (Fig. 1D). On the contrary, we did not observed any occurrence of fibrosis the normal control or vehicle groups (Fig. 1D). The results indicated that the heart hypertrophic mouse model was successfully established.

3.2. KLF15 is downregulated in murine hypertrophic heart

After the establishment of the mouse model of cardiac hypertrophy, total protein and total RNA were extracted from heart specimen to analyze KLF15 expression level. The results (Fig. 2A, B and 2C) showed that KLF15 was noticeably down-regulated in hypertrophic mice heart at both protein and mRNA levels in cardiac hypertrophy mouse model comparatively to the vehicle group. The present results suggest that KLF15 may be involved in cardiac hypertrophy and may therefore potentially play a part in heart failure.

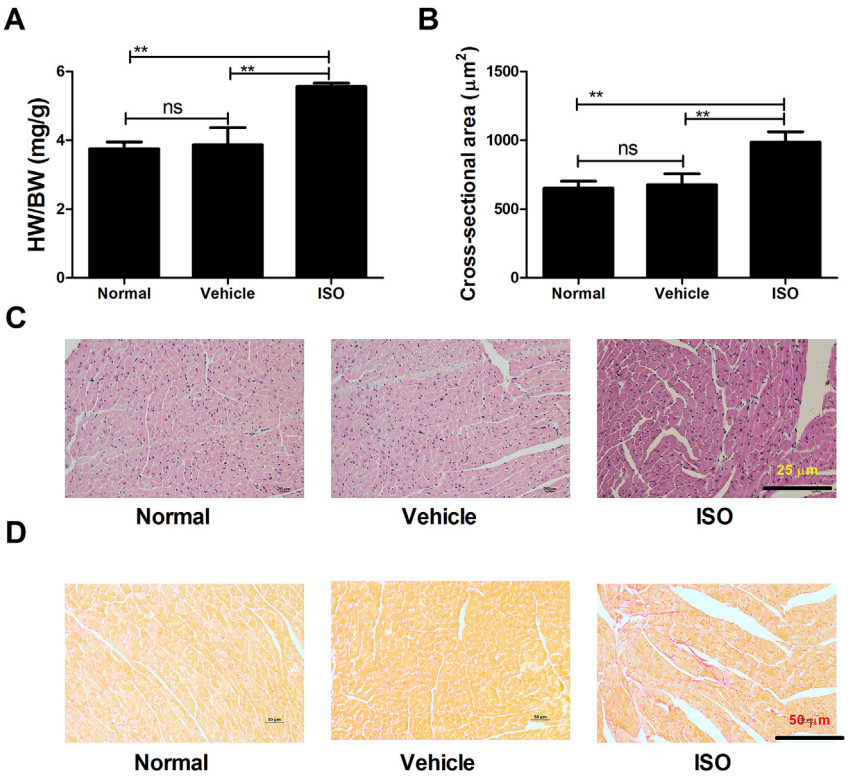

Fig. 1. Isoproterenol induces cardiac hypertrophy in mice. (**A**) Isoproterenol increased the heart weight to body weight ratios in mice with hypertrophic hearts compared to mice in the normal or vehicle groups. (**B**) Isoproterenol increased the cross-sectional heart section area in mice with hypertrophic hearts compared to mice in the normal or vehicle groups. (**C**) H&E staining indicated thickening of cardiomyocytes in mice with hypertrophic hearts compared to mice in the normal or vehicle groups. (**D**) Picrosirius Red staining indicated the occurrence of fibrosis in mice with hypertrophic hearts compared to mice in the normal or vehicle groups. Images were taken at 200× magnification. Error bars represents the standard deviation. $^{**}p < 0.01$. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

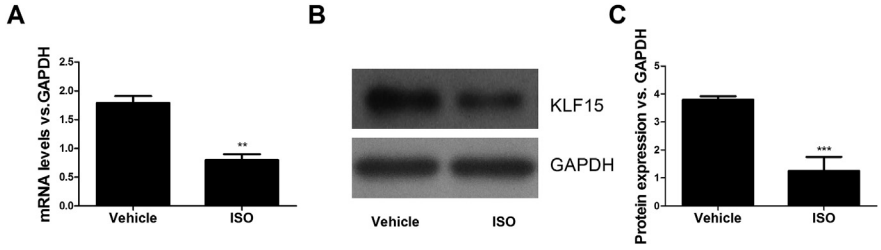

Fig. 2. The expression of KLF15 is decreased in mice with hypertrophic hearts. (**A**) Real time PCR analysis of mRNA expression of KLF15 in normal, vehicle and ISO groups. (**B**) Western blot analysis of protein expression of KLF15 in the heart of normal, vehicle and ISO groups. (**C**) Densitometry analysis of bands obtained from western blotting. The experiments were performed in triplicate and only representative images were presented. Error bars represents the standard deviation. $^{**}p < 0.01$, $^{***}p < 0.001$.

3.3. KLF15 overexpression represses ISO-induced cardiac hypertrophy

In order to explore the functional role of KLF15 in cardiac hypertrophy, we established hypertrophic mouse models used KLF15 knockdown (KD) or KLF15 overexpression (OE) transgenic mice. Cardiac hypertrophy was induced by administration of transgenic or wild type littermates mice with ISO for 5 weeks as indicated above. The results revealed that heart weight (HW)/body weight (BW) and cross-sectional area (Fig. 3A and B) were increased in wild type littermates mice and KLF15 knockdown transgenic mice subjected to ISO administration. On the contrary, the hearts of KLF15-transgenic mice subjected to ISO did not experience significant pathological changes (Fig. 3C–D). KLF15-transgenic mice were less affected by ISO administration and exhibited improved cardiac function even if hypertrophy was developed. The above observations showcased that KLF15 overexpression could be a potential way for protecting against the development of cardiac hypertrophy.

3.4. KLF15 overexpression reduces ISO-induced cardiac fibrosis

Fibrosis is a principal component caused by heart hypertrophy. To assess the cardiac fibrosis in hypertrophic hearts and uncover the possible effect of KLF15 overexpression on cardiac fibrosis, histopathological analyses using Picrosirius red staining were performed. As shown in Fig. 4, KLF15 overexpression repressed cardiac fibrosis in transgenic mice overexpressing KFL15 while the non-transgenic and KFL15 knockdown mice administered with ISO (Fig. 4A). In addition, the extent of fibrosis was more pronounced in the KFL15 knockdown group administered with ISO compared with other groups. These findings implied that KLF15 overexpression

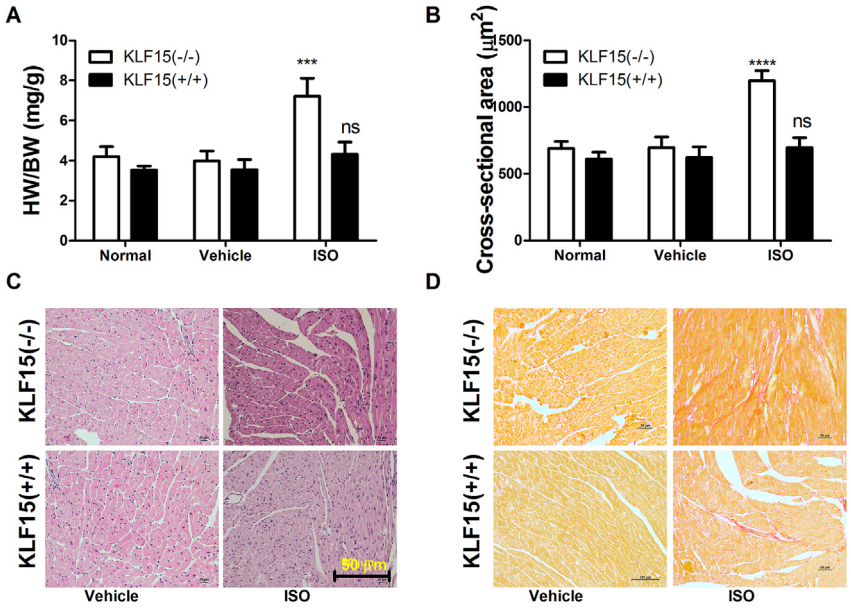

Fig. 3. The overexpression of KLF15 attenuates ISO-induced cardiac hypertrophy. (**A**) Isoproterenol increased the heart weight to body weight ratios in KLF15 knockdown mice with hypertrophic hearts compared to mice in the normal or vehicle groups. On the contrary, in mice overexpressing KLF15, the heart weight to body weight ratios were reduced compared with the knockdown mice. (**B**) Isoproterenol increased the cross-sectional heart section area in KLF15 knockdown mice with hypertrophic hearts compared to mice in the normal or vehicle groups. On the contrary, in mice overexpressing KLF15, the cross-sectional heart section was reduced compared with the knockdown mice. (**C**) H&E staining indicated thickening of cardiomyocytes in KLF15 knockdown mice with hypertrophic hearts compared to mice in the KLF15 overexpression submitted to ISO treatment or those transgenic mice in the vehicle group. KLF15 overexpression attenuated the thickening of cardiomyocytes. (**D**) Picrosirius Red staining indicated the increased occurrence of fibrosis in mice with hypertrophic hearts compared to mice in the vehicle group and those overexpressing KLF15. Images were taken at 200× magnification. Error bars represents the standard deviation. ***p < 0.001, ****p < 0.0001. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

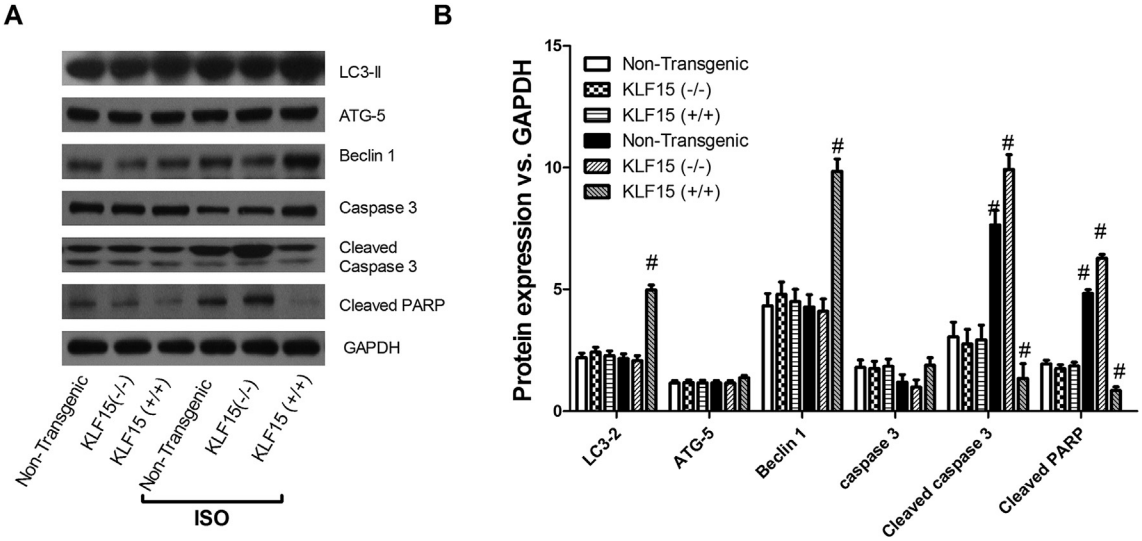

Fig. 4. The overexpression of KLF15 induced autophagy and inhibited apoptosis in hypertrophic hearts. (**A**) Western blot analysis of autophagy and apoptosis associated protein in the heart of normal, vehicle and ISO groups. (**B**) Densitometry analysis of bands obtained from western blotting. The experiments were performed in triplicate and only representative images were presented. Error bars represents the standard deviation. #p < 0.001.

protects against cardiac fibrosis in hypertrophic hearts.

3.5. *KLF15 overexpression inhibits apoptosis and induces autophagy in ISO-induced cardiac hypertrophy via Akt/mTOR signaling*

Autophagy and apoptosis are important biological processes commonly dysregulated in pathological states. To evaluate the extent of these processes in ISO-induced cardiac hypertrophy, we measured the protein expression of autophagy and apoptosis markers using western blotting. As depicted in Fig. 5, the expression of LC3-II, Atg5 and Beclin 1, which are autophagy markers, was not affected by ISO administration in KLF15 knockdown mice but was significantly up-regulated in KLF15 overexpression-transgenic mice subjected to ISO treatment when compared to untreated mice. On the contrary, apoptosis markers including cleaved-caspase3 and cleaved-PARP were higher in the hearts of non-transgenic mice and KLF15 knockdown transgenic mice subjected to ISO treatment but downregulated in KLF15 overexpression transgenic mice compared

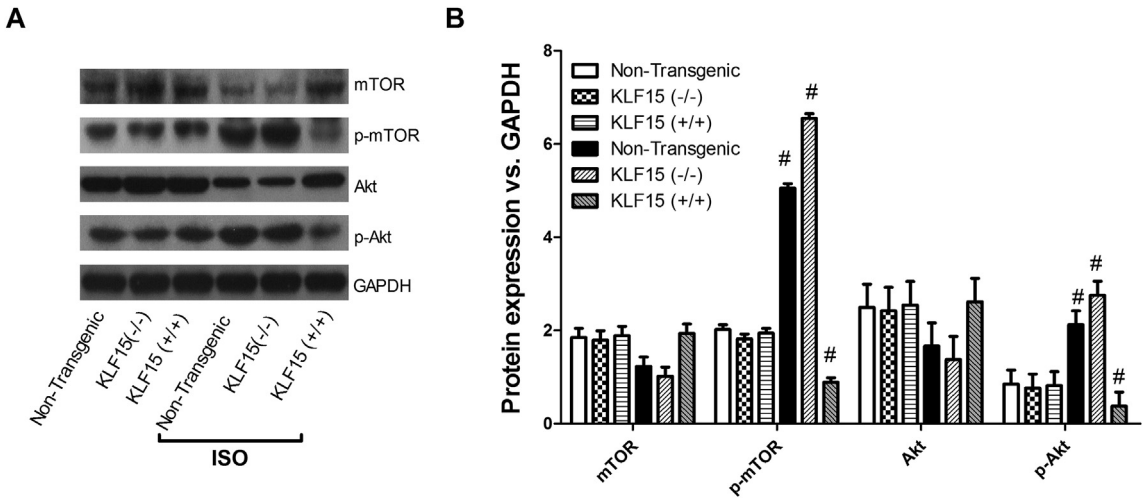

Fig. 5. The overexpression of KLF15 inhibited the Akt/mTOR signaling pathway in hypertrophic hearts. (**A**) Western blot analysis of mTOR, p-mTOR, Akt and p-Akt in the heart of KLF15 knockdown or overexpression mice treated with vehicle or ISO. (**B**) Densitometry analysis of bands obtained from western blotting. The experiments were performed in triplicate and only representative images were presented. Error bars represents the standard deviation. #p < 0.001.

to mice that were not subjected to ISO treatment. Similarly, the expression of p-Akt and p-mTOR was increased after induction of cardiac hypertrophy in the hearts of non-transgenic mice and KLF15 knockdown transgenic mice subjected to ISO treatment but downregulated in KLF15 overexpression transgenic mice compared to mice that were not subjected to ISO treatment. Taken together, these observations implied that KLF15 overexpression protects against pressure overload cardiac hypertrophy by inhibiting fibrosis and apoptosis and promoting autophagy, possibly by down-regulating Akt/mTOR signaling.

4. Discussion

Our work uncovered a physiological role of KLF15 in maintaining cardiac homeostasis, demonstrated the feasibility of targeting KLF15 to restore cardiac homeostasis in animals with cardiac hypertrophy. We showed that KLF15 was down-regulated in murine hypertrophic hearts. Then we used KLF15 overexpression or KLF15 knockdown transgenic mice to study the role of KLF15 in ISO-induced cardiac hypertrophy. The results showed that KLF15 overexpression inhibited ISO induced-cardiac hypertrophy and fibrosis. Despite the huge number of studies published on the involvement of KLFs various tissues and disease, surveys describing the roles of KLFs in the heart are limited. It was previously reported that KLF4 protects from ISO- and pressure-induced cardiac hypertrophy by regulating myocardin, fetal genes protein synthesis and cell enlargement [22–24]. The roles of KLF5 as an important effector of angiotensin II signaling in cardiac fibroblasts and as a key regulator of cardiac remodeling, hypertrophy and hypertension have been equally published [25–29]. Another KLF family member functioning in the heart is KLF11 [30]. Our present findings corroborate with previous studies showing that KLF15 negatively regulates cardiomyocyte hypertrophy by inhibiting GATA4 and myocyte enhancer factor 2 function [15–18,31–33].

The current knowledge of the molecular mechanism of KLF15 in cardioprotection is limited to a few number of studies. Notably, previous studies indicates that KLF15 robustly inhibits myocardin [16], a potent transcriptional activator. In addition, a recent study indicated that KLF15 is direct target of microRNA-133 which is involved in the metabolism of cardiac myocytes [32]. In mechanical- or metabolic factors-induced myocardial remodeling and myocardial fibrosis, it was shown that KLF15 exerts protective effect by modulating the expression of CTGF, TGF-β, and MRTF-A [18]. It was equally shown that KLF15 controls cardiac progenitor cell fate by regulating Wnt/β-catenin signaling in the postnatal heart [33]. Another significant mechanism of KLF15 is the regulation of its target genes by interacting with p300 [15]. In Angiotensin II induced-cardiac hypertrophy, overexpression of KLF15 in mouse heart prevented the development of cardiac hypertrophy by repressing the expression of MRTF-A and MRTF-B [17]. In the present study, we add to the scientific knowledge the point that the attenuation of cardiac hypertrophy exerted by KLF15 may be mediated via inhibition of the Akt/mTOR signaling. We also showed that KLF15 inhibits cell apoptosis and induces autophagy in hypertrophic heart.

5. Conclusion

KLF15 attenuates ISO-induced cardiac hypertrophy in mice by reducing fibrosis, inhibiting cell apoptosis and inducing autophagy probably by inhibiting the Akt/mTOR axis. The present finding not only contributes to the knowledge of KLF15 molecular mechanism.

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