Biochemical and Biophysical Research Communications 456 (2015) 250–256

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

# An alpha-adrenergic agonist protects hearts by inducing Akt1-mediated autophagy

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ARTICLE INFO

*Article history:*
Received 14 November 2014
Available online 25 November 2014

*Keywords:*
Autophagy
Cardioprotection
Adrenergic overload
Apoptosis

ABSTRACT

Alpha-adrenergic agonists is known to be protective in cardiac myocytes from apoptosis induced by beta-adrenergic stimulation. Although there has been a recent focus on the role of cardiac autophagy in heart failure, its role in heart failure with adrenergic overload has not yet been elucidated. In the present study, we investigated the contribution of autophagy to cardiac failure during adrenergic overload both *in vitro* and *in vivo*. Neonatal rat cardiac myocytes overexpressing GFP-tagged LC3 were prepared and stimulated with the alpha1-adrenergic agonist, phenylephrine (PE), the beta-adrenergic agonist, isoproterenol (ISO), or norepinephrine (NE) in order to track changes in the formation of autophagosomes *in vitro*. All adrenergic stimulators increased cardiac autophagy by stimulating autophagic flux. Blocking autophagy by the knockdown of autophagy-related 5 (ATG5) exacerbated ISO-induced apoptosis and negated the anti-apoptotic effects of PE, which indicated the cardioprotective role of autophagy during adrenergic overload. PE-induced cardiac autophagy was mediated by the PI3-kinase/Akt pathway, but not by MEK/ERK, whereas both pathways mediated the anti-apoptotic effects of PE. Knock down of Akt1 was the most essential among the three Akt family members examined for the induction of cardiac autophagy. The four-week administration of PE kept the high level of cardiac autophagy without heart failure *in vivo*, whereas autophagy levels in a myocardium impaired by four-week persistent administration of ISO or NE were the same with the control state. These present study indicated that cardiac autophagy played a protective role during adrenergic overload and also that the Akt pathway could mediate cardiac autophagy for the anti-apoptotic effects of the alpha-adrenergic pathway.



## 1. Introduction

Heart failure has been one of the leading causes of mortality worldwide in spite of recent advances in medical science. A number of neurohormonal factors are activated in congestive heart failure and positively or negatively contribute to progression of the disease state. Norepinephrine is a major neurotransmitter in the sympathetic nervous system and acts on the heart by binding to the cardiac cell surface adrenergic family of G-protein-coupled receptors, alpha- and beta-adrenergic receptors [1]. Elevations in norepinephrine in plasma have been correlated with the severity and poor prognosis of congestive heart failure [1]. Since adult cardiac muscle cells are terminally differentiated and have almost lost their proliferative capacity, the maintenance of cardiac cell fate is critical for normal cardiac function. We previously reported that activation of the alpha-adrenergic pathway protected cardiac myocytes from apoptosis induced by beta-adrenergic activation [2]. Clinically, therapeutic interventions by beta-adrenergic receptor blockers, but not alpha- adrenergic receptor blockers, were shown to favorably alter the natural history of heart failure [3], which is consistent with our findings and supports cardiac cell fate being a good target as a therapeutic strategy for congestive heart failure.

Autophagy, a highly conserved cellular mechanism for homeostasis from yeast to plants and animals, is an intracellular process involving the bulk degradation and recycling of cytosolic, long-lived proteins and organelles. Autophagy can be rapidly increased in response to the lack of any type of essential nutrient as a cytoprotective mechanism [4,5]. Class 1 phosphoinositide 3-kinase (PI3K), the mammalian target of rapamycin (mTOR), has been shown to regulate these nutrient signals, and inhibitors of mTOR are known to universally induce autophagy in yeast and animals [4]. During this process, targeted cytoplasmic constituents are

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http://dx.doi.org/10.1016/j.bbrc.2014.11.067
0006-291X/

isolated within a double-membrane vesicle known as an autophagosome, which fuses with lysosomes to form acidic single-membrane autolysosomes, in which lysosomal proteases degrade the inner autophagosomal membrane and cargo [5].

Autophagy is also crucial for maintaining baseline heart function by removing abnormal proteins and organelles, especially mitochondria [6]. The conditional deletion of autophagy-related genes 5 (Atg5) in the adult heart was reported to lead to the accumulation of damaged mitochondria and progression of heart failure [7]. We recently reported that the impaired autophagic degradation of damaged mitochondria by cytosolic p53 reduced the cardiac functional reserve in aged hearts [8]. Furthermore, autophagy has been implicated in several cardiac pathologies including ischemia, ischemia–reperfusion, pressure overload, and congestive heart failure [9–12]. Apoptosis, necrosis, and autophagy have simultaneously been observed in the failing human heart [13]. Dying cardiomyocytes in animal models of dilated cardiomyopathy were found to contain several autophagic vacuoles including degraded mitochondria and myelin-like figures, a typical characteristic of autophagy [11]. Cardiac apoptosis is regarded as a key mechanism that contributes to the transition from compensated hypertrophy to a failing heart under persistent hemodynamic stress [14]. Some of the common cardiac pro-apoptotic stressors in heart failure, such as reactive oxygen species and increases in cytosolic-free Ca+, have also been shown to stimulate cardiac autophagy [13]. Signaling pathways associated with Beclin1 and the Bcl-2 family or Atg5 constitute a point of cross-talk between apoptosis and autophagy [15]. However, the functional relationship between apoptosis and autophagy in the failing heart remains controversial [16,17]. In addition, although most recent studies suggested that autophagy may be a cardioprotective response, the pathological role of cardiac autophagy remains unclear [12].

In the present study, we demonstrated that cardiac autophagy increased in the persistent adrenergic stress and acted as a protective response against apoptosis in cardiac myocytes. We also showed that the Akt pathway mediated cardiac autophagy during anti-apoptotic alpha-adrenergic overload.

## 2. Material and methods

### 2.1. Assessment of autophagy

Primary ventricular cardiac myocytes were prepared and transfected with GFP-LC3 as previously described [2]. The cDNA of GFP-LC3 was kindly provided by Dr. T. Yoshimori (Osaka University, Osaka, Japan). The autophagosomes were quantified via fluorescence imaging of GFP-LC3 puncta in cells or tissues using Z-stack images under FV1000 fluorescence microscope (Olympus, Tokyo). To quantify the autophagic flux, cells were treated in the absence or presence of the lysosomotropic alkalinizing agent Cq as previously described [18]. Ultrathin cardiac specimens were examined using transmission EM (Hitachi H-7000, Tokyo) [8]. Proteins were extracted from cultured cardiac myocytes using RIPA buffer supplemented with Protease Inhibitor Cocktail (Santa Cruz, CA), which was subjected to western blotting. The blots were visualized by chemiluminescence, and the signals were analyzed using computer-assisted planimetry with NIH Image J.

### 2.2. In situ nick end-labeling

Terminal deoxynucleotidyl transferase mediated nick-end labeling of fragmented nuclei (TUNEL assay) for the detection of apoptosis was performed according to the manufacturer's instructions (Roche, Basel). The apoptotic index was calculated as previously described [2].

### 2.3. RNA interference

siRNAs were transiently transfected using Lipofectamine RNAiMAX (Invitrogen, CA) according to the product protocol. Gene silencing via RNA interference were purchased from Invitrogen. siRNAs were transiently transfected into myocytes using Lipofectamine RNAiMAX (Invitrogen) according to the product protocol. Sequences of siRNAs were described in Supplemental information.

### 2.4. Mice experiments in vivo

GFP-LC3 transgenic mice (strain GFP-LC3 No. 53, C57BL/6J background) were obtained from the RIKEN BioResource Center. An osmotic minipump (2004 model, Alzet, CA) was implanted subcutaneously into mice, which delivered chronic infusions of PE (15 mg/kg/day), ISO (15 mg/kg/day), or saline for 4 weeks. Hemodynamic assessments were performed using transthoracic echocardiography and cardiac tissue was harvested for histological and western blotting examinations. All animal studies were approved by the Bioethics Committee of Kyoto Prefectural University of Medicine.

### 2.5. Statistical analysis

Values shown are means ± SD. The significance of differences between groups was evaluated by ANOVA followed by Tukey's multiple comparison test. Values of $p < 0.05$ were considered significant.

## 3. Results

### 3.1. Adrenergic overload induced autophagy in cardiac myocytes

We prepared cultured neonatal cardiac myocytes transfected with GFP-LC3. Since the 16-kDa processed isoform of LC3 was recruited from the cytoplasm to autophagosomal membranes, punctate GFP-LC3-labeled structures represented autophagosomes [18]. The transfection efficiency achieved was at least 40–60%, which was confirmed by control transfection with the plasmid expressing pEGFP-C1 (data not shown). Twenty-four hours of stimulation with NE, the alpha1-adrenergic agonist, PE, and the beta-adrenergic agonist, ISO, markedly increased the number of punctate GFP-LC3-labeled autophagosomes in cardiac myocytes (Fig. 1A).

These increases in autophagosomes were enhanced by the treatment with chloroquine, an inhibitor of lysosome–autophagosome fusion, and negated by the administration of the Class III PIK inhibitor, 3-methyladenine (3-MA), which indicated that autophagic flux was stimulated in these cardiac myocytes (Fig. 1B). Electron microscopy also showed that both PE and ISO induced the accumulation of vacuoles containing some high density structures, a typical feature of autophagosomes, in cardiac myocytes (Fig. 1C). These results clearly indicated that adrenergic overload induced cardiac autophagy *in vitro*.

### 3.2. Autophagy preceded apoptosis in cardiac myocytes treated with the beta-adrenergic agonist, isoproterenol

To investigate the contribution of autophagy to apoptosis during adrenergic overload, we contrasted the time course of the number of GFP-LC3-positive cells with that of TUNEL-positive cells in cardiac myocytes stimulated with ISO. The number of GFP-LC3-positive

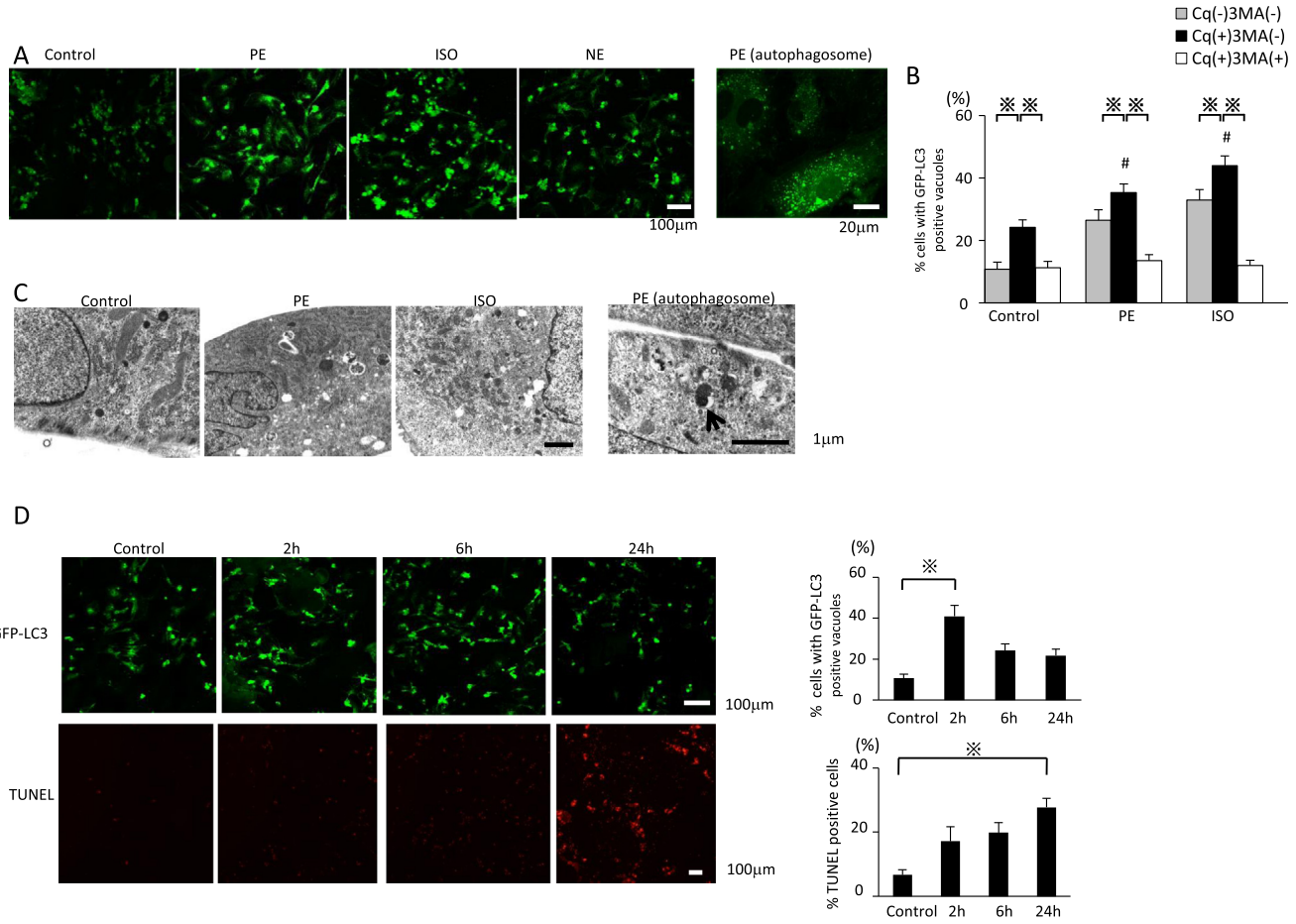


**Fig. 1.** Adrenergic overload induced autophagy in cardiac myocytes. (A) Fluorescent microscopy for GFP-LC3. Cultured neonatal cardiac myocytes overexpressing LC3-GFP were stimulated with the indicated adrenergic agonists for 2 h. (B) Autophagic flux was evaluated by Cq and 3MA in the indicated order. Results are means ± SD of 6 independent experiments. (C) Electron microscopy assessment of cardiac myocytes treated with the indicated adrenergic agonists for 2 h. The arrow head indicates the typical morphology of autophagosomes. Scale bar; 1 μm. (D) Autophagy preceded apoptosis in cardiac myocytes treated with the beta-AR agonist, isoproterenol. Top; representative photographs of cardiac myocytes overexpressing GFP-LC3. Bottom; representative photographs of TUNEL staining. A quantitative analysis of the % TUNEL-positive cells or % cells with GFP-LC3 vacuoles in cardiac myocytes stimulated with ISO for the indicated times. Results are means ± SD of 6 independent experiments. $^{*}p < 0.01$, $^{\#}p < 0.01$ vs control.

cardiac myocytes was increased from 2 h after the stimulation and its withdrawal started after 24 h, whereas the number of TUNEL-positive cardiac myocytes increased at least 24 h after the stimulation. Quantitative analysis also revealed that autophagy preceded apoptosis in cardiac myocytes stimulated with ISO (Fig. 1D).

### 3.3. Autophagy acted as cardioprotection during adrenergic stress

We previously reported that an alpha-adrenergic agonist protected cardiac myocytes from apoptosis [2]. TUNEL staining revealed that a 24-h stimulation with PE significantly inhibited increases in the number of red fluorescent-positive cells induced by ISO in cardiac myocytes (Fig. 2A). PE and ISO had diametrically opposite actions against cardiac apoptosis, whereas both agonists increased cardiac autophagy from the early phase of adrenergic overload (Fig. 1A). In order to clarify the role of autophagy in cardiac cellular fate during adrenergic overload, we prepared cardiac myocytes in which autophagy was blocked using RNAi for autophagy-related protein 5 (ATG5), a key regulator of autophagy (Fig. 2B). Blocking autophagy negated the inhibitory effects of PE on the increase in % TUNEL-positive cardiac myocytes, which indicated that autophagy acted as a cardioprotective response. The blockade of autophagy slightly increased the % TUNEL-positive cells in cardiomyocytes stimulated with ISO, which also suggested the anti-apoptotic role of autophagy (Fig. 2C). These results indicated that autophagy acted as a protective response against apoptosis during adrenergic overload in cardiac myocytes.

### 3.4. Akt and ERK pathways differentially regulated autophagy during alpha-adrenergic agonist induced-cardioprotection

The phosphorylation of the PI3K-Akt and MEK-1/2-ERK-1/2 kinase cascades has been identified as a survival pathway in cardiac myocytes [14]. TUNEL staining revealed that both the PI3 kinase inhibitor, wortmannin (WT) and ERK-specific inhibitor, PD98059 (PD) significantly negated the anti-apoptotic effects of PE (Fig. 3A), which indicated that both pathways mediated these anti-apoptotic effects. To investigate the autophagy pathway by which the alpha-adrenergic agonist induced cardioprotection, we administered WT or PD to cardiac myocytes which was transfected with GFP-LC3 and stimulated with PE (Fig. 3B). PI3K-Akt activates the mammalian target of rapamycin (mTOR), the inhibition of which has been shown to induce autophagy in several kinds of cells. Administration of PD had no effect on the number of punctate GFP-LC3 labeled autophagosomes in cardiac myocytes. However, WT significantly negated the effect of PE, which indicated that Akt mediated the induction of autophagy stimulated by PE. This result was distinct from the universal pathway in which PI3/Akt

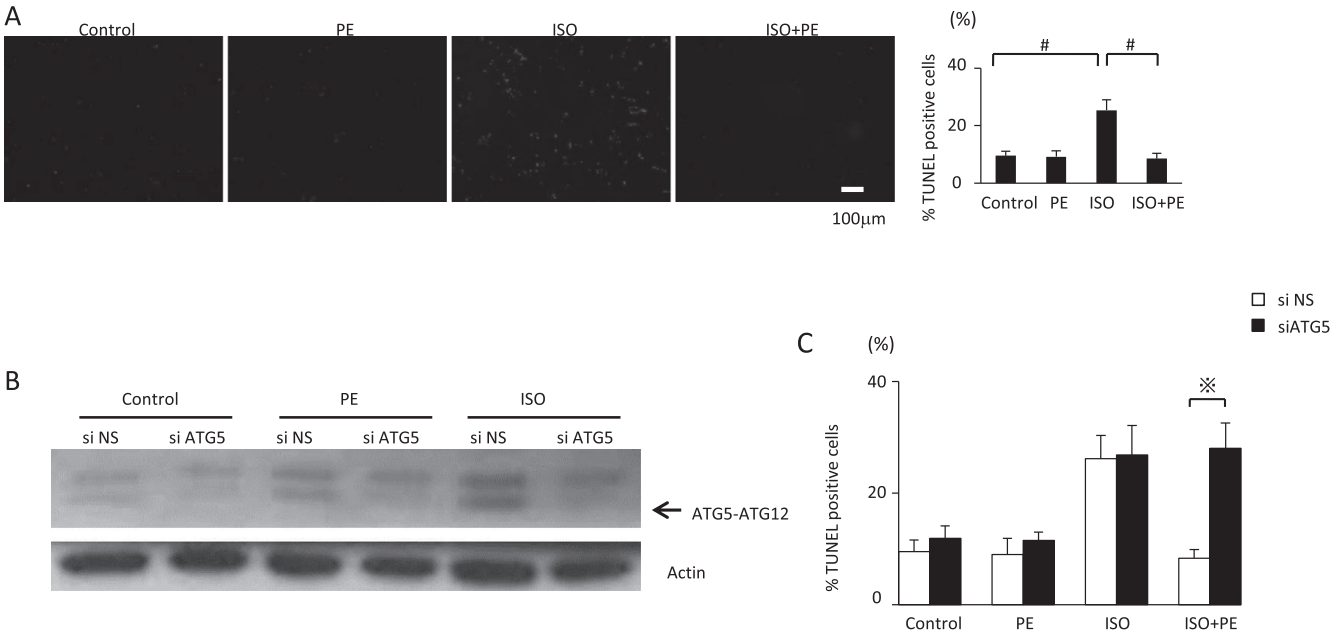


**Fig. 2.** Autophagy acted as cardioprotection during adrenergic overload. (A) Representative photographs for TUNEL staining of cardiac myocytes stimulated with the indicated reagents for 24 h. PE inhibited ISO-induced apoptosis in cardiac myocytes. RNAi for ATG5 was effective in cardiac myocytes. (B) Representative photographs of western blots. (C) The inhibition of autophagy by siATG5 increased the number of apoptotic cells in cardiac myocytes. Results are means ± SD of 6 independent experiments. $^{*}p < 0.01$, $^{\#}p < 0.05$.

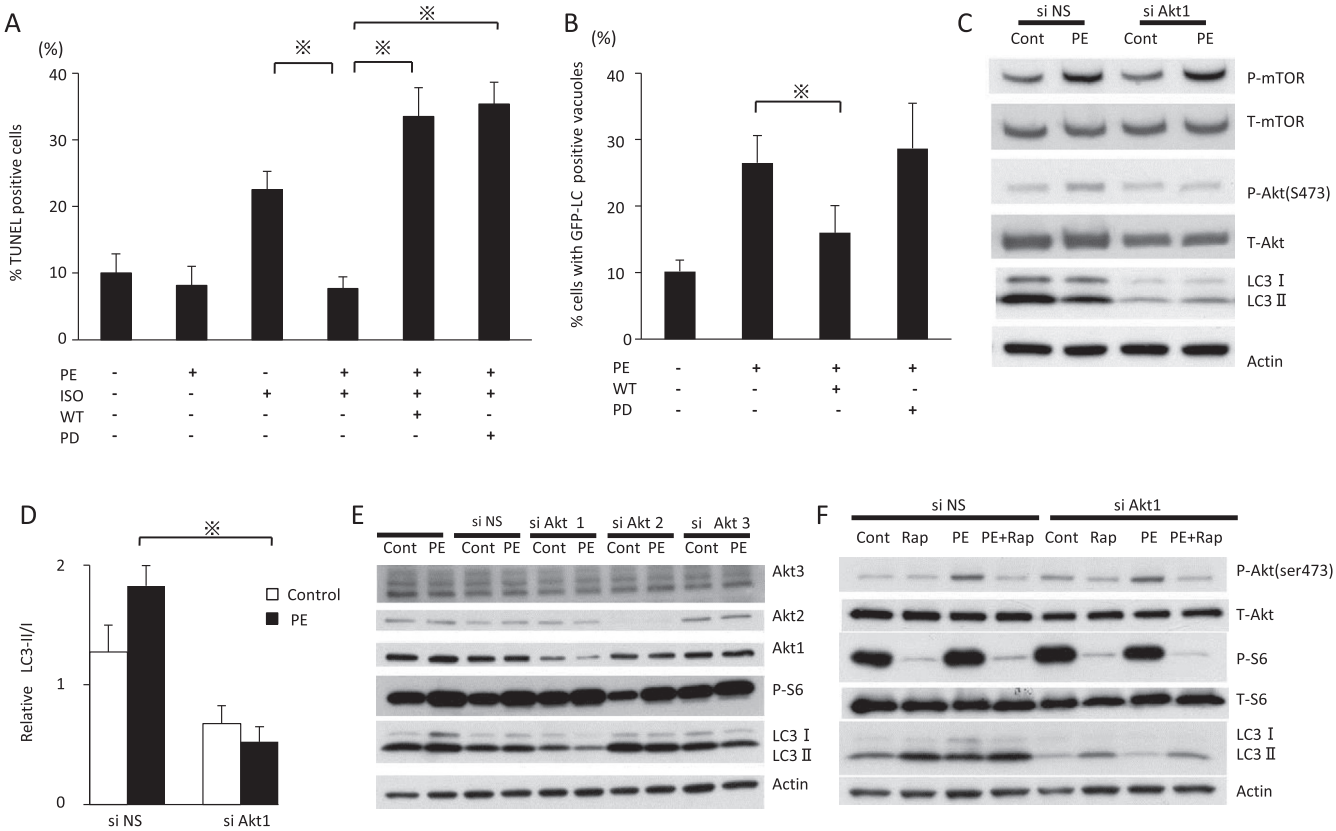


**Fig. 3.** The Akt1 pathway mediated alpha-adrenergic agonist induced-cardiac autophagy. (A) % TUNEL-positive cells in cardiac myocytes stimulated in the indicated order. Results are means ± SD of 6 independent experiments. $^{*}p < 0.05$. (B) Quantitative analysis of the % cells with GFP-LC3 vacuoles in cardiac myocytes with indicated orders. Results are means ± SD of 6 independent experiments. $^{*}p < 0.05$. (C) Representative photographs of western blots of cardiac myocytes with knockdown of Akt or NS. $n = 4$. (D) Quantitative analysis of LC3 in (C). Results are means ± SD of 4 independent experiments. $^{*}p < 0.01$. (E) Knockdown of Akt1 suppressed LC3 in alpha AR-stimulated cardiac myocytes. Representative photographs of western blots. $n = 4$. (F) Knockdown of Akt1 inhibited cardiac autophagy, which was mediated by an mTOR-independent pathway. Representative photographs of western blots. $n = 4$.

was reported to inhibit autophagy via the mTOR axis in several cell types [4]. To confirm the effect of Akt pathway on PE-mediated cardiac autophagy, we prepared cardiac myocytes in which the PI3K-Akt was inhibited using the RNAi method (Fig. 3C and D). Western blotting showed that the inhibition of Akt1 significantly repressed the expression of LC3 II/I, which supported the idea that Akt inhibited, but did not induce autophagy in cardiac myocytes during alpha-adrenergic overload.

### 3.5. The Akt1 pathway contributed to alpha-adrenergic agonist induced-cardiac autophagy

The Akt family has 3 main isoforms: Akt1, Akt2, and Akt3 [19]. All these isoforms are expressed in the myocardium and act differentially depending on the kinds of stimuli. To identify the Akt isoform that was mainly responsible for inducing cardiac autophagy, cardiac myocytes transfected with RNAi for each isoform were prepared and stimulated with PE. Western blotting showed that only RNAi for Akt1 inhibited the expression of LC3 II/I in cardiac myocytes stimulated with PE (Fig. 3E). The inhibition of mTOR by rapamycin induced autophagy in control cardiac myocytes with no adrenergic stimulation (Fig. 3F), which indicated that an ordinary mTOR-dependent autophagy pathway also exist in cardiac myocytes. On the other hand, there were no changes in the expression of the S6 kinase level in PE-stimulated myocytes (Fig. 3E and F), which confirmed the existence of an mTOR-independent pathway under the autophagic induction mechanism concerned with alpha-adrenergic overload.

### 3.6. The four-week administration of an alpha-adrenergic agonist increased cardiac autophagy with no sign of heart failure in vivo

Single injection with PE, ISO, and NE into mice *in vivo* increased the autophagic levels in myocardium at 2 h after the stimulation (Suppl. Fig. 1), which was consistent with our previous reports with LPS or Chloroquine [18]. To assess the effects of the chronic overload in the sympathetic nervous system as like failing heart state, a mouse model with osmotic pumps that enabled persistent adrenergic stimulation was prepared (Fig. 4A). A four-week overload of ISO or NE significantly induced fibrosis in the myocardium, which was confirmed by the results obtained with Masson Trichrome staining (Fig. 4B). Echocardiography revealed that chronic overload with ISO induced left ventricular (LV) hypertrophy, a typical sign of the early phase of heart failure on hemodynamic overload. Furthermore, the four-week administration of NE caused severe heart failure with apparent cardiac systolic dysfunction and LV thinning (Fig. 4C). In contrast to the cardiac impairments observed in mice administrated ISO or NE, the four-week administration of PE did not induce any apparent signs of heart failure including myocardial fibrosis, LV hypertrophy or thinning, or apparent cardiac dysfunction (Fig. 4B and, C).

We then examined the levels of cardiac autophagy during persistent adrenergic overload *in vivo* using GFP-LC3 mice. In contrast to control mice, ISO and NE appeared to slightly increase the number of basement green fluorescent, but not punctate GFP-LC3-labeled autophagosomes in the myocardium. However, the chronic administration of PE increased the number of green dots in the myocardium (Fig. 4D), which is a typical feature of autophagy [18]. A quantitative analysis of western blotting also showed that the four-week administration of PE significantly increased the LCII/I ratio in the myocardium (Fig. 4E), which indicated an increase in cardiac autophagy. These results indicated that persistent alpha-adrenergic overload increased cardiac autophagy with no signs of heart failure, thereby suggesting the cardioprotective role of autophagy mediated through the alpha-adrenergic pathway during chronic overload in the sympathetic nervous system *in vivo*.

## 4. Discussion

The present study demonstrated that cardiac autophagy increased in the early phase of adrenergic overload and acted as a cytoprotective response against apoptosis in cardiac myocytes. The results of the *in vivo* experiments also suggested that the increase in autophagy following persistent alpha-adrenergic stimulation may have inhibited the progression of heart failure. We also showed that the Akt pathway mediated cardiac autophagy during anti-apoptotic alpha-adrenergic overload, indicating the existence of an mTOR-independent autophagy pathway in cardiac myocytes.

We showed that all adrenergic stimulators, including anti-apoptotic alpha- and pro-apoptotic beta-adrenergic agonists, increased cardiac autophagy in the early phase of the administration (Fig. 1). Blocking autophagy using RNAi for Atg5 negated the anti-apoptotic effects of PE, which indicated that autophagy acted as a cardioprotective response (Fig. 2). Blocking autophagy slightly increased apoptosis in cardiomyocytes stimulated with ISO, which also suggested the cardioprotective effects of autophagy. We showed that the activation of autophagy clearly preceded the induction of apoptosis in cardiac myocytes stimulated with ISO (Fig. 1D). The upregulation of autophagy may be compensatory by removing damaged mitochondria and proteins in the early stages of myocardial stress. However, once the balance in the cellular fate leans to death, these cells may undergo apoptosis and die. A previous study using the $Atg5^{flox/flox}$; MLC2v-$Cre^{+}$ mouse demonstrated that autophagy inhibited beta-adrenergic stress-induced cardiac cell death [7]. Another study reported that the administration of isoproterenol inhibited cardiac autophagy for 10 min [20]. These findings, including the results of the present study, indicate that autophagy competed with pro-apoptotic actions and acted as a cellular protective response in cardiac myocytes under beta-adrenergic stress. The *in vivo* results of the present study showed that the persistent activation of autophagy may inhibit the progression of heart failure (Fig. 4). In contrast to our results, the inhibition of massive autophagy by the downregulation of Beclin 1 was previously reported to attenuate cardiac injury induced by ischemia–reperfusion [10]. Further understandings focusing the amount and the timing of controlling cardiac autophagy during persistent cardiac stress should be elucidated.

The alpha-adrenergic pathway is a potent hypertrophic factor that is mediated by though the G-alpha-linked receptor and controls downstream signaling pathways including ERK MAP kinase and PI3/Akt signaling. We previously reported the ERK- and Akt-mediated anti-apoptotic actions of alpha-adrenergic activation [14]. However, the ERK and Akt pathways had opposite effects on the regulation of cardiac autophagy during alpha-adrenergic overload in the present study (Fig. 3). Although PI3/Akt/mTOR-dependent autophagy pathway was well known, our experiments with the inhibition of Akt suggested the existence of another signaling pathway for autophagy. On the other hand, the inhibition of mTOR by rapamycin induced autophagy in control cardiac myocytes with no adrenergic stimulation (Fig. 3F), which indicated that an ordinary mTOR-dependent autophagy pathway may also exist in cardiac myocytes, similar to other cell types. Recent studies have reported that some amino acid signals and small molecule enhancers of cytostatic effects induced autophagy through signal transduction pathways other than the mTOR axis [21,22]. Thus, the co-existence of mTOR independent- and dependent-autophagy pathways may be possible in cardiac myocytes. The results of the present study also indicate that an mTOR-independent Akt pathway may be dominant for cardiac autophagy during adrenergic overload.

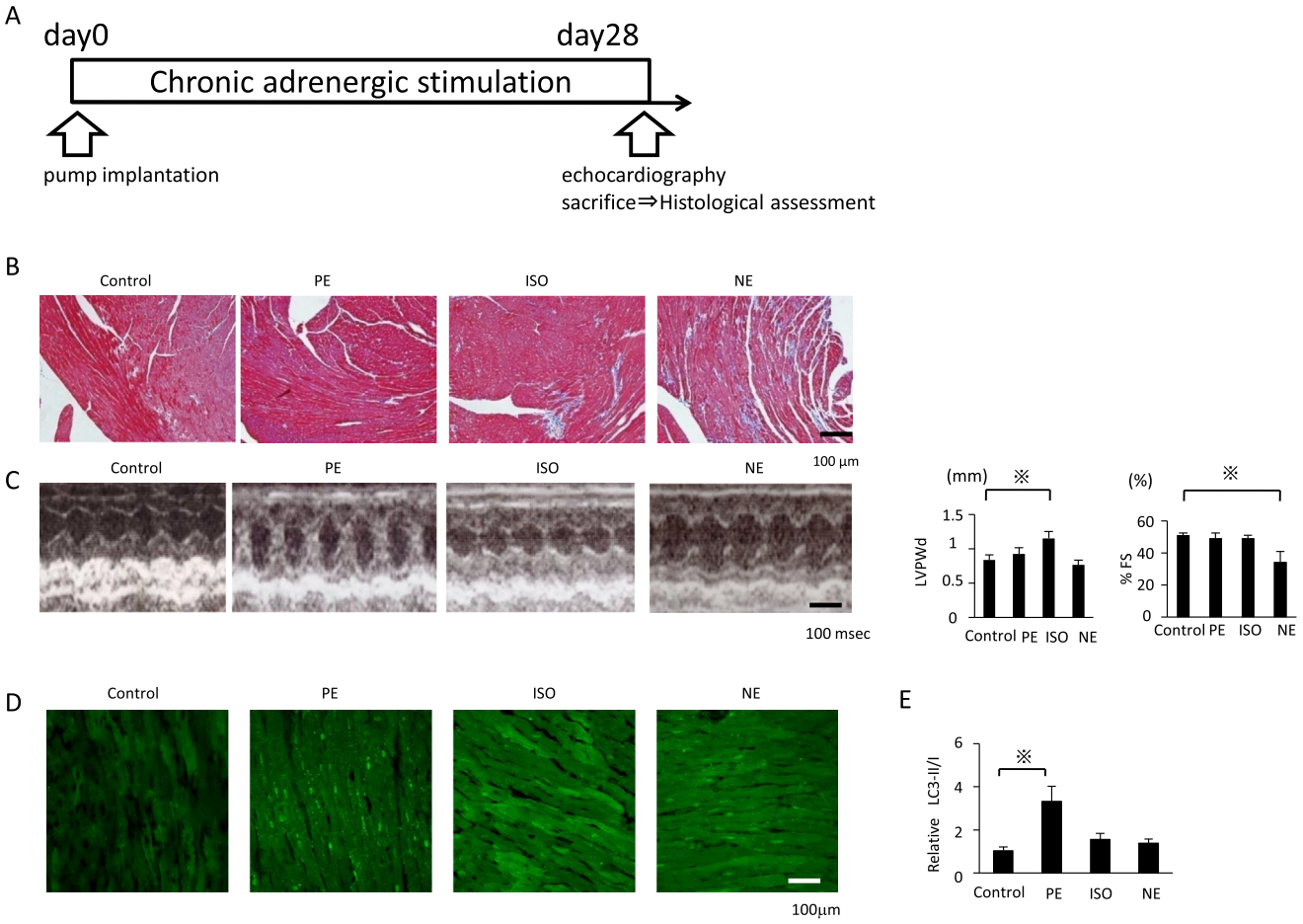


**Fig. 4.** The four-week administration of an alpha-adrenergic agonist increased cardiac autophagy with no sign of heart failure *in vivo*. (A) Experimental protocol of the *in vivo* study with mice. (B) Representative photographs of the histological assessments. Masson Trichrome staining of chronic models of adrenergic overload. Both the four-week administration of ISO and NE induced fibrotic changes in the heart, in spite of the absence of changes by PE. (C) The results of echocardiograms of mouse hearts. β-, but not α-adrenergic overload for 4 weeks induced cardiac remodeling with left ventricular hypertrophy. Results are means ± SD of 6 independent experiments. $^{*}p < 0.05$. (D) Chronic overload with an alpha-AR agonist stimulated cardiac autophagy *in vivo*. Representative fluorescent photographs of the heart with GFP-LC3 transgenic mice stimulated in the indicated order. Only PE increased the number of GFP-LC3-positive dots in the myocardium. E. Quantitative analysis of relative LC3-II/I by western blotting. Results are means ± SD of 4 independent experiments. $^{*}p < 0.05$.

The alpha-adrenergic agonist that activated cardiac autophagy in the present study is well known to induce cardiac hypertrophy. In hemodynamic overload, heart failure is preceded by cardiac remodeling with hypertrophy [23]. The activation of autophagy by rapamycin has been reported to prevent cardiac hypertrophy induced by thyroid hormone treatments [24]. Pressure overload due to transverse aortic constriction also revealed that autophagic activity was reduced during hypertrophic responses [7]. The distinct actions of the alpha-adrenergic pathway from other hypertrophic stimulators can be elucidated by two differential regulations of the autophagy pathway with ERK and Akt, as demonstrated in the present study (Fig. 3). The role of autophagy during cardiac hypertrophy preceding heart failure remains to be elucidated.

Although all three Akt family members, Akt1, Akt2, and Akt3, are expressed in the myocardium, Akt1 and Akt2 are dominant. Previous studies indicated that Akt1 was essential for mediating protective responses to an ischemia-preconditioning stimulation [19], whereas Akt2 was required to maintain normal cardiac glucose metabolism [25]. Akt1 has also been reported to directly inhibit autophagy in some cancer cells [26]. The present study showed that Akt1 was the most important isoform for activating autophagy in PE-induced cardioprotection. A better understanding of Akt1 signaling would support the results of the present study with the mTOR-independent autophagy pathway in the progression of heart failure.

In conclusion, we demonstrated that cardiac autophagy increased during adrenergic overload, and acted as a cardioprotective response in the progression of heart failure. The mTOR-independent Akt pathway was dominant for cardiac autophagy in alpha-adrenergic overload. Thus, further investigations on the mTOR independent pathway for cardiac autophagy will lead to a better understanding of the progression of heart failure.

## Acknowledgments

This work was supported in part by a Grant-in-Aid for scientific research from the Ministry of Education, Science, and Culture of Japan (to E. Iwai-Kanai and S. Matoba). We thank Dr. T. Yoshimori (Osaka University, Osaka, Japan) for kindly providing the GFP-LC3 DNA and Dr. R.A. Gottlieb (San Diego State University) for scientific advice.

## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.11.067.

## References


[1] T. Vago, M. Bevilacqua, G. Norbiato, et al., Identification of alpha 1-adrenergic receptors on sarcolemma from normal subjects and patients with idiopathic dilated cardiomyopathy: characteristics and linkage to GTP-binding protein, Circ. Res. 64 (1989) 474–481.

[2] E. Iwai-Kanai, K. Hasegawa, M. Araki, et al., α- and β-adrenergic pathways differentially regulate cell type-specific apoptosis in rat cardiac myocytes, Circulation 100 (1999) 305–311.

[3] S.A. Hunt, W.T. Abraham, M.H. Chin, et al., Focused update incorporated into the ACC/AHA 2005 guidelines for the diagnosis and management of heart failure in adults. A report of the American College of Cardiology Foundation/American Heart Association Task Force on Practice Guidelines, Circulation 119 (2009) (2009) e391–e479.

[4] B. Ravikumar, C. Vacher, Z. Berger, et al., Inhibition of mTOR induces autophagy and reduces toxicity of polyglutamine expansions in fly and mouse models of Huntington disease, Nat. Genet. 36 (2004) 585–595.

[5] N. Mizushima, Autophagy: process and function, Genes Dev. 21 (2007) 2861–2873.

[6] R.A. Gottlieb, A.B. Gustafsson, Mitochondrial turnover in the heart, Biochem. Biophys. Acta 2011 (1813) 1295–1301.

[7] A. Nakai, O. Yamaguchi, T. Takeda, et al., The role of autophagy in cardiomyocytes in the basal state and in response to hemodynamic stress, Nat. Med. 13 (2007) 619–624.

[8] A. Hoshino, Y. Mita, Y. Okawa, et al., Cytosolic p53 inhibits Parkin-mediated mitophagy and promotes mitochondrial dysfunction in the mouse heart, Nat. Commun. 4 (2013) 2208.

[9] A. Hamacher-Brady, N.R. Brady, R.A. Gottlieb, Enhancing macroautophagy protects against ischemia/reperfusion injury in cardiac myocytes, J. Biol. Chem. 281 (2006) 29776–29787.

[10] Y. Matsui, H. Takagi, X. Qu, et al., Distinct roles of autophagy in the heart during ischemia and reperfusion: roles of AMP-activated protein kinase and Beclin 1 in mediating autophagy, Circ. Res. 100 (2007) 914–922.

[11] Y. Tanaka, G. Guhde, A. Suter, et al., Accumulation of autophagic vacuoles and cardiomyopathy in LAMP-2-deficient mice, Nature 406 (2000) 902–906.

[12] H. Zhu, P. Tannous, J.L. Johnstone, et al., Cardiac autophagy is a maladaptive response to hemodynamic stress, J. Clin. Invest. 117 (2007) 1782–1793.

[13] S. Kostin, L. Pool, A. Elsasser, et al., Myocytes die by multiple mechanisms in failing human hearts, Circ. Res. 92 (2003) 715–724.

[14] E. Iwai-Kanai, K. Hasegawa, Intracellular signaling pathways for norepinephrine- and endothelin-1-mediated regulation of myocardial cell apoptosis, Mol. Cell. Biochem. 59 (2004) 163–168.

[15] K. Nishida, S. Kyoi, O. Yamaguchi, et al., The role of autophagy in the heart, Cell Death Differ. 16 (2009) 31–38.

[16] S. Shimizu, T. Kanaseki, N. Mizushima, et al., Role of Bcl-2 family proteins in a non-apoptotic programmed cell death dependent on autophagy genes, Nat. Cell Biol. 6 (2004) 1221–1228.

[17] M.C. Maiuri, E. Zalckvar, A. Kimchi, et al., Self-eating and self-killing: crosstalk between autophagy and apoptosis, Nat. Rev. Mol. Cell Biol. 8 (2007) 741–752.

[18] E. Iwai-Kanai, H. Yuan, C. Huang, et al., A method to measure cardiac autophagic flux in vivo, Autophagy 4 (2008) 322–329.

[19] S.P. Kunuthur, M.M. Mocanu, B.A. Hemmings, et al., The Akt1 isoform is an essential mediator of ischaemic preconditioning, J. Cell Mol. Med. 16 (2012) 1739–1749.

[20] U. Pfeifer, J. Four, W. Wilhelm, et al., Short-term inhibition of cardiac cellular autophagy by isoproterenol, J. Mol. Cell. Cardiol. 19 (1987) 1179–1184.

[21] T. Kanazawa, I. Taneike, R. Akaishi, et al., Amino acids and insulin control autophagic proteolysis through different signaling pathways in relation to mTOR in isolated rat hepatocytes, J. Biol. Chem. 279 (2004) 8452–8459.

[22] S. Sarkar, E.O. Perlstein, S. Imarisio, et al., Small molecules enhance autophagy and reduce toxicity in Huntington's disease models, Nat. Chem. Biol. 3 (2007) 331–338.

[23] M. Inoko, Y. Kihara, I. Morii, et al., Transition from compensatory hypertrophy to dilated, failing left ventricles in Dahl salt-sensitive rats, Am. J. Physiol. 267 (1994) H2471–H2482.

[24] J.A. Kuzman, T.D. O'Connell, A.M. Gerdes, Rapamycin prevents thyroid hormone-induced cardiac hypertrophy, Endocrinology 148 (2007) 3477–3484.

[25] B. DeBosch, N. Sambandam, C. Weinheimer, et al., AKT2 regulates cardiac metabolism and cardiomyocyte survival, J. Biol. Chem. 281 (2006) 32841–32851.

[26] H. Takeuchi, Y. Kondo, K. Fujiwara, et al., Synergistic augmentation of rapamycin-induced autophagy in malignant glioma cells by phosphatidylinositol 3-kinase/protein kinase B inhibitors, Cancer Res. 65 (2005) 3336–3346.