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Autophagy-mediated degradation is necessary for regression of cardiac hypertrophy during ventricular unloading

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ABSTRACT

Cardiac hypertrophy occurs in response to a variety of stresses as a compensatory mechanism to maintain cardiac output and normalize wall stress. Prevention or regression of cardiac hypertrophy can be a major therapeutic target. Although regression of cardiac hypertrophy occurs after control of etiological factors, the molecular mechanisms remain to be clarified. In the present study, we investigated the role of autophagy in regression of cardiac hypertrophy. Wild-type mice showed cardiac hypertrophy after continuous infusion of angiotensin II for 14 days using osmotic minipumps, and regression of cardiac hypertrophy was observed 7 days after removal of the minipumps. Autophagy was induced during regression of cardiac hypertrophy, as evidenced by an increase in microtubule-associated protein 1 light chain 3 (LC3)-II protein level. Then, we subjected cardiac-specific Atg5-deficient (CKO) and control mice (CTL) to angiotensin II infusion for 14 days. CKO and CTL developed cardiac hypertrophy to a similar degree without contractile dysfunction. Seven days after removal of the minipumps, CKO showed significantly less regression of cardiac hypertrophy compared with CTL. Regression of pressure overload-induced cardiac hypertrophy after unloading was also attenuated in CKO. These results suggest that autophagy is necessary for regression of cardiac hypertrophy during unloading of neurohumoral and hemodynamic stress.

1. Introduction

Cardiac hypertrophy occurs in patients with systemic hypertension and valvular diseases such as aortic stenosis. Cardiac hypertrophy is an early milestone during the clinical course of hemodynamic stress-induced heart failure and by itself is an independent risk factor for subsequent cardiac morbidity and mortality [1]. Therefore, cardiac hypertrophy is an early therapeutic target.

The heart undergoes a reduction in size in response to various stresses including malnutrition and decreased hemodynamic load. Various antihypertensive agents, aortic valve replacement and left ventricular assist device support reduce left ventricular (LV) hypertrophy [2–4]. Regression of LV hypertrophy in response to antihypertensive treatment significantly improves cardiovascular disease outcome and long-term prognosis [2]. Regression of hypertrophy is a major therapeutic target to treat patients with cardiac hypertrophy. However, the effect of antihypertensive treatment on cardiac hypertrophy is not satisfactory. Thus, it is necessary to identify the cellular and molecular mechanisms underlying regression of cardiac hypertrophy in order to develop novel and effective therapeutics to treat patients with cardiac hypertrophy.

The cardiac mass is determined by the balance between protein synthesis and degradation. The major pathways for protein degradation are the ubiquitin–proteasome system and autophagy [5]. In the ubiquitin–proteasome system, the signal for protein degradation is the covalent attachment of ubiquitin to the target protein, which is then degraded by the 26S proteasome. During autophagy,

Abbreviations: CKO, cardiac-specific Atg5-deficient mice; CTL, control mice; TAC, thoracic transverse aortic constriction; HW, heart weight; LV, left ventricle; LVW, left ventricle weight; IVSd, end-diastolic interventricular septal thickness; LVPWd, end-diastolic left ventricular posterior wall thickness; LVIDd, end-diastolic left ventricular internal dimension; LVIDs, end-systolic left ventricular internal dimension; FS, fractional shortening; MLC2v, myosin light chain 2v; LC3, microtubule-associated protein 1 light chain 3.

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an isolation membrane sequesters cytoplasmic proteins and organelles, to form the autophagosome [6]. The autophagosome fuses with the lysosome to become an autolysosome and degrade the materials contained within it. Autophagy plays an important role in various cardiovascular diseases including hypertrophy, heart failure and ischemic heart diseases. We have previously reported that the deletion of Atg5, an essential molecule for autophagosome formation, in mouse cardiomyocytes in adulthood results in an increase in cardiomyocyte size [7]. Recently, it has been reported that autophagy is activated during regression of cardiac hypertrophy in mice after unloading of pressure overload [8]. Thus, we hypothesized that autophagy is involved in regression of cardiac hypertrophy during unloading of the heart.

In this study, we used angiotensin II infusion by the minipumps followed by the removal of the minipumps or thoracic transverse aortic constriction followed by de-constriction as a model to study the molecular mechanisms underlying regression of cardiac hypertrophy. Using cardiac specific Atg5-deficient mice, we showed that autophagy is necessary for regression of angiotensin II- or pressure overload-induced cardiac hypertrophy. Thus, autophagy may be a therapeutic target to treat patients with cardiac hypertrophy.

2. Materials and methods

2.1. Mice

We crossed $Atg5^{flox/flox}$ mice [9] with mice expressing the *Cre* recombinase under the control of the myosin light chain 2v (MLC2v) promoter [10], to obtain $Atg5^{flox/flox}$;$MLC2vCre^{+/-}$ (CKO) mice and $Atg5^{flox/+}$;$MLC2vCre^{-/-}$ (CTL) mice. $Atg5^{flox/+}$;$MLC2vCre^{-/-}$ (CTL) mice were used as controls. This study was carried out under the supervision of the Animal Research Committee of Osaka University and in accordance with the Japanese Act on Welfare and Management of Animals (No. 105).

2.2. In vivo assessment of regression of cardiac hypertrophy

We implanted subcutaneous osmotic minipumps (Alzet, model 1002) to administer angiotensin II (Calbiochem) or saline to 8 to 11-week-old male mice for 2 weeks, and then removed the minipumps. For the pressure-overloaded model, we performed thoracic transverse aortic constriction (TAC) to induce cardiac hypertrophy as previously described [7]. We subjected 8 to 11-week-old male mice to mild TAC (mTAC) using a 23-gauge needle for aortic constriction. The suture at the transverse aorta was removed 10 days after mTAC (deTAC). We performed echocardiography on unsedated mice and imaged the heart in the two-dimensional parasternal short-axis view, and an M-mode echocardiogram of the midventricle was recorded at the level of the papillary muscles [7]. Fractional shortening (FS) and LV mass index was calculated as $100 \times$ (end-diastolic LV internal dimension (LVIDd) – end-systolic LV internal dimension (LVIDs))/LVIDd and $1.05 \times$ [(LVIDd + end-diastolic interventricular septal thickness (IVSd) + end-diastolic LV posterior wall thickness (LVPWd))^3 – (LVIDd)^3], respectively. Non-invasive measurements of blood pressure were performed on mice anaesthetized with 2.5% avertin using a blood pressure monitor for rats and mice Model MK-2000 (Muromachi Kikai), according to the manufacturer's instructions.

2.3. In vivo assessment of protein synthesis

We administered ^{14}C-labeled leucine (Moravek) by intraperitoneal injection at a dose of 0.1 mCi/kg of body weight 30 min before sacrifice. Whole hearts were lysed in 1 ml of Soluene-350 (Perkin Elmer), and added to 15 ml of Ultima Gold (Perkin Elmer), and the mixture was examined by a liquid scintillation analyzer.

2.4. Western blot analysis and proteasome activity analysis

Western blots were developed with the ECL Plus kit or ECL Advance kit (Amersham Biosciences Corp.). The quantification of signals was performed by densitometry of scanned autoradiographs with the aid of Image J (ver.1.44p). The following antibodies were used for the immunoblot analysis: rabbit polyclonal LC3B-specific antibodies, rabbit polyclonal phospho-S6 ribosomal protein (Ser240/244)-specific antibodies, and rabbit monoclonal S6 ribosomal protein (5G10)-specific antibody (Cell Signaling Technology), mouse monoclonal GAPDH-specific antibody (Abcam), rabbit polyclonal ubiquitin-specific antibody (DakoCytomation), and donkey anti rabbit IgG antibody (GE healthcare).

We evaluated proteasome activity in homogenates of hearts using the 20S Proteasome Activity Assay Kit (Chemicon).

2.5. Statistical analysis

Results are shown as the mean ± S.E.M. Paired data were evaluated by Student's *t*-test. A one-way analysis of variance (ANOVA) with Bonferroni post hoc test was used for multiple comparisons. A value of $p < 0.05$ was considered statistically significant.

3. Results

3.1. Establishment of a model to study the regression of cardiac hypertrophy

We implanted subcutaneous osmotic minipumps to administer angiotensin II to wild-type C57B6/J mice for 2 weeks, and then ventricular unloading was introduced by removal of the minipumps. Infusion of 800 ng/kg of body weight/min of angiotensin II for 2 weeks resulted in significant increases in the ratios of heart weight (HW)-to-tibia length and LV weight (LVW)-to-tibia length compared to those in the saline-infused group, suggesting that angiotensin II induced LV hypertrophy (Fig. 1A). However, infusion of 1000 ng/kg of body weight/min of angiotensin II did not induce a significant increase in the ratio of HW-to-tibia length, but a decrease in body weight (data not shown). Therefore, in the following experiments, we infused 800 ng/kg of body weight/min of angiotensin II to induce cardiac hypertrophy. Both 3 and 7 days after removal of the minipumps, significant decreases in the ratios of HW-to-tibia length and LVW-to-tibia length were observed compared to those before removal of the minipumps (Fig. 1B). Thus, discontinuation of angiotensin II infusion can induce regression of LV hypertrophy.

3.2. Autophagy is activated during regression of angiotensin II-induced cardiac hypertrophy

We examined autophagic activity during regression of LV hypertrophy. The conversion of LC3-I to LC3-II is an essential step for autophagosome formation [11]. The protein level of LC3-II in tissue homogenates prepared from angiotensin II-infused mouse hearts 6 h after removal of the minipumps was significantly higher than that from saline-infused hearts (Fig. 2A). This suggests that the level of autophagy may increase in the heart during regression of LV hypertrophy. To assess the autophagic flux, we administered a lysosomal inhibitor, bafilomycin A1, by intraperitoneal injection at a dose of 3 μmol/kg of body weight 30 min before sacrifice. Treatment with bafilomycin A1 led to an increase in the protein levels of LC3-II in angiotensin II- and saline-infused hearts com-

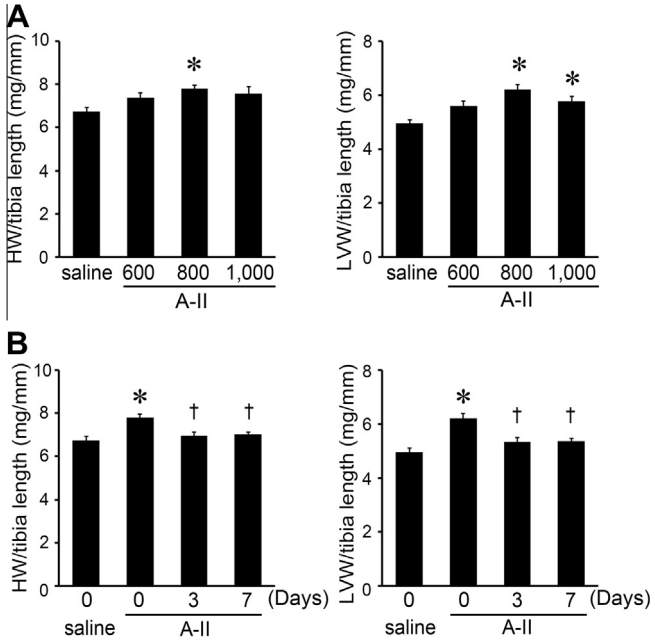

Fig. 1. Regression of angiotensin II-induced cardiac hypertrophy in wild-type C57B6/J mice. (A) Cardiac hypertrophic responses 2 weeks after angiotensin II infusion using minipumps. Each group was treated with saline, 600, 800, or 1,000 ng/kg of body weight/min of angiotensin II for 2 weeks. (B) Regression of cardiac hypertrophy after removal of the minipumps. Days indicate the duration after removal. Day 0 indicates that the parameters are assessed before removal of the minipumps. A-II, angiotensin II. Values represent the mean ± S.E.M. of data from 9 to 11 mice in each group. $^{*}p < 0.05$ versus saline-treated group, $^{\dagger}p < 0.05$ versus angiotensin II-treated group before removal of the minipumps (Day 0).

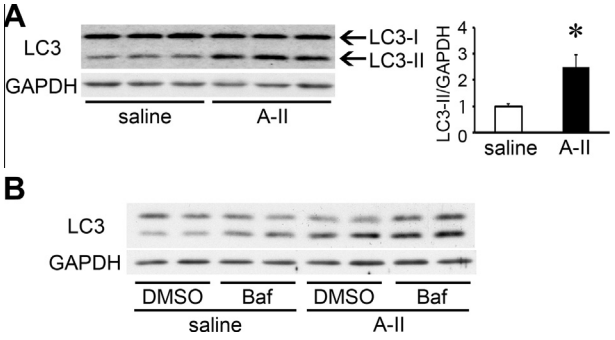

Fig. 2. Autophagic activity during regression of cardiac hypertrophy induced by angiotensin II in wild-type C57B6/J mice. (A) Western blot analysis of LC3 in the heart 6 h after removal of the minipumps. Right panel represents densitometric analysis of the ratio of LC3-II to GAPDH. (B) Western blot analysis of LC3 in the heart injected with bafilomycin A1 30 min before sacrifice. A-II, angiotensin II; Baf, bafilomycin A1. Values represent the mean ± S.E.M. of data from 6 to 9 mice in each group. $^{*}p < 0.05$ versus saline-treated group.

pared to those in corresponding control hearts (Fig. 2B), indicating that autophagic flux was accelerated during regression of LV hypertrophy.

3.3. Autophagy is not essential for angiotensin II-induced cardiac hypertrophy

To examine the role of up-regulation of autophagy during regression of LV hypertrophy, we employed cardiac-specific Atg5-deficient mice (CKO) in this study [7]. The CKO mice showed no differences in physiological and echocardiographic parameters compared to those in CTL at basal level (data not shown), as we previously reported [7]. Two weeks after administration of angiotensin II, there was no significant difference in blood pressure among angiotensin II- or saline-administered CKO and CTL mice (Table S1). Angiotensin II-administrated CKO and CTL mice exhibited significant increases in IVSd, LVPWd, FS, and LV mass index and a significant decrease in LVIDs, compared to those in saline-infused mice (Fig. 3A, Table S1). LVIDd showed no significant difference among 4 groups. The ratios of HW-to-tibia length and LVW-to-tibia length were increased in angiotensin II-administrated CKO and CTL mice, compared to saline-infused mice (Fig. 3B, Table S1). Lung and liver weight did not change after angiotensin II infusion (data not shown). These data indicate that angiotensin II induced concentric LV hypertrophy without cardiac dysfunction both in CTL and CKO mice. The extent of these changes in echocardiographic and physiological parameters induced by the administration of angiotensin II was not significantly different between CTL and CKO groups. Angiotensin II induced an increase in cardiomyocyte cross-sectional area in CKO and CTL hearts, but the extent of the increase was similar between CKO and CTL (Fig. 3C). These results indicate that autophagy is not essential for angiotensin II-induced cardiac hypertrophy. The ratio of intramuscular fibrotic area was significantly increased by angiotensin II infusion, but there was no significant difference in fibrotic area between CKO and CTL mice (Fig. S1A).

3.4. Autophagy is involved in regression of angiotensin II-induced cardiac hypertrophy after unloading

We examined the effect of *Atg5* ablation on regression of LV hypertrophy. Seven days after removal of the minipumps, blood pressure in all groups was similar (Table S2). Echocardiographic and physiological analysis showed that LVPWd, LV mass index, the ratios of HW-to-tibia length and LVW-to-tibia length of angiotensin II-infused CKO mice were significantly larger than all other groups (Fig. 3D, E, Table S2). IVSd in angiotensin II-infused CKO mice was larger than saline-administered mice and tended to be increased compared to angiotensin II-infused CTL mice. LVIDd, LVIDs and FS were not significantly different among all groups. Cardiomyocyte cross-sectional area on heart sections from angiotensin II-infused CKO was significantly larger than those from other groups (Fig. 3F). Lung and liver weight did not change during regression of LV hypertrophy (data not shown). The ratio of fibrotic area remained larger in angiotensin II-infused CKO and CTL mice than corresponding saline-infused mice (Fig. S1B). The protein expression level of LC3-II was reduced both in saline- or angiotensin II-infused CKO hearts compared to corresponding CTL hearts after 2-week infusion and 1 week after removal of the minipumps (Fig. S2A). Thus, regression of LV hypertrophy after discontinuation of angiotensin II infusion was attenuated in autophagy-deficient hearts, and cardiac function was not altered by autophagy-deficiency in this process.

We examined the role of the ubiquitin–proteasome system in regression of LV hypertrophy. There was no difference in polyubiquitinated protein level among all groups (Fig. S2B). Proteasome activity also showed no significant difference among all groups after 2-week infusion or 1 week after removal of the minipumps (Fig. S2C). Protein synthesis is mainly regulated by the activation of S6 to initiate translation. The ratio of phospho-S6 to total-S6 was not different between CTL and CKO mice both 2 weeks after infusion of angiotensin II and 1 week after removal of the minipumps (Fig. S2D). To estimate protein synthesis 6 h after removal of the minipumps, we administered ^{14}C-labeled leucine by intraperitoneal injection 30 min before sacrifice. The incorporation of ^{14}C-labeled leucine into LV was not significantly different among

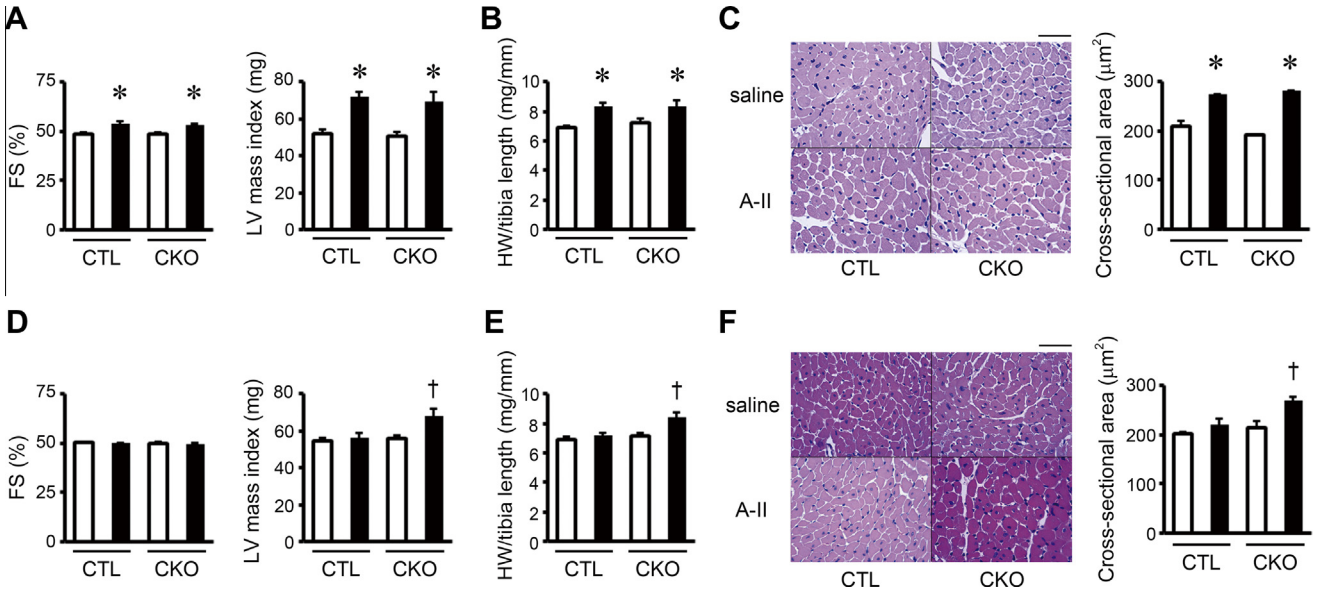

Fig. 3. Cardiac hypertrophy induced by angiotensin II infusion and its regression 1 week after minipump removal. (A–C) Cardiac hypertrophy induced by angiotensin II infusion for 2 weeks in CKO and CTL mice. (D–F) Regression of cardiac hypertrophy 1 week after removal of the minipumps of angiotensin II in CKO and CTL mice. (A, D) Echocardiographic analyses. (B, E) The ratio of HW to tibia length. (C, F) Left panel, microscopic hematoxylin–eosin stained sections of LV. Scale bar represents 50 μm. Right panel, cross-sectional area of cardiomyocytes. These were measured by tracing the outline of 100 myocytes in each section. Open and closed box indicate saline- and angiotensin II-treated group, respectively. Values represent the mean ± S.E.M. of data from 7 to 11 mice (A, B, D, E) or 3 mice (C and F) in each group. $^{*}p < 0.05$ versus saline-infused groups. $^{\dagger}p < 0.05$ versus all other groups.

saline-treated and angiotensin II-treated CKO and CTL mice (Fig. S2E).

3.5. Autophagy is involved in regression of pressure overload-induced cardiac hypertrophy

Then, we employed another model of LV hypertrophy, which is mediated through pressure overload by TAC. As we previously reported [7], Atg5-deficient mice exhibit cardiac dysfunction after ordinary TAC using a 26 gauge needle to induce aortic constriction. In this study, we adopted mild TAC (mTAC) using a 23 gauge needle so as not to induce cardiac dysfunction even in CKO mice. Ten days after mTAC, the pressure gradient between right and left arms was significantly increased in mTAC-operated groups compared to sham-operated groups (Fig. 4A), but there was no difference between mTAC-operated CKO and CTL mice. CKO mice showed no significant decrease in cardiac contractility estimated by FS 10 days after mTAC (Fig. 4B). mTAC induced significant increases in LV mass index and the ratios of HW-to-tibia length and LVW-to-tibia length (Fig. 4B, C). The induction of cardiac hypertrophy was similar between CKO and CTL mice. These results indicate that mTAC did not induce cardiac dysfunction, but cardiac hypertrophy in Atg5-deficient heart, and thus, our mTAC model is appropriate to assess regression of LV hypertrophy in Atg5-deficient mice. We released pressure overload to LV by removing the suture for constriction at the transverse aorta 10 days after mTAC (deTAC). One week after deTAC, the pressure gradient in deTAC-operated groups showed no significant difference with sham-operated groups (Fig. 4D). There was no significant difference in FS among all groups (Fig. 4E). One week after deTAC, LV mass index, the ratios of HW-to-tibia length, and LVW-to-tibia length showed no significant difference between sham-operated and deTAC-operated CTL mice. However, these parameters in deTAC-operated CKO mice were significantly larger compared to those in all other groups (Fig. 4E, F). These results indicate that deTAC induced regression of mTAC-induced LV hypertrophy in CTL mice, but not in CKO mice.

4. Discussion

Pressure overload and angiotensin II are the most common mediators for cardiac hypertrophy. Our data suggest that autophagy plays a central role in regression of cardiac hypertrophy. On the other hand, autophagy is not essential for cardiac hypertrophy induced by various stress including angiotensin II or mTAC as well as TAC, as previously reported [7]. Beclin1 is a key regulator for autophagy. Recently, it has been reported that regression of cardiac hypertrophy after deTAC was attenuated in *beclin1*$^{+/-}$ mice [8]. However, there are many criticisms of this report: (1) the extent of hypertrophy after TAC in *beclin1*$^{+/-}$ mice was smaller than controls, (2) there was no significant difference in heart weight between *beclin1*$^{+/-}$ and control mice after deTAC, (3) sham-operated *beclin1*$^{+/-}$ and control mice showed no difference in the level of p62, an autophagy flux marker, suggesting no inhibition of autophagy in *beclin1*$^{+/-}$ hearts at basal level. Furthermore, Beclin1 is implicated in numerous biological processes, including development, endocytosis, cytokinesis, immunity, tumorigenesis, ageing and cell death [12]. Thus, it is difficult to elucidate the role of autophagy in regression of cardiac hypertrophy during unloading based on the study using *beclin1*$^{+/-}$ mice. Our report is the first report that provides definite evidence for the involvement of autophagy in regression of cardiac hypertrophy during unloading.

Regression of cardiac hypertrophy could be induced by down-regulation of protein synthesis or upregulation of protein degradation. In this study, there was no significant difference in phosphorylation level of S6 and the incorporation of ^{14}C-labeled leucine into LV between CKO and CTL. Furthermore, known signaling molecules involved in cardiac hypertrophy such as ERK1/2 and Akt showed no difference in their activation levels during regression of cardiac hypertrophy (data not shown). Thus, protein synthesis is unlikely to play a role in the attenuation of regression of cardiac hypertrophy in CKO mice after unloading.

The ubiquitin–proteasome system is an important protein degradation system. In this study, we showed that there was no significant difference in ubiquitin–proteasome system activity between

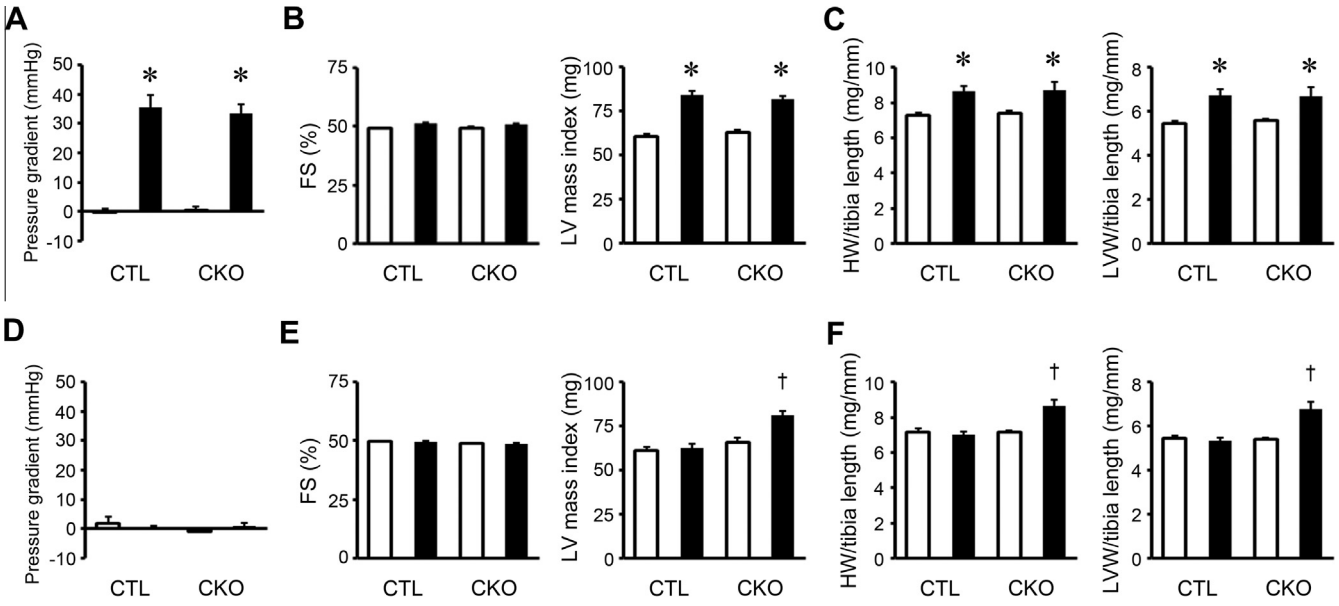

Fig. 4. Cardiac hypertrophy induced by mTAC and its regression 1 week after deTAC. (A and D) Pressure gradient between right and left arms of mice 10 days after mTAC (A) and 1 week after deTAC (D). (B and E) Trans-thoracic M-mode echocardiographic analyses, 10 days after mTAC (B) and 1 week after deTAC (E). (C and F) The ratio of HW to tibia length, and LVW to tibia length, 10 days after mTAC (C) and 1 week after deTAC (F). Open and closed box indicate sham- and mTAC-operated group, respectively (A–C) or sham- and deTAC-operated group, respectively (D-F). Values represent the mean ± S.E.M. of data from 6 to 12 mice in each group. $^{*}p < 0.05$ versus corresponding sham-operated group. $^{\dagger}p < 0.05$ versus all other groups.

CKO and CTL mice. Atrogin-1, also known as muscle atrophy F-box (MAFbx), and muscle-specific ring finger proteins (MuRF) are E3 ubiquitin ligases expressed specifically in skeletal and cardiac muscles and mediate muscle atrophy [13,14]. Atrogin-1 and MuRF-1 are known to negatively regulate the hypertrophic phenotypes of the heart [15,16]. Thus, one can hypothesize that atrogin-1 or MuRF-1 may be involved in regression of cardiac hypertrophy. However, the protein levels of atrogin-1 and MuRF-1 during regression of cardiac hypertrophy did not differ between CKO and CTL (data not shown). These data suggest that alteration of ubiquitin–proteasome system activity, atrogin-1 or MuRF-1 may not be involved in the observed phenotypes in CKO mice. Taken together, autophagy activation is indispensable for regression of cardiac hypertrophy during unloading of hemodynamic or neurohumoral stress.

The molecular mechanisms underlying the induction of autophagy during unloading remain to be elucidated. The forkhead box, class O (FoxO) family transcription factors promote autophagy in cardiomyocytes [17,18] and negatively regulate cardiac hypertrophy [19]. Furthermore, FoxO transcription factors promote atrophy in skeletal and cardiac muscle [18,20]. FoxO activity is upregulated during regression of cardiac hypertrophy after unloading by deTAC or by heterotopic cardiac transplantation [8,18]. Thus, FoxO transcription factors may be involved in autophagy induction during unloading. Since FoxO transcription factors regulate MuRF-1, atrogin-1 and calcineurin signaling [19–21], the role of FoxO in induction of autophagy during regression of cardiac hypertrophy is not yet conclusive. Myostatin, a member of the TGF-β superfamily, acts as a negative regulator of myocyte growth [22] and induces autophagy in skeletal muscle [23]. In the heart, myostatin negatively regulates cardiomyocyte growth mediated through Akt and NFAT3 signaling [24]. Myostatin is activated in heart failure patients after mechanical unloading with left ventricular assist device [25]. Thus, it is possible that myostatin activation may be involved in regression of cardiac hypertrophy. The precise mechanisms underlying the activation of autophagy during regression of cardiac hypertrophy have to be elucidated in future studies.

We have previously shown that inhibition of autophagy leads to the development of cardiac hypertrophy using temporally controlled cardiac-specific Atg5-deficient mice [7]. In addition, knockdown of Atg7 induced cardiomyocyte hypertrophy in isolated cardiomyocytes. Thus, autophagy has capability to alter the cardiac mass. However, it remains to be elucidated whether autophagy contributes to regression of cardiac hypertrophy by directly degrading a substantial amount of cardiac protein or by specifically degrading key molecules involved in protein synthesis or degradation.

In this study, we showed that autophagy plays a central role in regression of cardiac hypertrophy during unloading of hemodynamic and neurohumoral stress. Left ventricular hypertrophy is an independent risk factor for adverse consequences of cardiovascular disease. There is good clinical evidence that regression of cardiac hypertrophy reduces morbidity and mortality and improves prognosis [2]. Autophagy may be a novel therapeutic target to reverse cardiac hypertrophy.

Acknowledgments

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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.2013.10.135.

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