ATP6AP2 knockdown in cardiomyocyte deteriorates heart function via compromising autophagic flux and NLRP3 inflammasome activation

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INTRODUCTION

As a leading cause of morbidity and mortality, heart failure (HF) causes an enormous economic impact [1, 2]. Fully understanding the mechanisms would bring bright new therapeutic strategies for the treatment of heart failure.

Autophagy and mitophagy maintain intracellular homeostasis in cardiomyocytes. The loss of function of autophagy-related genes exacerbates pathological progression in myocardial infarction, atherosclerosis [3–5], doxorubicin-induced cardiomyopathy [6]. Nevertheless, some inconsistencies and controversial results remain about the influence of autophagy on remodeling in HF [7–10].

Hence, the exact role of autophagy in cardiac remodeling requires further study.

Emerging evidence has indicated that full activation of the NLRP3 inflammasome and secretion of IL-1β and IL-18 [11] participated in atherosclerosis, diabetes, heart failure [12–14]. Besides, many researchers have found that autophagy and NLRP3 inflammasome activation are mutually regulating processes [15–19]. So maintaining the average autophagic flux is essential for cell survival.

ATP6AP2 was initially found as a fragment related to proton pump V-ATPase, which is pivotal for maintaining the PH gradient and lysosome function in cells. Then, in 2002, ATP6AP2 was found to bind with prorenin and facilitate prorenin processing to renin and was misnamed as (pro)renin receptor (PRR) [20]. Several studies showed that ATP6AP2 participates in the pathogenesis of acute or chronic kidney diseases, hypertension, fibrosis, diabetes, and various other conditions [21–26]. Our previous research

Moderate autophagy can remove damaged proteins and organelles. In some inflammatory diseases, autophagy plays a protective role by inhibiting the NOD-like receptor family pyrin domain containing 3 (NLRP3). (Pro)renin receptor (PRR, or ATP6AP2) is a critical component of the V-ATPase required for autophagy. It remains controversial about ATP6AP2 in the pathological process. The impact of ATP6AP2 on NLRP3 inflammasome and autophagic flux remains unknown under pressure overload stress. This research explores the potential link between ATP6AP2, autophagic flux, and NLRP3. There was upregulation of ATP6AP2 from 5-day post-TAC, and this expression remained at a high level until 8-weeks post-TAC in wild mice. Meanwhile, autophagic flux switched from early compensatory activation to blocking in the heart failure phase. NLRP3 activation can be seen at 8-week post-TAC. Adenovirus-mediated knockdown of ATP6AP2(shR-ATP6AP2) accelerated the progress of heart failure. After TAC was induced, shR-ATP6AP2 significantly deteriorated heart function and fibrosis compared with the shR-Scr group. Meanwhile, there was an elevated expression of NLRP3 and autophagic flux blockade. A transgenic mouse(Tg) with cardio-restricted ATP6AP2/(P)RR overexpression was constructed. Although high expression in cardiac tissue, there were no spontaneous functional abnormalities under the basal state. Cardiac function, fibrosis, hypertrophy remained identical to the control TAC group. However, SQSTM1/P62 was reduced, which indicated the relief of autophagic flux blockade. Further, Neonatal rat ventricular myocyte (NRVMs) transfected with shR-ATP6AP2 showed more susceptibility than sh-Scr NRVMs to phenylephrine-induced cell death. More reactive oxygen species (ROS) or mito-ROS accumulated in the shR-ATP6AP2 group when phenylephrine stimulation. Blocking NLRP3 activation in vivo partly rescued cardiac dysfunction and fibrosis. In conclusion, ATP6AP2 upregulation is a compensatory response to pressure overload. If not effectively compensated, it compromises autophagic flux, leads to dysfunctional mitochondria accumulation, further produces ROS to activate NLRP3, eventually accelerates heart failure.
showed that ATP6AP2/PRR has high expression and participation in fibrosis in diabetic cardiomyopathy and alcoholic cardiomyopathy [25, 27]. However, other studies failed to confirm a relationship between high ATP6AP2 overexpression and pathological function [28, 29]. There were no spontaneous cardiac morphological abnormalities in Atp6ap2 transgenic overexpressed mice at baseline. Cardiac hypertrophy and cardiac or renal fibrosis did not show any differences between Tg and wild mice after isoproterenol infusion for 28 days. However, research involving genetic manipulation of ATP6AP2 in the hepatocyte, pancreatic β cells, podocytes, and cardiomyocytes [15–17, 30] revealed its lysosomal phenotypes. The regulative relationship between ATP6AP2, autophagic flux, and NLRP3 under pressure load is poorly understood. There is still much debate about the ATP6AP2 function, and more research is needed.

Consequently, we hypothesized ATP6AP2 could exert its protective role by promoting autophagic flux and further inhibiting NLRP3 activation. Our results firstly demonstrated that mice with ATP6AP2 knockdown in the heart compromise autophagic flux, activate NLRP3, and further promote maladaptive cardiac remodeling in the TAC model. Cellular ROS and mitochondrial ROS induced by ATP6AP2 knockdown accelerate NLRP3 activation.

RESULTS
Dynamic changes of ATP6AP2 in the progression toward heart failure
To confirm the changed expression of ATP6AP2 during the hypertrophy and heart failure phase, wild-type C57BL/6J mice were subjected to transverse aortic constriction (TAC) [18].
Measurements of peak flow velocity through the constricted aortic arch were similar on the 5th and 56th day past-TAC, which indicated the surgery was successful (Fig. S2ACDE). The echocardiography showed that LV ejection fraction (LVEF), LV fraction shortening (LVFS), LV internal diameter at end-systole (LVIDs) did not change significantly on the 5th day. (Fig. 1C, E, F, I). By the 56th day, LVEF and LVFS dropped by 35–40% (Fig. 1E, F). Meanwhile, LVIDs, HW/BW, and cardiomyocyte cross-sectional area increased considerably (Fig. 1D, G, H, I). Heart rate remained similar between groups (Fig. 1J).
ATP6AP2 expression began to increase on the 5th day of TAC and increased sharply from the 7th day. After that, the expression remained at a higher level (Fig. 1A, B). Immunohistochemical staining (IHC) of ATP6AP2 also confirmed this trend (Fig. 2H).

**General autophagy and mitophagy are transiently upregulated in the hypertrophic heart but are decreased during the decompensated heart failure**

Based on LC3, P62 (SQSTM1), Atg5, we first detected the changes in general autophagy at different time points after TAC. LC3B began to upregulate on the 5th and the 7th but returned to the basal level on the 14th day. However, it declined significantly after 28 days. SQSTM1/P62 increased from 7 days and was maintained at high levels (Fig. 2A, B).

The activation of autophagy flux or blockage of the degradation of autolysosome both can increase the expression of steady-state LC3B. To distinguish between the possibilities, we treated mice with Baf1 intraperitoneally, a late-stage autophagy inhibitor. After Baf1 treatment, the level of LC3B increased considerably 5 days after TAC compared with the TAC group (Fig. 2E). These results indicated autophagic flux was activated on the 5th day of TAC when the heart was still in compensated phase. PINK1 (PTEN-induced putative kinase 1) generally indicates the degree of mitophagy. Similar to previous research [19], our results showed cytosol PINK1 downregulated was still in compensated phase. PINK1 activation was activated on the 5th day of TAC when the heart was in the hypertrophy to heart failure in mice accompanying NLRP3 inflammasome expression upregulation.

**NLRP3 expression elevates combined with blocked autophagic flux in decompensatory heart failure**

Next, we want to explore the relationship between ATP6AP2, NLRP3 inflammasome, and autophagic flux during the TAC-induced early and late failure stages. Western blotting and IHC showed expression of NLRP3, caspase1, IL-1β, IL-18 increased in the myocardial tissue of TAC8W mice (Fig. 2D, H). ELISA results confirmed that serum IL-1β and TNF-α concentrations were significantly upregulated (Fig. 2F, G). Tissue fluorescence double staining showed that the colocalization of NLRP3 and ATP6AP2 increased (Fig. 2I), also the colocalization of NLRP3 and SQSTM1/P62 increased in TAC8W myocardium (Fig. 2J). These changes did not happen on TAC 5 days except for some increment of ATP6AP2 expression. Overall, these changes indicated that during decompensated heart failure, NLRP3 activated, accompanied by blockage of autophagic flux.

**Deletion of ATP6AP2 exacerbates progression of TAC-induced hypertrophy to heart failure in mice accompanying NLRP3 inflammasome expression upregulation**

To confirm whether the increased expression of ATP6AP2 exerts pathological effects or the upregulation is just a compensated increment with heart failure, and adenosine-monophosphor-limited vector for ATP6ap2 knockdown(shRNA-ATP6AP2) or control (shRNA-Src) was constructed. Western blot showed about 75—80% knockout efficiency (Fig. S3AB). Basal cardiac function showed no differences between mice receiving shRNA-ATP6AP2 or shRNA-Src. Four weeks after TAC, cardiac dysfunction was severe in shRNA-ATP6AP2-TAC. This effect was reflected by increased heart weight (Fig. 3A, Table. S3) and HW/BW, decreased LVEF and LVFS, and increased LVEDD, LVESD (Fig. 3B, D, E, F, G). Also, Masson’s trichrome showed prominent fibrosis in sh-ATP6AP2-TAC (Fig. 3C, H). IHC staining showed increased staining for NLRP3 in the sh-ATP6AP2-TAC group. (Fig. 3C, I). Meanwhile, tissue immunofluorescence(F) indicated increased SQSTM1/P62 in the sh-ATP6AP2-TAC group (Fig. 3C, J). Serum IL-1β and TNF-α showed apparent upregulation (Fig. 3K, L). The results indicated that the upregulation of ATP6AP2 under pressure overload might be a compensatory response.

**Moderately improved autophagic flux but similar fibrosis and cardiac dysfunction in Tg-ATP6AP2-TAC mice**

To further confirm the effect of ATP6AP2 upregulation in heart failure, we generated ATP6AP2 gain-of-function transgenic mice (Fig. S1). mRNA expression increased more than 80 folds, and protein expression increased about five folds (Fig. 4A-C) in the hearts of Tg-ATP6AP2 mice compared with wild littermates. There were no baseline differences in heart weight/body weight ratio (HW/BW; mg/g), the cardiac function between the two groups (Table S2).

After TAC was performed, the mRNA levels of Myh6, Myh7 did not show a difference between TAC-Tg and TAC-WT (Fig. 4D, E). Echocardiography showed LVEF were identical (Fig. 4F, G). Myocardite surface area was determined by HE staining; no difference was observed between TAC-Tg and TAC-WT (Fig. 4H, I). Also no significant difference in IHC staining for NLRP3, Caspase1, IL-1β, IL-18 (Fig. 4J, K, Fig. S2BFH). However, IHC/F indicated down-regulation of SQSTM1/P62 expression in TAC-Tg relative to TAC-WT (Fig. 4L). This suggested that ATP6AP2 overexpression reduced the inhibition of autophagic flux. The results indicated that the upregulation of ATP6AP2 may be compensated response to alleviate the blocked autophagic flux.

**sh-ATP6AP2 increases cell death partly through activation of NLRP3 inflammasome in vitro**

We have proved that ATP6AP2 knockdown increased NLRP3 expression in TAC-induced heart failure. In vitro research was performed to explore the mechanism. We firstly examined the ATP6AP2 expression in phenylephrine-induced primary isolated neonatal rat ventricular myocytes (NRVMs). NRVMs were stimulated with phenylephrine (100 μM) at different timepoints to mimic alterations in failing hearts in vivo. Phenylephrine stimulation for 6 h induced the upregulation of ATP6AP2, then continued to rise and maintained a high level throughout 48 h (Fig. S3C). Meanwhile, SQSTM1/P62 or NLRP3 did not show significant change when 6 h of phenylephrine but a marked upregulation after 48 h. Hence we selected phenylephrine stimulation for 6 h to mimic the early hypertrophy stage and 48 h to mimic the late-phase failure, respectively.

Long-time phenylephrine stimulation can induce cellular death (Fig. 5A, orange dots or white arrowheads indicated). This is similar to the previous study [7]. However, NRVMs transfected sh-ATP6AP2 showed more susceptibility than sh-Scr NRVMs to phenylephrine-induced cell death (Fig. 5A). This tendency was rescued after NLRP3 inhibitor MCC950 was given (Fig. 5B). Also, mRNA expression of IL-1β and IL-18 decreased (Fig. 5C, D). Next, we tend to further explore what mechanism ShR-ATP6AP2 causes upregulation of NLRP3.
Oxidative stress and mitochondrial ROS were required for NLRP3 inflammasome activation in ATP6AP2 knockdown cardiomyocytes

As ROS is recognized as one of the most common mechanisms in NLRP3 activation, we further detected whether oxidative stress involves shR-ATP6AP2 induced pathological phenotype. MitoSOX and DCFDA were used to measure mitochondrial ROS and total ROS in 48 h of phenylephrine stimulated NRVMs (Fig. 5E, F). Phenylephrine-induced upregulation of mito-ROS and total ROS compared with DMSO control. However, more ROS was produced
in the shR-ATP6AP2 + phenylephrine group (Fig. 5E–G). After blocking ROS with N-acetylcysteine (NAC), NLRP3 and Caspase1 downregulated considerably compared with phenylephrine + sh-ATP6AP2 (Fig. 5H, I). Also, TMRE by flow cytometry indicated mitochondrial membrane potential impaired by phenylephrine; shR-ATP6AP2/PRR aggravated this impairment. However, this change was rescued after blocking mitochondrial ROS with Mito-Tempo (Fig. 5J).

ATP6AP2 is required for the completion of autophagy and its knockdown inhibited V-ATPase-Driven lysosome acidification

Next, we tended to reveal cellular autophagy changes of shR-ATP6AP2 transfected NRVMs. We transduced an adenovirus plasmid encoding mCherry-GFP-LC3 into sh-Scr and sh-ATP6AP2 NRVMs. Using this reporter, we examined autophagic flux. BafA1, which inhibits autophagosomes-lysosome fusion [19] was used. BafA1 triggered a significant increase of autophagosomes indicated by yellow spots in the ShR-Scr group. In shR-ATP6AP2 NRVMs, more autophagosomes can be seen on the baseline. However, the increment of autophagosomes indicated by the yellow spots percentage after BafA1 in shR-ATP6AP2 was comparable to shR-ATP6AP2 (Fig. 6A, B). This indicated that shR-ATP6AP2 affected late autophagic flux.

Transmission electron microscopy (TEM) showed that the percentage of mitophagy in NRVMs increased after 6 h of phenylephrine stimulation. There was a significant accumulation of multivesicular vacuoles (Fig. 6C). Swollen or partially digested mitochondria can be seen around nuclear in large autophagic vacuoles. However, more undigested mitochondria can be seen in shR-ATP6AP2 + PE (Fig. 6C, E). Meanwhile, 48 h of phenylephrine inhibited mitophagy in NRVMs (Fig. 6D, F). However, the percentage of mitophagy was increased after the Ad-ATP6AP2 overexpression vector was transfected. Compared with the Ad-EGFP + phenylephrine group, more mitochondria were incorporated into autophagic vacuoles and digested in the PE + Ad-ATP6AP2 group.

Lysotracker Red probe (100 nmol/L for 30 min) showed that positive puncta in the cytosol were significantly reduced in the shR-ATP6AP2 group compared with shR-Scr. Bafilomycin A1 (BafA1) treatment (100 nmol/L for 4 h) served as a positive control (Fig. 6G). Treated shR-ATP6AP2 NRVMs with 3-methyladenine (an autophagy inhibitor) or rapamycin (an autophagy inducer), inhibited or enhanced the expression of the cytokine mRNA of IL-1β in NRVMs treated with 6 h of phenylephrine, respectively (Fig. 6H). These results also confirm ATP6AP2 exerts its role at the late stage of autophagic flux.

Phenotype changes in sh-ATP6AP2-TAC can be partly relieved by blocking NLRP3 inflammasome activation

We next examined whether the inhibition of NLRP3 can rescue the pathological cardiac phenotypes in TAC-operated shR-ATP6AP2 mice. MCC950 attenuated chamber dilation and cardiac dysfunction revealed decreased LVEDD, LVEDS compared with the sh-ATP6AP2-TAC 28 days after TAC (Fig. 7A–C). Also, HW/BW decreased after MCC950 was administrated (Fig. 7D). Masson’s trichrome indicated that administration of MCC950 resulted in the decrease of fibrosis at 28 days after TAC (Fig. 7E, F). We can see NLRP3 and caspase1 expression were significantly reduced after MCC950 was given compared with the sh-ATP6AP2-TAC group (Fig. 7E, G, H). HE staining and TUNEL also indicated MCC950 caused a reduction of cardiomyocyte area and apoptosis after MCC950 was given (Fig. 7E, I, J).

DISCUSSION

In the present study, we investigate the regulatory relation of ATP6AP2, autophagic flux, and NLRP3 inflammasome activation in the progression of cardiac hypertrophy and heart failure. We obtain the following principal findings. (i) Knockdown of ATP6AP2 deteriorates heart function in TAC-induced heart failure. (ii) Knockdown of ATP6AP2 compromises lysosomal acidification, hence impaired autophagic flux and mitophagy. (iii) NLRP3 inflammasome significantly increases in TAC-induced heart failure after ATP6AP2 knockdown. The NLRP3 inflammasome partially induces myocardial fibrosis and cardiac dysfunction. NLRP3 inhibition has a definite protective effect in ATP6AP2 knockdown-induced heart failure. (iv) Blocking cellular ROS and mitochondrial ROS of shR-ATP6AP2-transfected cardiomyocytes, can partially rescue the upregulation of NLRP3 and mitochondrial damage (Fig. 8).

The (pro)renin receptor ((P)RR), gene name ATP6AP2, was initially recognized as a potential regulator role in the renin-angiotensin system (RAS). It is ubiquitously expressed in the kidney, heart, brain and immune system, and so on [31]. It participates in a series of physiological processes, such as the cell cycle, autophagy, energy metabolism, and exerts a pathological role in fibrosis, hypertension, insulin resistance, pre-eclampsia, kidney injury, and cardiovascular diseases [32]. Nevertheless, there remains controversy about its function in pathological processes. Mice with cardiac-restricted overexpression of ATP6AP2 failed to produce morphological abnormalities under basal state and isoproterenol infusion [28, 29]. In physiological situations such as pregnancy, although high ATP6AP2 maternal plasma level, apparent tissue damage was absent [33]. Some research implements loss of function on ATP6AP2 through handle region peptide (HRP). However, there remains controversy about HRP efficiency [34, 35].

Despite two decades of studies on ATP6AP2, its function has yet to be fully revealed [20]. So far, there is some research about the role of ATP6AP2 in modulating lysosomal acidification as a component of V-ATPase. Most targeted tissues, cell homeostasis was compromised [15, 17, 30, 36, 37]. When ATP6AP2 knockout was specifically performed in cardiomyocytes or podocytes, mice developed lethal heart failure, nephrotic syndrome, and albuminuria. The most obvious changes in the cell are accumulated autophagic vacuoles and cytoskeletal changes [16, 30]. In humans, ATP6AP2 mutations failed to recognize any evidence of its role in the RAS [38]. Instead, links between ATP6AP2 missense mutations with autophagic defects emerged significantly [39, 40].

According to previous articles, in our study, autophagic flux was measured as objectively as possible [41–43]. Several combined methods were selected for a comprehensive evaluation. Our
results show ATP6AP2 upregulation can be seen from the hypertrophic phase until heart failure. ATP6AP2 knockdown blocked autophagic flux at late-phase autolysosome by compromising V-ATPase. This is consistent with the previous study [44]. We speculate this compensatory ATP6AP2 upregulation may exert its physiological pro-autophagic and protective role in pressure overload stress. The clinical application of recombinant BNP in severe decompensated heart failure inspired us. Even though serum BNP is at a high level, it still does not meet the compensatory needs in this situation. The exogenous input can
effectively improve symptoms because the concentration of endogenous BNP and exogenous input is not in the same order of magnitude.

Mitochondrial impairment and dysfunction can be provoked by prolonged and high-level cardiac stress. As one of the three most important processes for mitochondrial quality control, Mitophagy is adaptively induced to remove the damaged mitochondria and prevent oxidative damage [45]. Autophagy and mitophagy can inhibit apoptosis and postpone progression to heart failure to some extent [46, 47]. B. Wang et al. showed that enhancing mitophagy by the AMPKα2-PINK1-PARKIN pathway improved TAC-induced heart failure [19]. Our results revealed that ATP6AP2 knockdown or overexpression accordingly obstructed or facilitated autophagy flux. This result is consistent with previous research [15, 17, 30]. We further revealed that mitophagy is also mediated by ATP6AP2.

NLRP3 inflammasome pathways are closely related to most cardiovascular diseases. Cardiac fibrosis and multiple sclerosis severity can be limited by NLRP3 inflammasome inhibitor [48]. ASC−/− mice had decreased neointimal lesion, the neointimal formation was attenuated after vascular injury [49]. The acute or chronic inflammation aroused by cholesterol crystals or a high-cholesterol diet was limited in NLRP3 or IL-1β-deficient mice [12, 13, 50, 51]. Pressure overload, especially in the early phase, can trigger CaMKII-NLRP3 pathway activation in cardiomyocytes and recruit macrophages to promote fibrosis and cardiac dysfunction [14]. Mitochondrial DNA, if not be eliminated appropriately and timely by autophagy, can trigger IL-1β mediated myocarditis and dilated cardiomyopathy via TLR9 activation [8]. Increasing evidence indicates the crosstalk between autophagy and inflammasomes. Autophagy can downregulate NLRP3 inflammasome activation and exert protective effects [52–54]. Consistent with these researches, our results affirmed that NLRP3 promotes cardiac fibrosis and dysfunction in pressure overload stress. We further revealed that ATP6AP2 could promote autophagic flux and anti-inflammasome function.

Oxidative stress has been widely accepted to contribute to cardiac remodeling in many pathological processes such as cardiac hypertrophy, cell apoptosis [55]. Defective autolysosomes, damaged mitochondrial that not be timely degraded can be the source of ROS [56]. Excessive ROS can provoke doxorubicin cardiotoxicity, myocardial infarction, ischemia-reperfusion injury [12, 51, 57–60]. The high efficiency of autophagic flux may play a protective mechanism against excessive ROS in doxorubicin-induced cardiomyopathy [6, 61, 62]. Our in vitro results indicated that ATP6AP2 knockdown induced autophagic dysfunction and dysfunctional mitochondria accumulation; increased ROS and mitochondrial ROS further accelerated NLRP3 inflammasome. Our results are consistent with previous studies. Again we confirm autophagy has a suppressive role on NLRP3 by inhibiting oxidative stress.

In consideration of cardiac diseases diversity and severity, and there still exist some inconsistent implementation of assays to assess autophagy in different contexts, some variability was revealed in changed autophagic activity among different animal models. We tried to perform longitudinal experiments at different time points of the TAC model to sketch altered autophagic activity, including early hypertrophy and late heart failure stages. As ATP6AP2 and autophagy may play multifaceted and complex roles in disease pathogenesis, much work still needs to be done.

In conclusion, our research shows that mice with ATP6AP2 knockdown in cardiomyocytes compromising autophagic flux confers a more obvious inflammasome activation phenotype and further promote maladaptive cardiac remodeling in the model of pressure overload stress. Cellular ROS and mitochondrial ROS induced by ATP6AP2 knockdown accelerate NLRP3 activation. The clinical research CANTOS involving IL-1β neutralizing antibody canakinumab in coronary disease has encouraging findings. It predominantly decreases myocardial infarction [63]. This may give us some inspiration that NLRP3 inflammasome inhibition can be particularly effective to prevent this maladaptive remodeling in situations where the autophagic flux is not fully compensated in late heart failure. Cell impairment triggered by ATP6AP2 depletion argues against using ATP6AP2/PRR inhibitors for treatment.

METHODS

Neonatal rat ventricular myocyte isolation (NRVMs)
Hearts from 1–3-day-old rat pups of Sprague-Dawley were isolated and digested using collagenase II (C8150, Solarbio). The cell suspension was preplated in the culture flask and adhered to for 1.5 h at differential speed. Through this procedure, purified myocytes can be obtained. Then plated on gelatin-coated plates at a density of 4 × 10^4 /cell/cm^2 overnight at 37 °C. The final concentration of 100 μM L-Buthiomyocardinidine was added to the plating medium containing 10% FBS. Cardiomyocyte purity was about 95%. NRVMs were continued to culture in a complete medium for 3 days (high-glucose DMEM containing 5% FBS, 100 units/ml penicillin, and 95%. NRVMs were continued to culture in a complete medium for 3 days (high-glucose DMEM containing 5% FBS, 100 units/ml penicillin, and 100 μg/ml streptomycin), experiments were initiated on the fourth day. Adenovirus expressing the constitutively active ATP6AP2 (Ad-ATP6AP2) or EGFP (Ad-EGFP) and Sh-ATP6AP2, Sh-Scr was applied to the culture at a multiplicity of infection (MOI) of 50 for 24 h.

Drug treatment
Phenytoine (PE 100 μM/μL) was added to the Cardiomyocytes medium at a series of time points, followed by carbonyl cyanide p-trichloromethoxyphenyl hydrazono CCCP (20 μM) treatment to induce mitophagy. Some may be pretreated with or without a small molecular inhibitor of NLRP3-MCC950 (5 μM) [12], antioxidant N-acetylcysteine NAC (10 mM), Mitto-Tempo (500 μM). In some experiments, mice were injected MCC950 (5 mg/kg/day) diluted in PBS via intraperitoneal injection [64] or Vehicle control (PBS).

Recombinant adenovirus vectors
Recombinant adenovirus vectors-mediated ATP6AP2 knockdown was designed by Gene-Pharma (Shanghai Gene Pharma Co. Ltd, Shanghai, China). We synthesized interfering sequences targeting ATP6AP2-S'-GCTCGAGATCCGTGGTTTCA-3'. Adenovirus containing rat ATP6AP2 cDNA (gene ID: 302526) sequence or control transgene Ad-EGFP was synthesized and cloned into pDC315 with the EcoRI and SalI sites as previously described using the AdMax system [65]. Adenovirus is administered through the tail vein in 10^7 doses per mouse. The expression time of the adenovirus vector in vivo was about 2–4 weeks, and we gave the same dose of virus again 2 weeks later.
Immunoblot analysis

Forty milligrams Ventricular tissue was homogenized in 300 ul protein lysis buffers (P0013B, beyotime). Protease inhibitors (PMSF36978, Thermo ScientificTM) and Phosphatase inhibitors were added before use. Centrifugation was performed at 10,000–14,000 x g for 3–5 min after full lysis. The supernatant was taken for subsequent Western.

For NRVMs, remove the culture medium and wash with cold PBS. Add lysate in a ratio of 150 ul per well of the six-well plate. Use a micropipette to blend a few times to make full contact with the cell lysate. Protein concentration was measured. Equal amounts of protein (30 ug) were loaded for electrophoresis and transferred onto PVDF membranes. Blots were blocked in 1×TBST with 5% milk before incubation overnight at 4 °C with 1:1000 ATP6AP2 (ab64957), 1:5000 GAPDH (protein tech,10494-1-AP), 1:1000 caspase-1p20/p10 polyclonal.
antibody (protein tech, 22915-1-AP), 1:1000 IL-1β (affinity, AF5130), 1:1000 NLRP3 (protein tech, 19771-1-AP). Blots were washed with 1xTBST, 1:10000 secondary antibody conjugated with HRP was incubated at room temperature for 1 h, washed with 1xTBST, ECL substrate (Immobilon ECL, WBULS0500) was used for detecting, membranes were visualized by GE AI600 via chemiluminescence and analyzed with ImageJ.

Quantitative RT-PCR
Total RNA from tissue and culture cells was isolated using TRIZOL reagent. RNA was reverse transcribed with Prime Script RT Reagent Kit (Takara; RR037A). qRT-PCR was performed via Light Cycler 480 SYBR Green I Master (Roche; 04887352001). Expression levels of RNA were normalized to GAPDH. Primers for mouse gene expression are shown in the table.
Fig. 6 ATP6AP2 is required for the completion of autophagy and its knockdown inhibits V-ATPase-driven lysosome acidification. A, B Representative fluorescent images are shown. Manually quantitative analysis indicates the mean number of autophagosomes (yellow) and autolysosomes (red) per cell. Statistical analysis was conducted by Kruskal–Wallis one-way ANOVA with Dunn post-hoc test. **P < 0.01. C, D Autophagy and mitophagy levels showed by transmission electron microscopy in NRVMs transfected with shR-ATP6AP2 knockdown vector or Ad-ATP6AP2 overexpression vector. Representative images of normal mitochondria and mitochondria in-taken by vacuole structure can be seen. E, F immunofluorescence images of LysoTracker Red staining in shR-Scr and shR-ATP6AP2 NRVMs treated with DMSO or bafilomycin A1 for 60 min. H the effect of autophagy inducer or inhibitor on IL-1β mRNA expression in NRVMs while sh-ATP6AP2 was transfected. GAPDH was used as the loading control. Statistical analysis was conducted by Kruskal–Wallis one-way ANOVA with Dunn post-hoc test. At least four independent experiments were conducted. *P < 0.05, **P < 0.01.

(Supplementary Table 1). Relative quantitation was determined by the 2−ΔΔCT method.

ELISA

The mouse blood was collected in a clean test tube, coagulated at room temperature, and centrifuged at 2000 × g for 15 min. The serum was collected and stored at −80 °C after dividing. For cell culture supernatant, the supernatant was collected and stored at −80 °C after centrifugation at 1200 × g for 15 min. The mouse blood was collected in a clean test tube, coagulated at room temperature, and centrifuged at 2000 × g for 15 min. The serum was collected and stored at −80 °C after dividing. For cell culture supernatant, the supernatant was collected and stored at −80 °C after centrifugation at 1200 × g for 15 min.

Transverse aortic constriction surgery (TAC)

TAC surgery was performed on anesthetized mice 8–12 weeks of age as described previously [66]. Mice were randomized into either a sham group or a TAC group by a random number table. Briefly, the operating field was disinfected with 75% alcohol, and surgical tools were sterilized. The heating pad was maintained at 37 ± 1 °C. 2% isoflurane was used to maintain anesthesia. Mice were placed in the supine position, shaved fur with hair clippers. The transverse aortic arch was visualized through median sternotomy. A 27-gauge blunt needle was used to yield a 0.4 mm narrow in diameter, 7.0 silk suture ligature was performed. 6.0 silk suture ligature was used to close the rib cage and skin. Sham-operated mice performed the same operation except for constriction of the aortic arch. Warm-maintaining measure after the operation is conducive to recovery.

Ultrasound echocardiography

Ultrasound echocardiography (Veo 2100, VisualSonics, Toronto, Canada) with a transducer frequency of 40 Hz was used. Five percent isoflurane was inhaled to induce anesthesia and 1.5% isoflurane for anesthesia maintenance during the operation. The heart rate of the mice was maintained between 450 and 500 beats/min. The heart structure was recorded by M-mode ultrasound at the papillary muscle section. Analysis was performed using the software Vevo2100. Parameters including LVEDD, LVESD, LVID, LVEF, LVFS (%) were used to evaluate the changes in cardiac function by investigators blind to mice genotype.

Immunohistochemistry and immunofluorescence microscopy analysis

Ventricles were fixed in 4% paraformaldehyde (PFA) for 24 h, dehydrated by gradient alcohol, transparent by xylene, and embedded in paraffin. Tissues were cut into sections of 5 μm thickness, deparaffinization, rehydrated to complete the removal of paraffin. Hematoxylin and eosin (H&E) staining were used for histological analysis. Fibrillar collagen was detected with Masson’s trichrome. Additionally, sections were blocked protein and endogenous enzymes, antigen retrieval, primary for ATP6AP2 (1:200, Abcam, ab64957), NLRP3 (1:200, protein tech,19971-1-AP), Caspase1 (1:200, protein tech, 22915-1-AP), IL-1β (1:200, abcam, af113301), IL-18 (protein tech,10663-1-AP) were performed for 24 h at 4 °C. After washing with TBST, sections were incubated with HRP conjugated goat-rabbit IgG or AlexaFlour-conjugated secondary antibody (Abcam, ab150077, ab150079). Analysis was performed by Image-J software for quantification of fibrosis by investigators blind to mice genotype.

Reactive oxygen species (ROS) measurement-DCFDA

Isolated NRVMs were suspended in DMEM completed medium and exposed to phenylephrine after transfection with adenovirus-mediated Scramble (sh-Scr) or ATP6AP2 (sh-ATP6AP2) and stimulated with MCC950 (5 μM). DCFDA/H2DCFDA-Cellular ROS Assay Kit (ab113851, Abcam) was used to quantitatively assess reactive oxygen species in live-cell samples according to the manufacturer’s instructions.

Mitochondrial ROS detection

MitoSOX Mitochondrial Superoxide Indicator (40778550, YEASEN) was used to detect mitochondrial ROS. NRVMs were seeded on cover slides of 12-well plates and transfected with shR-ATP6AP2 or shR-Scr virus vector for 24 h at MOI = 50 on the 4th day. The medium was changed to a complete culture medium and continued for 48 h of PE stimulation or DMSO control. Then added 37 °C pretreated MitoSOX (500 nM) and incubated for 20 min. After PBS cleaning, the nuclei were stained with DAPI. Imaging with fluorescence microscopy (Ex = 597NM, Em = 599 nm).

Ad-mCherry-GFP-LC3B adenosival vector to detect autophagy

Ad-mCherry-GFP-LC3B (C3011, Beyotime) was used to detect the autophagy flow of infected cells according to the manufacturer’s instructions. Isolated NRVMs were plated in 24 orifice plates. Sh-Scr, shR-ATP6AP2(MOI = 50), and Ad-mCherry-GFP-LC3B adenosival vector (MOI = 30) infection were performed after NRVMs reached 70% confluence. About 24 h after infection, the culture medium containing the virus was removed, 2 ml fresh complete culture medium was added to each well, and the growth status and fluorescence protein expression of cells were observed after 24 h. 48 h further culture. Fluorescence microscopy was used to observe LC3B fluorescence changes.

Flow cytometry-TMRE

NRVM cells were transfected with sh-ATP6AP2 or sh-Scr, pretreated with mito-tempo, and then stimulated with phenylephrine (100 umo/L) or DMSO for 6 h. TMRE-Mitochondrial Membrane Potential Assay Kit (ab113852, Abcam) was used to quantitatively changes in mitochondrial membrane potential in live cells by flow cytometry according to the manufacturer’s instructions.

ICC/IF

Cell survival and death were assessed with fluorescent dyes (SYTO ™ Orange s11368, and SYTO™ Green, S7578). To show that phenylephrine can induce oxidative stress injury and Shr-ATP6AP2 can aggravate phenylephrine-induced oxidative stress injury, NVCM was labeled with double fluorescent dye to distinguish live from dead cells. After brief incubation with SYTO Orange nucleic acid stain, the nucleic acids of dead cells fluoresce bright orange when excited with the 547 nm. SYTO Green is a cell-permeant nucleic acid stain that preferentially labels live-cell nuclei. The nucleic acids of live cells fluoresce bright green when excited with the 517 nm. NRVMs were infected with sh-Scr or shR-ATP6AP2, then phenylephrine stimulation for 36 h, after that incubated with SYTOX Orange and SYTOX Green at a concentration of 5 μM for 30 min at 37 °C. NRVMs were visualized by a fluorescence microscope.

Lysosomal PH measurement

lysosomal PH was quantitatively detected by Lyso-Tracker Red (C10416, Beyotime) according to the manufacturer’s instruction. Isolated NRVMs were plated in 24 orifice plates. ShR-Scr, shR-ATP6AP2(MOI = 50) for 24 h. The cell culture medium was removed, and 37 °C preincubated LysoTracker Red staining solution was added, and the cells were co-incubated at 37 °C for 20 min. LysoTracker Red staining solution was removed, and a fresh cell culture solution was added. They were then observed under a fluorescence microscope.
TUNEL assay in tissue was performed according to the manufacturer’s directions (TUNEL Andy Fluor TM 488 Apoptosis Kit, A050, ABP biosciences).

Cardio-restricted ATP6AP2 overexpression transgenic mice
Tg mice expressing ATP6AP2 (H11-CAG-LSL-ATP6AP2-polyA Cas9-KI) were generated under the direction of the murine -CAG promoter by using CRISPR/Cas9 technology (Supplementary Fig. 1). The brief process is as

**TUNEL**

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Supplementary information is available at cell death discovery.

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AUTHOR CONTRIBUTIONS
LL designed the experiment and generated the data for the manuscript. YJC, YL, and SNL wrote part of the manuscript. SKV and FY provided technical support.
LLW and YWZ edited the manuscript. HXL and YDS performed statistical analyses of the data.

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**COMPETING INTERESTS**
The authors declare no competing interests.

**ETHICS**
The animal protocols described in the animal experiments were reviewed and approved by the Animal Ethical and Welfare Committee of Shandong University (No. 21136).

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