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Original article

MAFbx/Atrogin-1 is required for atrophic remodeling of the unloaded heart☆

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ABSTRACT

Mechanical unloading of the failing human heart induces profound cardiac changes resulting in the reversal of a distorted structure and function. In this process, cardiomyocytes break down unneeded proteins and replace those with new ones. The specificity of protein degradation via the ubiquitin proteasome system is regulated by ubiquitin ligases. Over-expressing the ubiquitin ligase MAFbx/Atrogin-1 in the heart inhibits the development of cardiac hypertrophy, but the role of MAFbx/Atrogin-1 in the unloaded heart is not known. *Mechanical unloading, by heterotopic transplantation, decreased heart weight and cardiomyocyte cross-sectional area in wild type mouse hearts. Unexpectedly, MAFbx/Atrogin-1$^{-/-}$ hearts hypertrophied after transplantation ($n = 8$–10). Proteasome activity and markers of autophagy were increased to the same extent in WT and MAFbx/Atrogin-1$^{-/-}$ hearts after transplantation (unloading). Calcineurin, a regulator of cardiac hypertrophy, was only upregulated in MAFbx/Atrogin-1$^{-/-}$ transplanted hearts, while the mTOR pathway was similarly activated in unloaded WT and MAFbx/Atrogin-1$^{-/-}$ hearts. MAFbx/Atrogin-1$^{-/-}$ cardiomyocytes exhibited increased calcineurin protein expression, NFAT transcriptional activity, and protein synthesis rates, while inhibition of calcineurin normalized NFAT activity and protein synthesis. Lastly, mechanical unloading of failing human hearts with a left ventricular assist device ($n = 18$) also increased MAFbx/Atrogin-1 protein levels and expression of NFAT regulated genes. MAFbx/Atrogin-1 is required for atrophic remodeling of the heart. During unloading, MAFbx/Atrogin-1 represses calcineurin-induced cardiac hypertrophy. Therefore, MAFbx/Atrogin-1 not only regulates protein degradation, but also reduces protein synthesis, exerting a dual role in regulating cardiac mass.*

1. Introduction

Mechanical unloading of the failing human heart induces profound cardiac changes resulting in the reversal of a distorted structure and function [1–3]. In heart failure patients fitted with a left ventricular assist device (LVAD), and in hemodynamically unloaded rodent hearts, pathways of protein synthesis and degradation are activated simultaneously [4,5]. A more complete understanding of this self-renewal of heart muscle cells should therefore be of clinical importance.

Protein turnover is the net result of protein synthesis and protein degradation, and ultimately controls cardiomyocyte size. Signaling pathways regulating protein synthesis have been studied in detail, particularly during conditions that lead to cardiac hypertrophy [6]. Surprisingly, activation of protein synthesis is also a feature of cardiac atrophy [4]. While pathways of protein degradation in the heart are the focus of several recent investigations [7,8], it is not known to what extent these pathways regulate atrophic remodeling of the unloaded heart. More importantly, it is not known why atrophic remodeling in the failing heart can result in improved function.

The importance of protein degradation in the heart has been recognized for some time [9], but only lately specific proteolytic systems have been investigated in detail. Much of what is known about protein degradation in the heart originates from studies in skeletal muscle. The two main pathways regulating protein degradation in muscle are the ubiquitin proteasome system (UPS) and autophagy [10–12], and both pathways are precisely controlled. The UPS is regulated at several

Abbreviations: AMCM, adult mouse cardiomyocytes; Atg, autophagy-related gene; CnA, calcineurin A; CsA, cyclosporine A; eIF3f, eukaryotic initiation factor 3f.

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different steps, the most critical being the ubiquitination of target proteins by ubiquitin ligases [13].

Ubiquitin ligases are essential in regulating protein degradation in muscle. The muscle-specific ligases atrophy F-box protein (MAFbx) [14], also known as Atrogin-1 [15], and Muscle RING finger protein 1 (MuRF1) were originally identified by their transient upregulation during skeletal muscle atrophy. Mice lacking *MAFbx/Atrogin-1* and *MuRF1* are resistant to denervation-induced skeletal muscle atrophy while overexpression of MAFbx/Atrogin-1 in myotubes induces atrophy [14]. *MAFbx/Atrogin-1* and *MuRF1* are transcriptionally upregulated in various models of skeletal muscle atrophy including hindlimb suspension, immobilization, denervation, cancer, diabetes, fasting, and renal failure [14,15]. Many investigations have focused on the transcriptional regulation of the ligases in muscle, and the pathways that are involved in this process. Main pathways include PI3kinase-Akt, p38, and p300, all of which converge on Foxo transcription factors [16].

As in skeletal muscle, Foxo transcription factors, which are activated during atrophic remodeling of the heart [17], regulate cardiac expression of *MAFbx/Atrogin-1* and *MuRF1* [18]. Although much less attention has been given to the ligases in the heart, the following is already known. Overexpression of *MAFbx/Atrogin-1* in the heart reduces physiologic and pathologic cardiac hypertrophy in vivo [19,20]. MuRF1 overexpression in cardiomyocytes prevents hypertrophy in vitro [21], and mice deficient in *MuRF1* develop enhanced cardiac hypertrophy in response to pressure overload [22]. Additionally, MuRF1 is required for the reversal of cardiac hypertrophy [23]. These studies establish the importance of ubiquitin ligases in regulating cardiac hypertrophy; however, the role of MAFbx/Atrogin-1 and MuRF1 in regulating atrophic remodeling of the heart has not yet been investigated.

In the current study, we examine the role of MAFbx/Atrogin-1 and MuRF1 in mechanical unloading of the heterotopically transplanted heart. We demonstrate that *MuRF1* is dispensable for mechanical unloading-induced cardiac atrophy. Unexpectedly, *MAFbx/Atrogin-1*$^{-/-}$ hearts not only are resistant to mechanical unloading-induced atrophy, but they also hypertrophy in response to a decreased load. Protein degradation was not significantly altered in transplanted (unloaded) *MAFbx/Atrogin-1*$^{-/-}$ hearts, however protein synthesis rates were drastically elevated in *MAFbx/Atrogin-1*$^{-/-}$ cardiomyocytes. Further investigation into the mechanism behind this phenomenon revealed that calcineurin protein levels and NFAT activity were significantly increased in *MAFbx/Atrogin-1*$^{-/-}$ cardiomyocytes. Enhanced protein synthesis in *MAFbx/Atrogin-1*$^{-/-}$ cardiomyocytes resulted in hypertrophy, while inhibition of calcineurin restored protein synthesis rates to normal. The MAFbx/Atrogin-1–calcineurin axis is also regulated in failing hearts unloaded with a left ventricular assist device. The results of our animal studies are replicated in the failing human heart. We show that MAFbx/Atrogin-1 is required for atrophic remodeling of the unloaded heart by keeping protein synthesis in check, and suggest that MAFbx/Atrogin-1 expression aids in the process of reverse remodeling of the failing heart induced by mechanical unloading.

2. Methods

An expanded Methods section is available in the Data Supplement.

2.1. Experimental animals

MAFbx/Atrogin-1$^{-/-}$, *MuRF1*$^{-/-}$, and respective WT littermate male mice (8–10 weeks old), were used for in vivo unloading experiments (heterotopic transplantation of the heart) and for the isolation of adult cardiomyocytes. Experiments involving the use of animals were approved by the IACUC of The University of Texas Health Science Center at Houston.

2.2. Heterotopic transplantation of the mouse heart

Unloading of the heart was induced by isogenic heterotopic transplantation of mouse hearts as described previously and in further detail in the Data Supplement [24].

2.3. Cell culture

Adult mouse cardiomyocytes isolated from hearts of *MAFbx/Atrogin-1*$^{-/-}$ mice and their WT littermates were used for the pulse chase experiments and immunocytochemistry, explained in detail in the Data Supplement.

2.4. Human subjects

Paired cardiac tissue samples were obtained from 18 patients (ranging in age from 43 to 67 years) with idiopathic dilated cardiomyopathy (16 males, 2 females) referred to the Texas Heart Institute for heart transplantation and placed on left ventricular assist device (LVAD) support for a mean duration of 123 ± 20 days. Tissue from the left ventricular apex was obtained during LVAD implantation and again during LVAD explantation or at the time of death. Tissue samples were immediately frozen in liquid nitrogen and stored at −80 °C for molecular analyses. Human subjects gave informed consent and the study protocol was approved by the Committee for the Protection of Human Subjects of St. Luke's Episcopal Hospital in Houston, Texas, and by The University of Texas Medical School at Houston.

2.5. Statistical analysis

Results are expressed as means ± SEM. Analysis was performed using two-tailed, unpaired Student's *t*-test or one-way ANOVA with Tukey post hoc test. A value of $P < 0.05$ was considered significant.

3. Results

3.1. MAFbx/Atrogin-1, not MuRF1, is required for atrophic remodeling of the heart

The ubiquitin ligases MAFbx/Atrogin-1 and MuRF1 are critical regulators of skeletal muscle size and mass [14,15]. Expression of both ligases increases with muscle unloading and the absence of either ligase suppresses unloading-induced skeletal muscle atrophy [14]. Although protein degradation pathways in skeletal muscle and in the heart are thought to be the same, it is not clear if they are similarly regulated. Therefore we first determined whether unloading of the mouse heart increased *MAFbx/Atrogin-1* and *MuRF1* expression. Heterotopic transplantation in isogenic animals causes mechanical unloading of the heart and is a model of cardiac atrophy [25,26]. In wild type (WT) mice, mechanical unloading of the heart induces cardiac atrophy after seven days, as demonstrated by a significant decrease in heart weight (Fig. 1A). The expression of *MAFbx/Atrogin-1* and *MuRF1* was increased in the transplanted heart after mechanical unloading (Fig. 1B). These results suggest that, as in skeletal muscle, the ubiquitin ligases MAFbx/Atrogin-1 and MuRF1 may also be important regulators of protein degradation during atrophic remodeling of the heart.

To test the hypothesis that MAFbx/Atrogin-1 and MuRF1 are required for atrophic remodeling of the mechanically unloaded heart we subjected WT, *MAFbx/Atrogin-1*$^{-/-}$, and *MuRF1*$^{-/-}$ littermate mice to isogenic heterotopic transplantation [25,26]. Surprisingly, *MuRF1*$^{-/-}$ hearts atrophied to the same extent as WT hearts after seven days of transplantation (unloading) (Supplemental Fig. 1). The heart weight, myocyte diameter, and myocyte cross sectional area of *MuRF1*$^{-/-}$ transplanted (unloaded) hearts did not significantly differ from WT transplanted (unloaded) hearts (Supplemental Figs. 1B–D). These data