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Changes in cell autophagy and apoptosis during age-related left ventricular remodeling in mice and their potential mechanisms

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ABSTRACT

Cardiac structures and functions change with advanced age, but the underlying mechanisms are not well understood. Autophagy and apoptosis play important roles in the process of cardiac remodeling. This study was designed to explore changes in cell autophagy and apoptosis during age-related left ventricular remodeling and to determine whether the mitogen-activated protein kinase (MAPK) pathway is an underlying mechanism. Eight 5-month-old (adult group) and eight 24-month-old male C57bl/6 mice (aged group) were studied. The heart mass index, left ventricular mass index and hydroxyproline content of both groups were compared. Western Blotting was used to quantitate the protein expression of microtubule-associated protein 1 light chain 3 (LC3), Beclin-1, caspase-3, B-cell leukemia-2 (Bcl-2) and MAPKs in the left ventricles of adult and aged mice. Our results showed that the heart mass index, left ventricular mass index and hydroxyproline content in the left ventricles of the aged mice were increased significantly compared with the adult mice, indicating that left ventricular remodeling occurs with aging. The expression of LC3 and Beclin-1 in the left ventricles of aged mice were decreased significantly compared to adult mice. Meanwhile, the level of myocardial caspase-3 in adult mice remained the same in aged mice, and the level of myocardial Bcl-2 increased significantly in aged mice. There were no differences in the expression level of myocardial extracellular signal-regulated kinase 1/2 (ERK1/2), activated/phospho-ERK1/2, c-Jun N-terminal kinase 1/2 (JNK1/2) and p38 between aged and adult mice. However, the expression of myocardial activated/phospho-JNK1/2 increased significantly in aged mice, while activated/phospho-p38 decreased significantly. These findings indicate that autophagy decreases without a concurrent change in apoptosis during age-related left ventricular remodeling in mice. The MAPK pathway may be involved in the regulation of age-related left ventricular remodeling by modulating autophagy.

1. Introduction

Cardiac remodeling during aging includes cardiac myocyte loss, reactive hypertrophy of the remaining cells and increased interstitial tissues [1,2]. These changes may result in a decline in the biological and physiological functions of the heart. It has been suggested that the aging-induced cardiac changes render the heart more susceptible to ischemic damage [3]. Autophagy and apoptosis play important roles in cardiac remodeling and myocardial ischemia/reperfusion injury [4–8]. However, the related autophagy and apoptosis changes in aged hearts and their underlying mechanisms are poorly understood.

Autophagy, an evolutionarily conserved process of lysosome-dependent degradation of damaged proteins and organelles, plays a pivotal role in the maintenance of the cellular environment in the heart [9]. Effective autophagy in cardiomyocytes is necessary for normal metabolism and cellular survival. The inability of autophagy to completely remove damaged structures results in the progressive accumulation of "garbage", including cytosolic protein aggregates and defective mitochondria. The rate of autophagosome formation and the proteolytic activity of lysosomes decline with

Abbreviations: MAPK, mitogen-activated protein kinase; ERK1/2, extracellular signal-regulated kinase 1/2; JNK1/2, c-Jun N-terminal kinase 1/2; LC3, microtubule-associated protein 1 light chain 3; Bcl-2, B-cell leukemia-2.

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aging [10]. Manabu Taneike et al. reported that inhibition of autophagy in the heart induced age-related cardiomyopathy [11]. Although functional autophagy is crucial for normal cardiomyocyte function, the level of autophagy in aged hearts has not been completely described.

There is evidence showing that the effects of aging predispose cardiomyocytes to apoptosis [12], but the apoptosis changes in aged hearts and the underlying mechanisms are not well understood. Caspase-3 is a critical executioner of apoptosis because it is either partially or totally responsible for the proteolytic cleavage of many key proteins [13]. B-cell leukemia-2 (Bcl-2), a well-known anti-apoptotic protein, was originally identified as the product of a human lymphoma oncogene [14]. Bcl-2 is now recognized as a representative of a family of proteins that regulate cell death [15]. The abundance of Bcl-2 and other members of the same family in myocardial tissue may be one of the crucial factors determining cell fate after cardiac ischemia. The expression level and role of Bcl-2 in the aging heart are not yet clearly defined.

Mitogen-activated protein kinases (MAPKs) are important signaling proteins involved in the regulation of cell responses to various stresses, including ischemia and hypoxia [16]. MAPKs are also known to regulate cell autophagy and apoptosis [17–19]. Three MAPKs have been identified to date: extracellular signal-regulated kinase 1/2 (ERK1/2), c-Jun N-terminal kinase 1/2 (JNK1/2) and p38. In the myocardium, ischemia activates all three MAPKs. There is evidence that the roles of ERK1/2 in ischemia are protective and that JNK1/2 and p38 are involved in cell death pathways [20–22]. However, the expression differences of these proteins in aged hearts have not yet been well described.

Therefore, we designed this study to determine the expression of microtubule-associated protein 1 light chain 3 (LC3), Beclin-1, caspase-3 and Bcl-2 in the left ventricular myocardium to reflect the levels of cell autophagy and apoptosis during cardiac remodeling in aged mice. We also measured the activity of MAPK proteins in the left ventricular myocardium of aged mice. This study may provide insight into age-related myocardial autophagy and apoptosis changes and their potential mechanisms. Additionally, this study may provide an initial step into understanding the age-related decrease of myocardial ischemia tolerance.

2. Materials and methods

The Experimental Animal Ethical Committee at Sun Yat-sen University approved the animal protocol. All reagents were obtained from Sigma Chemicals (St. Louis, MO, USA) unless otherwise specified in the text.

2.1. Mice and myocardial tissue sample collection

Eight 5-month-old male C57bl/6 mice (the adult group) and eight 24-month-old male C57bl/6 mice (the aged group) were obtained from the Experimental Animal Center of Sun Yat-sen University (Guangzhou, China). After arrival, the mice were kept for 3 days in an air-conditioned room at 25 °C with a 12-h light/12-h dark cycle and free access to tap water and food. The mice were weighed and sacrificed under anesthesia with 10% chloral hydrate (300 mg/kg). Hearts were rapidly excised by middle thoracotomy, rinsed in normal saline, blotted briefly against filter paper to absorb surface water and then weighed. The atrial myocardium and right ventricular myocardium were removed, and the left ventricular myocardium including the interventricular septum was kept and weighed. The heart mass index (heart mass/body mass, mg/g) and left ventricular mass index (left ventricular mass/body mass, mg/g) were calculated. After being weighed, the left ventricles were cut into two (upper, lower) sections. The upper sections were used for a hydroxyproline content assay and the lower sections were quickly frozen in liquid nitrogen and stored at −80 °C until being used for Western Blot analysis. The entire procedure was performed in cold conditions.

2.2. Left ventricular collagen content assay

Left ventricle myocardial hydroxyproline content was determined as an index of the amount of collagen, which reflects the level of myocardial fibrosis. The left ventricular specimens were minced and then homogenized for 2 min at 4 °C in appropriate quantities of deionized water to yield a 10% mixture (wt/vol). The hydroxyproline content of homogenates was assayed as we have previously described [23]. Briefly, left ventricular samples were homogenized and hydrolyzed in 6 N HCl at 110 °C for 18 h. The hydrolysate was then filtrated through a 0.45-mm Millipore filter. Chloramine T was then added to the filtered hydrolysate. The mixture was then treated with 400 mmol/L paradi-methylamino-benzaldehyde and incubated at 60 °C for 30 min. Finally, samples were assayed spectrophotometrically at 560 nm against a blank sample and compared with a standard curve. The hydroxyproline content of the left ventricles was calculated as mg/g wet weight.

2.3. Western Blot analysis

Left ventricles were minced and homogenized in ice-cold buffer with pH 7.5 containing 20 mM Tris–HCl, 1 mM EDTA, 5 mM $MgCl_2$, 1 mM phenylmethylsulfonyl fluoride, 2 mM sodium orthovanadate and 20 μg/ml aprotinin. The prepared samples were subjected to Western Blotting as previously described [23]. Briefly, 40 μg of protein per lane were loaded onto and separated with a 10% or 12% SDS–polyacrylamide gel. The proteins were then transferred onto a polyvinylidene difluoride (PVDF) membrane at 4 °C. The membrane was probed first with primary antibodies (monoclonal rabbit anti-GAPDH at 1:2000 dilution; monoclonal rabbit anti-LC3A at 1:1000 dilution; monoclonal rabbit anti-Beclin-1 at 1:1000 dilution; monoclonal rabbit anti-caspase-3 at 1:1000; monoclonal mouse anti-Bcl-2 at 1:1000 dilution; polyclonal rabbit anti-ERK1/2 at 1:1000 dilution; monoclonal mouse anti-phosphorylated-ERK1/2 at 1:1000 dilution; polyclonal rabbit anti-JNK1/2 at 1:1000; polyclonal mouse anti-phosphorylated-JNK1/2 at 1:1000 dilution; polyclonal rabbit anti-p38 at 1:1000; and polyclonal rabbit anti-phosphorylated-p38 at 1:1000 dilution) for 1 h at room temperature and then with a horseradish peroxidase-conjugated goat anti-rabbit (1:3000) or goat anti-mouse (1:3000) IgG secondary antibody for 1 h at room temperature.

Anti-GAPDH, anti-LC3A, anti-Beclin-1 and anti-caspase-3 antibodies were obtained from Cell Signaling Technology, Inc. (Beverly, MA, USA). Anti-Bcl-2, anti-ERK1/2, anti-phosphorylated-ERK1/2, anti-JNK1/2, anti-phosphorylated-JNK1/2, anti-p38 and anti-phosphorylated-p38 antibodies and horseradish peroxidase-conjugated goat anti-rabbit or goat anti-mouse IgG secondary antibodies were obtained from Santa Cruz (Santa Cruz, CA, USA). The probed protein bands were visualized with an enhanced chemiluminescence reaction. The protein bands were densitometrically analyzed by an ImageQuant 5.0 densitometer (Amersham Biosciences, Piscataway, NJ, USA). The results were then normalized to the mean value of the adult group.

Notably, phosphorylation of both threonine and tyrosine residues that are separated by one amino acid in MAPKs activates these protein kinases [24–26]. The antibodies used in this study specifically detect MAPKs that are phosphorylated at those sites according to the companies' data and therefore detect the activated MAPKs.

2.4. Statistical analysis

Data are presented as the means ± SD. The adult and aged groups were compared by unpaired *t*-tests using the SPSS 11.0 software for Windows. $P < 0.05$ was considered significant.

3. Results

The heart mass index and left ventricular mass index of the 24-month-old mice were significantly greater compared with those of the 5-month-old mice ($P < 0.001$) (Table 1). Hydroxyproline content in the left ventricle was increased in aged mice compared with adult mice ($P < 0.001$) (Table 1).

LC3A (including LC3A-I and LC3A-II) and Beclin-1 expression in the left ventricular myocardium of aged mice decreased significantly compared with adult mice ($P = 0.006$ for LC3A-I, $P = 0.003$ for LC3A-II, $P = 0.007$ for Beclin-1). Meanwhile, the value of the LC3A-II/LC3A-I ratio was reduced in aged mice compared to adult mice ($P < 0.001$). However, the level of caspase-3 in the left ventricular myocardium of aged mice was unchanged compared to that of adult mice ($P = 0.18$). Notably, the hearts of aged mice expressed significantly more Bcl-2 protein than those of adult mice ($P < 0.001$) (Fig. 1).

Table 1
Body mass, heart mass, left ventricle mass, heart mass index, left ventricular mass index and hydroxyproline content of the adult and aged mouse groups.

	Adult group	Aged group	*P* value
BM (g)	27.80 ± 1.62	25.93 ± 2.88	0.134
HM (mg)	124.25 ± 4.37	135.50 ± 5.63	0.001
LVM (mg)	99.13 ± 3.56	109.50 ± 5.52	0.001
HMI (mg/g)	4.48 ± 0.21	5.26 ± 0.37	<0.001
LVMI (mg/g)	3.57 ± 0.22	4.25 ± 0.27	<0.001
Hydroxyproline (mg/g)	4.51 ± 0.25	6.26 ± 0.25	<0.001

Values are means ± SD ($n = 8$). BM: body mass, HM: heart mass, LVM: left ventricle mass, HMI: heart mass index, LVMI: left ventricular mass index.

There were no differences in myocardial ERK1/2 protein expression or the phosphorylation levels between adult and aged mice ($P = 0.58$ for ERK1, $P = 0.39$ for ERK2, $P = 0.69$ for ERK1 phosphorylation, $P = 0.72$ for ERK2 phosphorylation). The protein expressions of JNK1/2 and p38 in aged hearts were not different from those in adult mice ($P = 0.83$ for JNK1, $P = 0.75$ for JNK2, $P = 0.46$ for p38). However, phospho-JNK1/2 was significantly increased in aged mouse hearts compared with adult mouse hearts ($P = 0.002$ for phospho-JNK1, $P < 0.001$ for phospho-JNK2). The expression of myocardial phospho-p38 was significantly decreased in aged mice compared with adult mice ($P = 0.001$) (Fig. 2).

4. Discussion

Heart aging is accompanied by various anatomical, ultrastructural, mechanical and biochemical changes, which can compromise the adaptive reserve capacity of the aging heart and render the heart more susceptible to ischemia. Our present study shows that heart mass index and left ventricular mass index increase in aged mice. This finding is consistent with previous studies [27,28]. The present study also demonstrates that myocardial collagen content increases in aged mice. Together, our results indicate that left ventricular remodeling occurs during mouse aging.

Given the postmitotic nature of cardiomyocytes, the efficient removal of dysfunctional mitochondria is critical for the maintenance of cell homeostasis because damaged organelles cannot be diluted by cell proliferation. The only known mechanism whereby mitochondria are turned over is through autophagy [29]. Cardiac-specific autophagy-deficient mice show no obvious phenotype up to 10 weeks of age. However, these mice begin to die after the age of 6 months, with significantly increased left ventricular dimensions and decreased fractional shortening of the left ventricle compared with control mice [30].

Our results demonstrate that LC3A-I, LC3A-II, Beclin-1 and the LC3A-II/LC3A-I ratio are all decreased in the left ventricular myocardium of aged mice compared to adult mice, indicating that

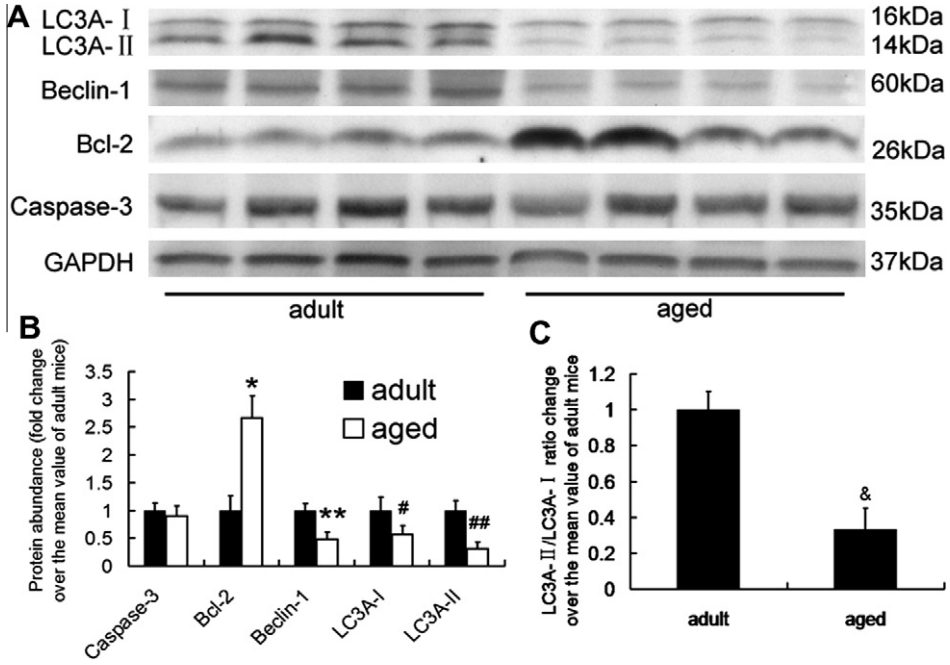

Fig. 1. Caspase-3, Bcl-2, Beclin-1, LC3A-I and LC3A-II protein abundance in adult and aged mouse hearts. (A) A representative autoradiogram displaying the protein bands. (B) A graphic representation of caspase-3, Bcl-2, Beclin-1, LC3A-I and LC3A-II protein abundances quantified by integrating the volume of the autoradiogram bands. Values are expressed as fold changes over the mean value of the adult mouse group and presented as the means ± SD ($n = 8$). (C) A graphic representation of the LC3A-II/LC3A-I ratio in adult and aged mouse hearts. $^{*}P < 0.001$ compared with adult mice, $^{**}P = 0.007$ compared with adult mice, $^{\#}P = 0.006$ compared with adult mice, $^{\#\#}P = 0.003$ compared with adult mice, $^{\&}P < 0.001$ compared with adult mice.

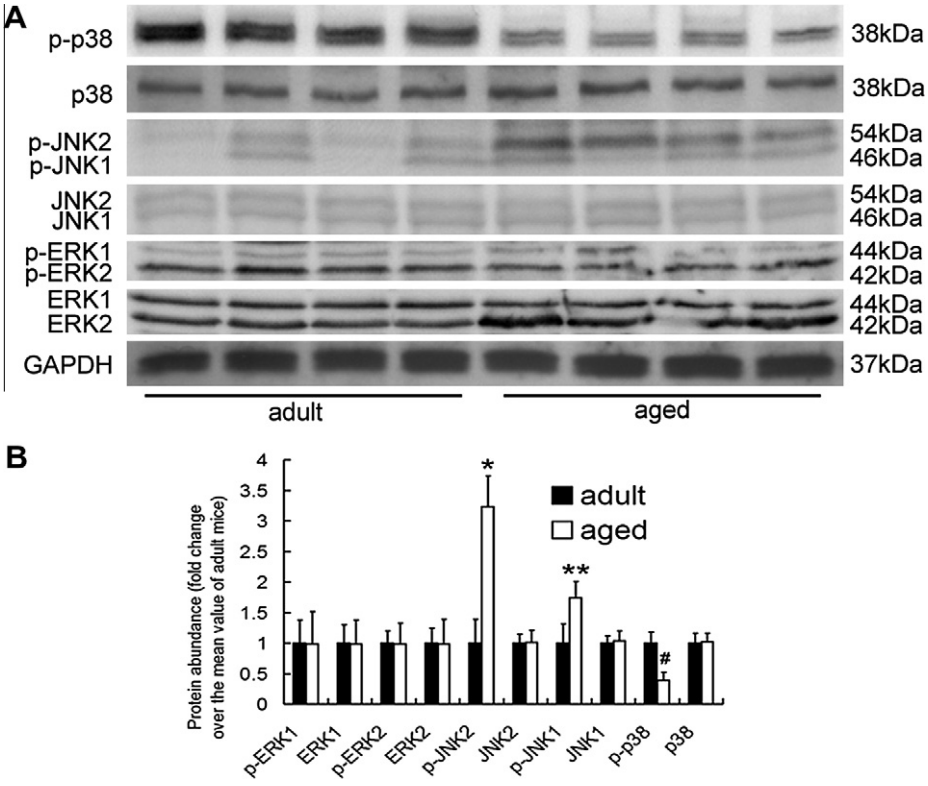

Fig. 2. ERK1/2, phospho-ERK1/2, JNK1/2, phospho-JNK1/2, p38 and phospho-p38 protein abundance in adult and aged mouse hearts. (A) A representative autoradiogram displaying the protein bands. (B) A graphic representation of ERK1/2, JNK1/2, p38, phospho-ERK1/2, phospho-JNK1/2 and phospho-p38 protein abundances quantified by integrating the volume of the autoradiogram bands. Values are expressed as fold changes over the mean value of the adult group and presented as the means ± SD ($n = 8$). $^{*}P < 0.001$ compared with adult mice, $^{**}P = 0.002$ compared with adult mice, $^{\#}P = 0.001$ compared with adult mice.

autophagy decreases with heart aging. Similarly, Hua et al. reported that levels of Beclin-1, Atg5 and the LC3-II/LC3-I ratio were decreased in aged hearts [6]. Over time, the age-related decline in the protective autophagy rate enhances oxidative stress, decreases ATP production, and results in the collapse of the cellular catabolic machinery and cell death [31]. Accordingly, we speculate that reduced autophagy is associated with left ventricular remodeling during aging and that therapies that can up-regulate autophagy activity may be of great value in alleviating cardiac remodeling of the aged heart.

Our results show that the expression of caspase-3, a critical executioner of apoptosis, is not significantly different between aged and adult mice, suggesting that apoptosis does not change during age-related left ventricular remodeling. We also demonstrate that the expression of the Bcl-2 protein increases significantly in the aged mouse heart. Consistent with our study, Bcl-2 expression was shown to be increased in aged female CD1 mouse hearts, and protein expression of the Bcl-2 family was found to be enhanced in the end stage of heart failure [32,33]. The cellular protective role of Bcl-2 has been well established [34,35]. Thus, the increased expression of Bcl-2 in aged hearts shown in our study may be a compensatory response to prevent cell death, including apoptosis. Increased Bcl-2 expression may be responsible for the decreased autophagy seen in aged hearts because Bcl-2 is believed to inhibit Beclin-1-dependent autophagy [36].

MAPKs are known to regulate a wide array of critical cellular processes, such as cell growth and cell differentiation [37]. MAPKs are also involved in the myocardial response to stress, cell survival, cell autophagy and apoptosis, which are all fundamental processes of cardiac remodeling [17–19,37–39]. Our study also shows that myocardial phospho-JNK1/2 increases significantly in aged mice and that there are no differences in the amount of myocardial JNK1/2, ERK1/2 and phospho-ERK1/2 present between aged and adult mice. Similar to our results, a recent study showed that there was no significant difference in phospho-ERK1/2 in the hearts of 24–26-month-old C57/BL6 mice compared with that of 10–14-week-old mice (JNK was not examined in the study) [40]. Our study also shows that there is no difference in myocardial p38 protein expression between the aged and adult mice groups; however, the amount of activated p38 decreases in aged mouse hearts. Previous studies have shown that p38 expression is decreased in failing human hearts [41]. Over-expression of p38 rescues failing hearts and decreases myocyte apoptosis and infarct size after myocardial infarction in rats [42]. There is also evidence suggesting that endogenous p38 protects cardiomyocytes from cell death after ischemia [43]. Over-expression of p38 may have a direct effect on cardiac fibrosis because p38 dominant-negative mice develop massive cardiac fibrosis in response to pressure overload [44]. Taken together, our study suggests that the activation of JNK1/2 and the inactivation of p38 in the aged heart are involved in aging-related left ventricular remodeling. At the same time, we speculate that the changes in JNK1/2 and p38 activation are associated with reduced autophagy during aging-related cardiac remodeling and that the reduced autophagy and changes in JNK1/2 and p38 activation may contribute to decreased ischemia tolerance.

ERK1/2, JNK1/2, p38 and Bcl-2 have been shown to play a role in ischemic preconditioning-induced cardioprotection [45,46]. Autophagy is also required for the cardioprotection mediated by ischemia preconditioning [47]. Ischemic preconditioning is a phenomenon in which prior exposure to short episodes of ischemia induces protection against subsequent severe ischemia [48]. It has been suggested that it is more difficult to induce cardioprotection by ischemic preconditioning in aged hearts. Studies are needed to explore how the aging-induced changes in MAPKs and Bcl-2 expression and autophagy may relate to the decreased effectiveness of ischemic preconditioning.

In summary, we have shown that aging increases left ventricular mass index and collagen content, consistent with age-related cardiac remodeling. We also demonstrate that the expression of LC3 and Beclin-1 decreases while caspase-3 expression does not change in the aged heart, indicating that autophagy decreases without a concurrent change in apoptosis during age-related left ventricular remodeling. In addition, the expression of Bcl-2 and active JNK1/2 increases, while the expression of active p38 decreases in aged hearts. Because Bcl-2, JNK1/2 and p38 play roles in cell survival, autophagy, apoptosis and cardiac fibrosis, the aging-induced changes in expression of these molecules may contribute to cardiac remodeling and reduced autophagy. The reduced autophagy and the expression changes of these molecules above may contribute to decreased tolerance of ischemia seen in aged hearts.

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