Decreased autophagy: a major factor for cardiomyocyte death induced by β1-adrenoceptor autoantibodies

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Cardiomyocyte death is one major factor in the development of heart dysfunction, thus, understanding its mechanism may help with the prevention and treatment of this disease. Previously, we reported that anti-β1-adrenergic receptor autoantibodies (β1-AABs) decreased myocardial autophagy, but the role of these in cardiac function and cardiomyocyte death is unclear. We report that rapamycin, an mTOR inhibitor, restored cardiac function in a passively β1-AAB-immunized rat model with decreased cardiac function and myocardial autophagic flux. Next, after upregulating or inhibiting autophagy with Beclin-1 overexpression/rapamycin or RNA interference (RNAi)-mediated expression of Beclin-1/3-methyladenine, β1-AAB-induced autophagy was an initial protective stress response before apoptosis. Then, decreased autophagy contributed to cardiomyocyte death followed by decreases in cardiac function. In conclusion, proper regulation of autophagy may be important for treating patients with β1-AAB-positive heart dysfunction.

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Heart dysfunction is the terminal stage of various cardiovascular diseases, and it is characterized by a complicated etiology and high mortality. Recent studies indicate that cardiomyocyte death was a leading contributor to the development of heart dysfunction.1 Because systolic and diastolic function is directly affected by myocardial cell loss, understanding how cardiomyocyte death occurs will inform treatment strategies to prevent or treat heart dysfunction.

Since the 1990s, studies have revealed that diverse cardiovascular diseases are correlated to anti-β1-adrenergic receptor autoantibodies (β1-AABs).2,3 We reported that β1-AABs were induced by myocardial remodeling in heart dysfunction,4 and that its long-term presence significantly decreased cardiac function in vivo.5 β1-AABs also caused cell death of cultured adult rat ventricular myocytes and this was attributed to apoptosis.6 Recently, work from our laboratory7 and others8 indicated that β1-AABs induced myocardial apoptosis. However, β1-AAB-induced cardiomyocyte death was not completely reversed with the caspase inhibitor Z-VAD-fmk,6 indicating that other factors were involved in β1-AAB-induced cardiomyocyte death.

Presently, we observed that β1-AABs decrease myocardial autophagy that maintains cellular homeostasis.9 Deficiencies in autophagy allow the accumulation of damaged, denatured or aging proteins10 and organelles,11 and this will cause cell death. To date, the role of β1-AAB-induced changes in autophagy as related to cardiac function and cardiomyocyte death is unclear. Therefore, we characterized β1-AAB-induced changes in myocardial autophagy and identified a role for this in cardiac function and cardiomyocyte death. Our data will inform future studies of β1-AAB-positive heart dysfunction and suggest a treatment window for autophagy regulation.

Results

β1-AABs caused the decrease of cardiac function in passively immunized rats. Rats were passively immunized by injecting β1-AABs (2 μg/g), once every 10 days, for 80 days. Before each immunization, serum β1-AABs were measured and it increased 20 days after passive immunization. Serum β1-AABs remained stable until the end of the experiment in the β1-AAB group compared with the control group (Supplementary Figure S1a).

Meanwhile, cardiac function was measured 40 and 60 days after passive immunization, and there was no significant difference in left ventricular function between the immunized and control groups. However, animals had significantly decreased left ventricular systolic pressure (LVSP), maximal positive and negative values of the instantaneous first derivative of left ventricular pressure (+dP/dtmax and −dP/dtmax) and significantly increased left ventricular end diastolic pressure (LVEDP) 80 days after passive immunization in the β1-AAB group compared with the control group (Supplementary Figure S1b–e), indicating that a long-term

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Abbreviations: β1-AABs, anti-β1-adrenergic receptor autoantibodies; RNAI, RNA interference; LVSP, left ventricular systolic pressure; +dP/dtmax, maximal positive values of the instantaneous first derivative of left ventricular pressure; −dP/dtmax, maximal negative values of the instantaneous first derivative of left ventricular pressure; LVEDP, left ventricular end diastolic pressure; RAPA, rapamycin; CQ, chloroquine; 3-MA, 3-methyladenine; β1-AR-ECII, the second extracellular loop of β1-adrenoceptor; MAP1-LC3, LC3; microtubule-associated protein 1 light chain 3; PI3K, phosphatidylinositol-3-kinase; DMEM, Dulbecco’s modified Eagle’s medium; FBS, fetal bovine serum; CCK-8, cell counting kit-8

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presence of β1-AABs could cause a significant decrease in cardiac function.

**Decreased autophagy had a role in decreased cardiac function induced by β1-AABs.** In this study, Beclin-1, an important gene in autophagic regulation, and LC3, a protein marker for autophagy, were selected to measure autophagy. Significantly decreased LC3 and Beclin-1 protein were observed 20 days after passive immunization and lower levels persisted 40 and 80 days after passive immunization in the β1-AAB group (Figures 1a–c), indicating that β1-AABs may lead to decreased autophagy in myocardial tissues. To confirm the results, p62 protein, which was degraded within the autolysosomes, was used as a marker of autophagic flux. At 20 days after immunization, p62 protein in cardiac tissue increased significantly in the β1-AAB group compared with control groups and p62 remained relatively high at 40 and 80 days (Figures 1a and d), indicating a defect in autophagic flux in the presence of β1-AABs.

Rapamycin (RAPA), an mTOR inhibitor, is often used to upregulate autophagy. In this experiment, it was used to improve autophagy in rat myocardial tissue (Supplementary Figure S2). Significantly improved left ventricular function was observed in rats pretreated with rapamycin compared with those only passively immunized in the β1-AAB group (Figures 2a–d), suggesting that upregulating autophagy may reverse decreased cardiac function induced by β1-AABs and that insufficient autophagy caused by the long-term presence of β1-AABs may be associated with decreased cardiac function.

**β1-AABs caused the death of H9c2 cells.** Cell viability was measured to reflect the effects of β1-AABs stimulation of different durations. Significantly decreased cell viability was observed 12 h after β1-AABs stimulation and at a minimum of 36 h after β1-AABs stimulation (Figure 3a), indicating that β1-AABs may cause the death of myocardial cells.

LDH is released when cell membranes lyse, and it is an indicator of cell damage. Significantly increased LDH activity was observed at 6 h which lasted till the end of the experiment compared with the control group (Figure 3b), suggesting that β1-AABs may directly damage H9c2 cells.

**Decreased autophagy was critical for cardiomyocyte death.** LC3 and Beclin-1 were used to indicate autophagy, and Beclin-1 and LC3 protein and mRNA in H9c2 myocardial cells were measured 0, 12, 24, 36, and 48 h after β1-AABs stimulation using western blot and real-time PCR. In situ expression of LC3 protein was measured with immunostaining. Beclin-1 protein decreased 24 h after β1-AABs stimulation, dropped to a minimum at 36 h and recovered to normal at 48 h compared with the control group (Figure 4f). Both LC3 protein (see Figures 4a and b) and LC3 mRNA (see Figure 4e) were decreased 12 h after β1-AABs stimulation, and were minimal at 36 h, returning to normal at 48 h compared with the control group. Immunostaining revealed that green punctate fluorescent signals representing LC3 significantly decreased 36 h after β1-AABs stimulation, but recovered at 48 h (Figures 4g and h). Thus, β1-AABs could decrease myocardial autophagy.

In addition, p62 was used to reflect autophagic flux. Figures 4a and d show that p62 protein increased significantly 12 h after β1-AABs stimulation, peaked at 36 h, and then recovered at 48 h compared with controls. Meanwhile, chloroquine (CQ) was used to block autophagosome–lysosome fusion. Chloroquine rescued LC3 and p62 for 36 h after β1-AABs stimulation (Figures 4i–k), indicating that autophagic flux decreases in the presence of β1-AABs.
Cell viability decreased the most 36 h after β₁-AABs stimulation in H9c2 myocardial cells. Therefore, 36 h was selected as the time point for β₁-AABs treatment. Recombinant plasmid pcDNA3.1-Beclin-1 for upregulating autophagy (Supplementary Figures S3a−e) and recombinant plasmid expressing small interfering RNA targeting Beclin-1 (Beclin-1-shRNA) to inhibit autophagy (Supplementary Figures S4a−f) were transfected into H9c2 cells, followed by β₁-AABs stimulation for 36 h. Empty negative control plasmid for protein overexpression and shRNA expression did not influence autophagy (Supplementary Figures S3a−e, S4a−f) and cell viability (Supplementary Figures S3f and S4g). Cell viability increased in cells with upregulated autophagy, but decreased in cells with inhibited autophagy compared with mock cells (Figure 4l). To confirm these data, rapamycin or 3-methyladenine (3-MA) were used to upregulate or suppress autophagy, and cell viability of H9c2 myocardial cells was measured (Figure 4m). Two different ways to increase or inhibit autophagy yielded similar results, suggesting that β₁-AAB-induced decreases in autophagy have a role in cardiomyocyte death.

**Increased autophagy benefitted myocardial cells with early β₁-AABs stimulation.** Autophagy is well known as a stress response,13 so we observed changes in this stress over time, using earlier β₁-AABs stimulation. Cells were collected 0, 30 min, 1, 3, 6, and 12 h after stimulation and LC3 protein, LC3 mRNA, beclin-1 protein, beclin-1 mRNA, and p62 protein were measured. Autophagy increased early as LC3 protein (see Figures 5a and b), LC3 mRNA (see Figure 5e), beclin-1 protein (see Figures 5a and c), and beclin-1 mRNA (see Figure 5f) increased 30 min after β₁-AABs treatment and remained high for 1 and 3 h and recovered to normal at 6 h compared with controls. At 12 h, expression decreased. Meanwhile, p62 protein was declined at 30 min, remained low at 1 and 3 h, returned to normal at 6 h, and then increased at 12 h (Figures 5a and d). These data are consistent with results mentioned above, in which
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Figure 5  Effect of early β₁-AABs stimulation on autophagy in H9c2 cells and its importance on cardiomyocyte death. (a) Representative western blots of autophagy markers LC3, Beclin-1, and p62 at 0, 30 min, 1 h, 3 h, 6 h, and 12 h after β₁-AABs stimulation. (b–d) Quantification of western blot data from (a). Data are expressed as means±S.D. (n = 6 per group). *P<0.05; **P<0.01. (e and f) After exposure to β₁-AABs for different time, LC3 and Beclin-1 mRNA were measured by real-time PCR. Data are expressed as means±S.D. (n = 6 per group). *P<0.05; **P<0.01. (g) Western blot showed the protein expression of LC3, and p62 after 3 h β₁-AABs stimulation in H9c2 cells pretreated with/or without 20 μM chloroquine (CQ). (h and i) Quantification of western blot data from (g). Data are expressed as means±S.D. (n = 6 per group). *P<0.05, **P<0.01 versus Control; #P<0.05, ##P<0.01 versus β₁-AAB group. (j and k) Cardiomyocyte death was enhanced by inhibiting autophagy (Beclin1-shRNA or 3-MA) 6 h after β₁-AAB stimulation. Cell viability was determined by CCK-8. (n=8, means±S.D.) #P<0.05 versus β₁-AAB group.

Figure 4  Change of autophagic flux induced by β₁-AABs in H9c2 myocardial cells and its effect on cardiomyocyte death. (a) H9c2 myocardial cells were treated with β₁-AABs or negative IgGs at 0, 12, 24, 36, and 48 h. Western blot indicated protein expression of LC3, Beclin-1, and p62 in H9c2 cells. (b–d) Quantification of western blot data from (a). Data are expressed as means±S.D. (n = 6 per group). *P<0.05; **P<0.01. (e and f) Real-time PCR was used to measure LC3 and Beclin-1 mRNA expression in H9c2 cells. Data are expressed as means±S.D. (n = 6 per group). *P<0.05; **P<0.01. (g) Representative images of immunofluorescence staining for LC3 (green) and DAPI (blue) in H9c2 cells. Scale bar was 10 μm. (h) A statistical analysis by counting LC3 puncta in 20 different fields. The number of LC3 puncta/cell was evaluated as the total number of dots (green) divided by the number of nuclei (blue) in each microscopic field. Data are expressed as means±S.D. (n = 6 per group). **P<0.01 versus Control; #P<0.05 versus β₁-AAB group. (i) H9c2 cells were pretreated with/or without 20 μM chloroquine (CQ, an inhibitor of autophagy), and immunoblotting assays were performed with LC3B or p62 antibodies at 36 h after β₁-AABs stimulation. (j and k) Quantification of western blot data from (i). Data are expressed as means±S.D. (n = 6 per group). *P<0.05 versus Control, **P<0.01 versus β₁-AAB group. (l and m) Change in cell viability 36 h after β₁-AABs stimulation when myocardial autophagy was upregulated by overexpressing Beclin-1/RAFA or inhibited by RNA interference against Beclin-1/3-MA. Data are expressed as means±S.D. (n = 6 per group). **P<0.01 versus Control; #P<0.05 versus β₁-AAB group.
autophagy decreased to the minimum 36 h after β1-AABs stimulation. Therefore, the presence of β1-AABs first induced increased autophagic flux in myocardial cells and this may deplete autophagic genes or proteins and cause insufficient autophagy.

To better interpret changes in LC3, chloroquine was used to block the fusion of the autophagosome with the lysosome, and Figures 5g–i show that pretreatment with chloroquine could upregulate LC3 and p62 significantly for 3 h after β1-AABs stimulation, indicating that early increases in LC3 offer efficient autophagic flux.

To confirm that β1-AAB induced early increases in autophagy on cardiomyocyte death, autophagy was inhibited with Beclin-1 using RNA interference technology or 3-MA. In addition, because early increased autophagy recovered to almost normal 6 h after stimulation, 6 h was chosen for observation to eliminate the effect of later decreases in autophagy and diminished cell viability. Data show that cell viability did not change 6 h after β1-AABs stimulation of myocardial cells, but cardiomyocyte death occurred when autophagy was inhibited (Figures 5j and k).

**Discussion**

The objective of this study is to explore the importance of β1-AAB-induced reduction of myocardial autophagy on cardiac function *in vivo*, and discuss changes in β1-AAB-induced autophagy over time and its significance on cardiomyocyte death. We observed that changes in β1-AAB-induced autophagy increased early and then decreased. The early increase was cardioprotective, but the later decrease in autophagy prompted cardiomyocyte death and reduced cardiac function *in vivo*.

Recently, autoantibodies against the second extracellular loop of β1-adrenoceptor (β1-AR-ECII) (β1-AABs) were detected in the sera of patients with idiopathic dilated cardiomyopathy, Chagas’ heart disease, and heart dysfunction caused by ischemic cardiomyopathy. In our previous study, the long-term presence of autoantibodies may contribute to decreased cardiac function in a rat model immunized with the peptide corresponding to the second extracellular loop of β1-adrenoceptor. In this study, we also observed that β1-AABs may directly cause cardiomyocyte death. To confirm these data, LDH was measured in myocardial cells because this is documented to leak from damaged cells. Similarly, in this study, we also observed that β1-AABs could decrease myocardial autophagy in a passively immunized rat model. Increased p62 protein indicated a defect in autophagy induced by β1-AABs. To observe the effect of decreased autophagy on cardiac function, the mTOR inhibitor rapamycin was used to upregulate autophagy. Data show that decreased cardiac function induced by β1-AABs was effectively reversed by enhanced autophagy. Thus, decreased myocardial autophagy is a major contributor to heart dysfunction.

Myocardial cells are a basic unit of cardiac systolic and diastolic function and when they are damaged or dead, contractile proteins in myocardial cells are degraded immediately, decreasing contractility. To identify a role for β1-AABs in myocardial cells, we purified IgG antibody in actively immunized rat serum to obtain β1-AABs. Next, a relatively stable H9c2 cell line was selected from embryonic rat heart tissues, and this line was used to observe the effects of β1-AABs on survival and autophagy of myocardial cells under the same conditions and time points. Data show that cardiac cell viability deceased at 12 h after β1-AABs stimulation and was minimal at 36 h, suggesting that β1-AABs may directly cause cardiomyocyte death. To confirm these data, LDH was measured in myocardial cells because this is documented to leak from damaged cells. Similarly, LDH activity in the cell culture medium significantly increased 6 h after β1-AABs stimulation, indicating damage. In conclusion, β1-AABs stimulation directly harmed the myocardial cell membrane, and caused cell death. This conclusion is consistent with previous results made by Jane-wit et al in an adult rat model.

Previously, β1-AABs were confirmed to induce apoptosis in cultured neonatal rat myocardial cells. Staudt’s group also pointed out that β1-AABs could cause apoptosis in adult isolated myocardial cells. Thus, we measured β1-AAB effects on myocardial apoptosis over time and found increased apoptosis 6 h after β1-AABs stimulation and a return to normal at 24 h. Next, the caspase inhibitor Z-VAD-fmk was used to inhibit apoptosis and we observed that cardiomyocyte death recovered to a certain extent 36 h after β1-AABs stimulation (Supplementary Figure S5), indicating that β1-AAB-induced apoptosis is involved in cardiomyocyte death. Because β1-AAB-induced cardiomyocyte death was not completely recovered via apoptotic inhibition, other mechanisms are at play. Thus, we studied H9c2 cells with β1-AABs at different time points and observed decreased autophagic flux 12 h after stimulation and this was minimal at 36 h, indicating that...
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data show that the early increase in LC3 means efficient autophagy. However, increased LC3 may cause cardiomyocyte death. Thus, we used recombinant plasmid pcDNA3.1-Beclin-1 and recombinant plasmid expressing small interfering RNA targeting Beclin-1 (Beclin-1-shRNA) to upregulate and inhibit myocardial autophagy. Autophagy is reported to be upregulated by increasing or suppressing Beclin-1 expression. Thus, H9c2 cells were transfected with recombinant plasmid pcDNA3.1-Beclin-1 to upregulate autophagy and we observed that cell viability was higher after β1-AABs stimulation in transfected cells compared with cells treated with only β1-AABs. In addition, Beclin-1 RNA interference plasmid was used to transfect H9c2 cells to inhibit autophagy and cell viability decreased after β1-AABs stimulation. However, beclin-1 could not only induce the autophagy, but also it could suppress autophagosome–lysosome fusion, so data are difficult to interpret when beclin-1 manipulation is used to modulate autophagy. Therefore, we measured cell viability of H9c2 myocardial cells pretreated with rapamycin to upregulate autophagy or 3-MA to suppress autophagy and both yielded similar results. Therefore, decreased autophagy promotes cardiomyocyte death and improvements in autophagy benefit cardiac function.

Autophagy is commonly regarded as a stress response. H9c2 myocardial cells were treated with β1-AABs at earlier time points (0, 30 min, 1, 3, and 6 h) and autophagy was found to first increase and then decrease. However, increased LC3 can be associated with either increased autophagic initiation or reduced degradation in the lysosome. To better distinguish between these two scenarios, chloroquine was applied and data show that the early increase in LC3 means efficient autophagic flux. So the increased autophagy after short-term β1-AABs stimulation is a stress response, and later depletion of autophagic genes and proteins decreases autophagy. Additional investigations are needed to learn whether β1-AABs directly induced these changes in myocardial cells. To verify the effect of early increased autophagy after β1-AABs stimulation on cardiomyocyte death, H9c2 cells were transfected with Beclin-1-shRNA or 3-MA to inhibit autophagy and we found that myocardial survival was significantly reduced after early β1-AABs stimulation due to inhibition of autophagy. In addition, comparing changes in apoptosis and autophagy over time, autophagy increased before apoptosis. This early increase of autophagy was cardioprotective but this effect gradually disappeared as autophagy decreased and cells died.

In conclusion, autophagy is a stress response before apoptosis in β1-AAB-induced cardiomyocyte death, and decreased autophagy becomes a subsequent reason for cardiac dysfunction caused by β1-AAB-induced cardiomyocyte death. Thus, autophagic regulation is more important than apoptosis for patients with β1-AAB-positive heart dysfunction.

Our study is limited because we only observed that a lack of autophagy caused by β1-AABs decreased cardiomyocyte death. However, the role of apoptotic changes with autophagic upregulation or inhibition in cardiomyocyte death induced by β1-AABs requires more study. In addition, validating whether β1-AAB-induced cardiomyocyte death could be completely reversed by Z-VAD-fmk plus rapamycin is unknown. Still, we conclude that decreased autophagy is a major factor for cardiomyocyte death induced by β1-AABs. Our preliminary observations may open new insights into the pathogenesis and prevention of β1-AAB-positive heart dysfunction.
Cell culture and transfection. Rat myocardial cell-derived cell line H9c2 was purchased from Cell Bank of China Science Academy (Shanghai, China). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Hyclone, SH30022.01B) containing 10% fetal bovine serum (FBS) (Sijjing, W0001, Hangzhou, China), 100 U/ml penicillin and 100 μg/ml streptomycin (Solarbio, P1400-100, Beijing, China), and incubated in the atmosphere with 5% CO2 at 37 °C. Cells were transfected with pcDNA3.1-Beclin-1 (Beclin-1 gene over-expressed, RefSeq Number: NM_053739.2) or Beclin-1-shRNA transfectant (Beclin-1 gene knocked down, RefSeq Number: NM_205739.2) using Lipofectamine 2000 (Invitrogen, 11668-027, Grand Island, NY, USA) according to the protocol supplied by the manufacturer. Approximately 3 μg of total RNA was extracted from cells and reverse transcribed to cDNA. The thermal profile of SYBR Green PCR included 30 s heat activation of the DNA template, 3 cycles of 95 °C for 20 s and 1 cycle of 95 °C for 30 s, 55 °C for 20 s, and 68 °C for 1 min, followed by 30 cycles of 95 °C for 20 s, 60 °C for 20 s, and 72 °C for 20 s. The last cycle was followed by a melting curve analysis to confirm the specificity of the PCR product.

Statistical analysis. Data are expressed as means ± standard deviation (S.D.). Statistical analysis was performed with SPSS software (version 15.0, SPSS Inc., Chicago, IL, USA). A Student's t-test was used to compare the means of two independent samples and one-way ANOVA was applied after a Bonferroni post hoc test for more than two samples. Data were considered as statistically significant when P < 0.05.

Conflict of Interest
The authors declare no conflict of interest.

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