The role of autophagy in cardiomyocytes in the basal state and in response to hemodynamic stress

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Autophagy, an evolutionarily conserved process for the bulk degradation of cytoplasmic components, serves as a cell survival mechanism in starving cells. Although autophagy has been observed in various heart diseases, including cardiac hypertrophy and heart failure, it remains unclear whether autophagy plays a beneficial or detrimental role in the heart. Here, we report that the cardiac-specific loss of autophagy causes cardiomyopathy in mice. In adult mice, temporally controlled cardiac-specific deficiency of Atg5 (autophagy-related 5), a protein required for autophagy, led to cardiac hypertrophy, left ventricular dilatation and contractile dysfunction, accompanied by increased levels of ubiquitination. Furthermore, Atg5-deficient hearts showed disorganized sarcomere structure and mitochondrial misalignment and aggregation. On the other hand, cardiac-specific deficiency of Atg3 early in cardiogenesis showed no such cardiac phenotypes under baseline conditions, but developed cardiac dysfunction and left ventricular dilatation one week after treatment with pressure overload. These results indicate that constitutive autophagy in the heart under baseline conditions is a homeostatic mechanism for maintaining cardiomyocyte size and global cardiac structure and function, and that upregulation of autophagy in failing hearts is an adaptive response for protecting cells from hemodynamic stress.

The autophagy and ubiquitin-proteasome pathways are responsible for the degradation of intracellular components. In autophagy, cytoplasmic proteins and dysfunctional organelles are sequestered in an autophagosome, a double-membrane vesicle, delivered to the lysosome by fusion and then degraded. The principal role of autophagy is to supply nutrients for survival. In addition, a low level of constitutive autophagy is also important for controlling the quality of proteins and organelles, in order to maintain cell function. Thus, autophagy functions as a cell-protective mechanism. However, autophagy also has a causative role in cell death; autophagic structures are present in dying cells in neurodegenerative diseases, myopathies and liver injury.

Autophagic vacuoles are found in cardiomyocytes in ischemic hearts, and in human and hamster cardiomyopathic failing hearts. Mice with a deficiency of lysosome-associated membrane protein-2, a model for Danon disease, show an accumulation of autophagic vacuoles and cardiomyopathy. Moreover, inhibition of autophagy has been reported in the progression of cardiac hypertrophy. The precise role of autophagy in the heart, however, remains to be elucidated.

To determine the role of basal constitutive autophagy in adult mouse hearts, we generated temporally controlled cardiac-specific Atg5-deficient mice. We crossed mice bearing an Atg5fl/+ allele with transgenic mice (MerCreMer) which express the Cre recombinase in a tamoxifen-inducible and cardiomyocyte-specific manner. The resulting Atg5fl/+MerCreMer+ mice were indistinguishable in appearance from age-matched control Atg5fl/+MerCreMer− littermates. In Atg5fl/+MerCreMer+ mice that had been treated with tamoxifen for 7 d, we observed an approximately 70% reduction in Atg5 protein levels in whole heart homogenates (Fig. 1a) and an approximately 90% reduction of Atg5 protein levels in a partially purified adult cardiomyocyte preparation (Fig. 1b). Successful recombination occurred 3 d after tamoxifen injection (Fig. 1c). Suppression of Atg5-dependent conversion of microtubule-associated protein 1 light chain 3 (LC3-I) to LC3-II (a phosphatidylethanolamine conjugate) and accumulation of the p62/sequestosome indicated a reduction in autophagy levels in tamoxifen-treated Atg5fl/+MerCreMer+ hearts (Fig. 1d). Echocardiographic analysis of tamoxifen-treated Atg5fl/+MerCreMer+ demonstrated left ventricular dilatation and severe contractile dysfunction (Fig. 1e,f). The heart-to-body and lung-to-body weight ratios were increased in tamoxifen-treated Atg5fl/+MerCreMer+ mice (Fig. 1f). Atg5-deficient hearts exhibited no abnormal histological findings that is, no myofibrillar disarray, vacuole formation or enhanced intermuscular fibrosis, but did show an increase in the cross-sectional area of cardiomyocytes (Fig. 2a–c).

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The Beneficial Role of Autophagy in Cardiomyocytes in the Basal State and in Response to Hemodynamic Stress

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Introduction

The autophagy and the ubiquitin-proteasome system are responsible for the degradation of intracellular components. In autophagy, cytoplasmic proteins or dysfunctional organelles are sequestered in an autophagosome, a double-membrane-vesicle, delivered to the lysosome by fusion and then degraded. The principal role of autophagy is to supply nutrients for survival. In addition, a low level of constitutive autophagy is also important for the quality control of proteins and organelles to maintain cell function. Thus, autophagy functions as a cell protective mechanism. In contrast, the presence of autophagic structures in dying cells in neurodegenerative diseases, myopathies and liver injury led to the hypothesis that autophagy has a causative role in cell death. Cardiomyocytes contain autophagic vacuoles in ischemic hearts, and in human or hamster cardiomyopathic failing hearts. The precise role of autophagy in the heart, however, remains to be elucidated.

Role of autophagy in the heart at the basal state

We, first, generated temporally controlled cardiac-specific Atg5 mice to determine the role of basal constitutive autophagy in adult mouse hearts. Mice bearing an Atg5 allele were crossed with transgenic mice (MerCreMer) expressing a Cre recombine in a tamoxifen-inducible and cardiomyocyte-specific manner. The resulting Atg5; MerCreMer mice were indistinguishable in appearance from age-matched control littermates bearing Atg5; MerCreMer. In Atg5; MerCreMer mice that had been treated with tamoxifen for 7 d, we observed an approximately 70% reduction in Atg5 protein levels in whole heart homogenates and suppression of the Atg5-dependent conversion of microtubule-associated protein 1 light chain 3 (LC3)-I to LC3-II (phosphatidylethanolamine-conjugate) and the accumulation of p62/sequestosome indicated a reduction in autophagy level in tamoxifen-treated Atg5; MerCreMer hearts. An echocardiographic analysis of tamoxifen-treated Atg5; MerCreMer demonstrated left ventricular (LV) dilatation and severe contractile dysfunction (Fig. 1). The heart-to-body and lung-to-body weight ratio significantly increased in tamoxifen-treated Atg5; MerCreMer hearts (Fig. 3a, b).

Polyubiquitinated protein levels and proteasome activity increased in tamoxifen-treated Atg5; MerCreMer hearts (Fig. 3a, b). Ultrastructural analyses of Atg5-deficient hearts revealed a dysorganized sarcomere structure, disalignment and aggregation of mitochondria, and the appearance of aberrant concentric membranous structures similar to those observed in Atg7-deficient livers (Fig. 3c), suggesting the importance of autophagy in the turnover of organelles. The increased activation of S6 kinase in Atg5-deficient hearts indicated enhanced protein synthesis, which may be involved in cardiac hypertrophy (Fig. 3a). The observed hypertrophy is not only due to the accumulation of abnormal proteins, but also to activation of molecular maneuvers engaged with cardiac hypertrophy. The accumulation of ubiquitinated proteins is known to induce endoplasmic reticulum (ER) stress. GRP78 and GRP94 protein accumulation of ubiquitinated proteins is known to induce ER stress. GRP78 and GRP94 protein accumulation of ubiquitinated proteins is known to induce ER stress. GRP78 and GRP94 protein accumulation of ubiquitinated proteins is known to induce ER stress. GRP78 and GRP94 protein accumulation of ubiquitinated proteins is known to induce ER stress. GRP78 and GRP94 protein accumulation of ubiquitinated proteins is known to induce ER stress. GRP78 and GRP94 protein accumulation of ubiquitinated proteins is known to induce ER stress. GRP78 and GRP94 protein accumulation of ubiquitinated proteins is known to induce ER stress.

Figure 1. Cardiac dysfunction in tamoxifen-treated Atg5; MerCreMer mice. Thoracic M-mode echocardiographic and physiological analyses. Representative images of tracings are shown.

Figure 2. Hypertrophic responses in tamoxifen-treated Atg5; MerCreMer mice. (a) Hematoxylin-Eosin-stained sections of LV. (b) Cross-sectional area of cardiomyocyte. Values represent the means ± s.e.m. of 3 to 8 mice in each group. *P < 0.05 versus all other groups.

Figure 3a, b. Ultrastructural analyses of Atg5-deficient hearts revealed a dysorganized sarcomere structure, disalignment and aggregation of mitochondria, and the appearance of aberrant concentric membranous structures similar to those observed in Atg7-deficient livers (Fig. 3c), suggesting the importance of autophagy in the turnover of organelles. The increased activation of S6 kinase in Atg5-deficient hearts indicated enhanced protein synthesis, which may be involved in cardiac hypertrophy (Fig. 3a). The observed hypertrophy is not only due to the accumulation of abnormal proteins, but also to activation of molecular maneuvers engaged with cardiac hypertrophy. The accumulation of ubiquitinated proteins is known to induce endoplasmic reticulum (ER) stress. GRP78 and GRP94 protein levels were significantly increased in Atg5-deficient hearts (Fig. 3d). The protein level of cleaved caspase-12, which mediates the ER-specific apoptotic pathway and the number of TUNEL-positive cells, identified as cardiomyocytes by α-sarcomeric actin staining, significantly increased in Atg5-deficient hearts.
Role of autophagy in the heart in response to pressure overload

We then attempted to confirm these observations using another line of cardiac-specific Atg5-deficient mice. Atg5<sup>fl<sub>x</sub>/fl<sub>x</sub></sup> mice were crossed with knock-in mice expressing Cre under the control of myosin light chain 2v (MLC2v) promoter to produce Atg5<sup>fl<sub>x</sub>/fl<sub>x</sub></sup>; MLC2v-Cre<sup>+</sup> mice. In these mice, Cre is expressed in cardiomyocytes after embryonic day eight. Atg5<sup>fl<sub>x</sub>/fl<sub>x</sub></sup>; MLC2v-Cre<sup>-</sup> littersmates were used as controls. In contrast to tamoxifen-treated Atg5<sup>fl<sub>x</sub>/fl<sub>x</sub></sup>; MLC2v-Cre<sup>-</sup>, Atg5<sup>fl<sub>x</sub>/fl<sub>x</sub></sup>; MLC2v-Cre<sup>+</sup> showed no cardiac hypertrophy or dysfunction, suggesting alternate compensatory mechanisms function to cancel the phenotypes. In Atg5<sup>fl<sub>x</sub>/fl<sub>x</sub></sup>; MLC2v-Cre<sup>+</sup>, the reduction in autophagy may be too acute for compensatory mechanisms to be effective. This helped clarify the role of autophagy in response to stress such as pressure overload.

Pressure overload by means of a thoracic transverse aortic constriction (TAC) induced cardiac hypertrophy 1 week after the operation and heart failure 4 weeks later in wild-type mice. While autophagy was suppressed in TAC-induced hypertrophied hearts, as detected by decreased LC3-II levels, it was up-regulated in failing hearts, as evidenced by increased LC3-II levels. To elucidate the role of autophagy in cardiac remodeling, we performed TAC operations on Atg5<sup>fl<sub>x</sub>/fl<sub>x</sub></sup>; MLC2v-Cre<sup>+</sup> hearts compared to the corresponding controls. The Atg5<sup>fl<sub>x</sub>/fl<sub>x</sub></sup>; MLC2v-Cre<sup>+</sup> showed severe cardiac dysfunction and LV dilatation 1 week after TAC (Fig. 4a) and died of heart failure thereafter. Pressure overload activated the S6 kinase in the heart, but the activation of p70-S6 kinase was greater in TAC-operated Atg5<sup>fl<sub>x</sub>/fl<sub>x</sub></sup>; MLC2v-Cre<sup>-</sup> than that in controls. Proubiquitinated protein and the expression levels of GRP94 and GRP78 were significantly increased in TAC-operated Atg5<sup>fl<sub>x</sub>/fl<sub>x</sub></sup>; MLC2v-Cre<sup>+</sup> hearts (Fig. 4b). Proteasome activity in Atg5<sup>fl<sub>x</sub>/fl<sub>x</sub></sup>; MLC2v-Cre<sup>+</sup> was higher than in controls after TAC. These findings suggest that reduced autophagy resulted in the enhancement of both protein synthesis and proteasome-dependent protein degradation in pressure-overloaded hearts. The number of TUNEL-positive cardiomyocytes increased in Atg5<sup>fl<sub>x</sub>/fl<sub>x</sub></sup>; MLC2v-Cre<sup>-</sup> hearts after TAC.

Discussion

In the basal state, autophagy mediates the essential and continuous turnover of intracellular proteins and organelles in the heart. The downregulation of protein turnover could cause abnormal proteins to accumulate, promoting ER stress, leading to apoptosis and cardiac dysfunction. It is also possible that the accumulation of abnormal proteins or organelles may directly cause cardiac dysfunction. Basal autophagy could be a homeostatic mechanism for the maintenance of normal cardiac function and morphology.

As to the role of autophagy in the stress response, our results indicate that autophagy plays a beneficial role in the heart in response to pressure overload. Autophagy is necessary for accelerated protein turnover in remodeling hearts and important for preventing the accumulation of abnormal proteins or damaged organelles, which can disrupt cardiac function. Since autophagy is a mechanism for maintaining energy homeostasis during starvation, it is also possible that autophagy is necessary to compensate for increased energy demand during remodeling. Finally, autophagy may be an active adaptive intervention for protecting cardiomyocytes under stress by regulating cardiomyocyte death and function.