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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.

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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-

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