《Research Note》

Effects of Fasting and Refeeding on Expression of Atrogin-1/MAFbx in Cardiac Muscle of Broiler Chickens

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The expression of atrogin-1/MAFbx, a muscle-specific E3 ubiquitin ligase, is high under catabolic conditions that result in muscle atrophy. We previously showed that expression of atrogin-1/MAFbx was expressed in skeletal, smooth and cardiac muscles of chickens and its expression in skeletal and smooth muscles was regulated by nutritional conditions. In this study, the effects of fasting and refeeding on the expression of atrogin-1/MAFbx and other proteolytic-related genes, including 20S proteasome C2 subunit, m-calpain large subunit, and cathepsin B, in cardiac muscle of broiler chickens were investigated. Chickens were fasted for 24 h, and refed for 2 h. Atrogin-1/MAFbx mRNA expression was increased by fasting, but subsequent refeeding reduced the increase. The mRNA expression of the 20S proteasome C2 subunit and m-calpain large subunit mRNA was not affected by fasting or refeeding. Cathepsin B mRNA expression was increased by fasting, but unlike atrogin-1/MAFbx mRNA expression, the increase was not reduced by refeeding. These results demonstrate the muscle-specific ubiquitin ligase atrogin-1/MAFbx gene expression in cardiac muscle as well as skeletal and smooth muscles of chicken is regulated by food deprivation and nutritional supply.

Key words: atrogin-1/MAFbx, broiler, cardiac muscle, fasting, heart, refeeding

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Introduction

The ubiquitin-proteasome system is a major proteolytic pathway in various tissues, and involves ATP-dependent proteolytic of ubiquitin-conjugated proteins via a protein complex termed the 26S proteasome (Lecker *et al.*, 1999). Ubiquitin-mediated proteasomal degradation plays an important role in many biological processes and proteins that are regulated by the proteasome undergo rapid turnover in various cells. However, while the proteasome is the main mechanism of proteolysis in skeletal and cardiac muscle (Lecker *et al.*, 1999), proteolysis under catabolic conditions is due primarily to the activation of the ubiquitin-proteasome proteolytic pathway (Lecker *et al.*, 2004), in which proteins destined to be degraded are linked to a chain of ubiquitin molecules that targets them for rapid breakdown by the proteasome (Glickman and Ciechanover, 2002). Evidence suggests that expression of atrogin-1, an E3 ubiquitin ligase also known as MAFbx (muscle atrophy F-box), is high under catabolic conditions that result in muscle atrophy (Gomes *et al.*, 2001; Bodine *et al.*, 2001). It is also known to play a

critical role in the regulation of muscle proteolysis (Ohtsuka *et al.*, 2011).

Atrogin-1/MAFbx mRNA is expressed in mammalian cardiac muscle, and its expression increases during heart failure (Carvalho *et al.*, 2010), pressure overload (Razeghi *et al.*, 2006) and hypoxemia (Razeghi *et al.*, 2006). We previously reported that atrogin-1/MAFbx mRNA is expressed in the skeletal, smooth, and cardiac muscles of chickens (Nakashima *et al.*, 2013), and that its expression is increased by fasting in skeletal (Nakashima *et al.*, 2005) and smooth muscles (Nakashima *et al.*, 2013) and decreased by subsequent refeeding in skeletal (Nakashima *et al.*, 2005) and smooth muscle (Nakashima *et al.*, 2013). However, the response of atrogin-1/MAFbx expression during food deprivation or altered nutritional supply remains largely unknown in chicken cardiac muscle.

To clarify this issue, in the present study, we examined the effects of fasting and subsequent refeeding on atrogin-1/MAFbx mRNA level in the cardiac muscle of broiler chickens.

Materials and Methods

Animal Preparation and Experimental Protocol

One-day-old male broiler chicks (chunky) were supplied by a local commercial hatchery (Komatsu Hatchery, Nagano, Japan), and were housed in an electrically-heated battery

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brooder where they were provided with water and a commercial starter diet (Toyohashi Co. Ltd., Aichi, Japan) *ad libitum* for 11 days. On day 11, birds of similar body weight were selected and housed in wire-bottomed aluminum cages where they were given free access to a commercial starter diet and water for 3 days. On day 14, chicks were divided into three groups: the fed group, the food-deprived group and the refed group. Fed chickens were maintained as described above. Food-deprived chicks had food removed 24 h before they were killed. Refed chicks were food-deprived for 24 h and then refed for 2 h. All experimental procedures were conducted in accordance with the guidelines established by the Animal Care and Use Committee of the National Institute of Livestock and Grassland Science.

RNA Isolation and Real-time PCR

The heart was rapidly excised, frozen in liquid nitrogen, and stored at -80°C . Total RNA was extracted using TRIzol Reagent (Invitrogen Life Technologies) according to the manufacturer's instructions. Complementary DNA was synthesized from total RNA using random hexamer primers (TaKaRa, Tokyo, Japan) and ReverTra Ace[®] (TOYOBO, Tokyo, Japan). The sequences of the forward and reverse primers were as follows: chicken atrogin-1/MAFbx, forward, 5'-CCA ACA ACC CAG AGA CCT GT-3', and reverse, 5'-GGA GCT TCA CAC GAA CAT GA-3'; chicken 20S proteasome C2 subunit, forward: 5'-AAC ACA CGC TGT TCT GGT TG-3' and reverse: 5'-CTG CGT TGG TAT CTG GGT TT-3'; chicken m-calpain large subunit, forward, 5'-ACA TCA TCG TGC CCT CTA CC-3', and reverse, 5'-GAG ATC TCT GCA TCG CTT CC-3'; chicken cathepsin B, forward, 5'-CAA GCT CAA CAC CAC TGG AA-3', and reverse, 5'-TCA AAG GTA TCC GGC AAA TC-3'; and chicken GAPDH, forward, 5'-CCT CTC TGG CAA AGT CCA AG-3', and reverse, 5'-CAT CTG CCC ATT TGA TGT TG-3'. The mRNA levels were measured by real-time PCR analysis using a LightCycler[®] instrument (Roche Diagnostics, Mannheim, Germany) and the QuantiTect SYBR Green PCR system (Qiagen, Tokyo, Japan). The expression of GAPDH was measured as an internal control.

Statistical Analysis

One-way analysis of variance was performed using the General Linear Models procedure in the Statistical Analysis System (SAS: Version 9.2; SAS Institute Inc., Cary, NC, USA). Significant differences between means were determined using Tukey's multiple comparison test. A P value < 0.05 was considered to be statistically significant. Each result represents the mean $\pm$ standard deviation (SD).

Results and Discussion

The effects of fasting and refeeding on the expression of atrogin-1/MAFbx mRNA in cardiac muscle (heart) were investigated. Although body weight was significantly lowered by fasting (fed, 435 ± 18 g versus fasted, 346 ± 5 g, $p < 0.05$), refeeding for 2 h did not restore the body weight of the fasted chickens to that of fed chickens (refed, 374 ± 17 g). On the other hand, heart weight unaffected by fasting or refeeding (fed, 3.81 ± 0.73 g, fasted, 3.46 ± 0.16 g, refed, 3.16 ± 0.17

Fig. 1. **Effects of fasting and refeeding on mRNA levels of atrogin-1/MAFbx in chicken cardiac muscle.** RNA quantification was performed as described in Materials and Methods. RNA quantification data are expressed as ratios of GAPDH levels in fed chickens, whose expression level was taken to be equal to 1. Values are means $\pm$ SD ($n=6$). Means not sharing the same superscript letter are significantly different ($p < 0.05$).

g). Atrogin-1/MAFbx mRNA levels were significantly increased by fasting in the cardiac muscle, and refeeding significantly suppressed this increase (Fig. 1). This finding is in agreement with those of previous studies showing that atrogin-1/MAFbx in skeletal muscle is induced by fasting and coordinately upregulated during fasting (Gomes *et al.*, 2001; Nakashima *et al.*, 2006; Cleveland and Evenhuis, 2010; Ohtsuka *et al.*, 2011). Fasting also stimulates expression of atrogin-1/MAFbx mRNA in rodent skeletal muscles (Lecker *et al.*, 2004; Dehoux *et al.*, 2004). It has been reported that fasting stimulates atrogin-1/MAFbx expression in the skeletal muscle of chickens (Ohtsuka *et al.*, 2011; Li *et al.*, 2011), indicating that fasting induces atrogin-1/MAFbx expression, and refeeding reduces its expression in the chicken cardiac and skeletal muscle. We also reported that fasting stimulates atrogin-1/MAFbx expression, and refeeding reduces its expression in the chicken skeletal (Nakashima *et al.*, 2006) and smooth (Nakashima *et al.*, 2013) muscles.

During fasting, the increase in atrogin-1/MAFbx gene expression occurs before skeletal muscle weight loss, and the level of atrogin-1/MAFbx mRNA expression is high during conditions of rapid protein degradation (Gomes *et al.*, 2001). In the present study, we examined the effects of fasting and refeeding on the expression of atrogin-1/MAFbx in chicken cardiac muscle. As shown in Fig. 2, the expression of atrogin-1/MAFbx in cardiac muscle was increased by fasting and decreased subsequently by refeeding. This study shows that fasting is associated with increased expression of atrogin-1/MAFbx in chicken cardiac muscle.

We previously reported that refeeding suppresses the expression of atrogin-1/MAFbx mRNA in skeletal muscle (Nakashima *et al.*, 2006). By contrast, Li *et al.* reported that refeeding suppresses atrogin-1/MAFbx expression at the mRNA level, but not at the protein level in chicken skeletal muscle (Li *et al.*, 2011). In the present study, refeeding

Fig. 2. Effects of fasting and refeeding on the mRNA levels of proteasome C2 subunit (A), m-calpain large subunit (B), and cathepsin B (C) in chicken cardiac muscle. RNA quantification was performed as described in Materials and Methods. RNA quantification data are expressed as ratios of GAPDH levels in fed chickens, whose expression level was taken to be equal to 1. Values are means $\pm$ SD ($n=5-6$). Means not sharing the same superscript letter are significantly different ($p<0.05$).

suppressed the expression of atrogin-1/MAFbx mRNA in chicken cardiac muscle. The present study shows that refeeding causes decreased expression of atrogin-1/MAFbx mRNA in chicken cardiac muscle.

Multiple proteolytic systems play key roles in various types of protein loss and muscle wasting. The intracellular proteolytic processes that occur in skeletal muscle are mediated by various proteases, such as lysosomal acidic cathepsins (Hall-Angeras *et al.*, 1991) and Ca^{2+} -dependent calpains (Goll *et al.*, 1991; Goll *et al.*, 1992). Proteins can also be degraded by the ATP-dependent ubiquitin-proteasome system (Coux *et al.*, 1996), an essential pathway for accelerated proteolysis that is activated in various animal models of muscle wasting (Mitch and Goldberg, 1996; Lecker *et al.*, 1999). However, the degradation system(s) that play a role in the breakdown of cardiac muscle proteins has yet to be determined. Despite the known involvement of multiple pathways in protein degradation, the effect of fasting and refeeding on the expression of these proteolytic systems in chicken cardiac muscle has yet to be elucidated at

the molecular level. Thus we examined the effects of fasting and refeeding on the expression of three proteolytic-related genes in cardiac muscle. As shown in Fig. 3, expression of the 20S proteasome C2 subunit (A) and m-calpain large subunit (B) was not affected by fasting or refeeding. By contrast, the expression of cathepsin B mRNA (C) was significantly increased by fasting and further increased by refeeding (Fig. 2-A, -B, and -C). We also show that in cardiac muscle, fasting stimulates the expression of cathepsin B, but not the expression of the 20S proteasome C2 subunit or the m-calpain large subunit. We also reported previously that refeeding suppresses the expression of atrogin-1/MAFbx mRNA, the 20S proteasome C2 subunit, and the m-calpain large subunit, as well as the expression of cathepsin B in chicken skeletal muscle, resulting in a decrease in proteolysis (Nakashima *et al.*, 2005). Here, we show that refeeding increases cathepsin B expression, but not the expression of the 20S proteasome C2 subunit the m-calpain large subunit in chicken cardiac muscle. These findings indicate that the expression of proteolytic-related genes in chicken cardiac

muscle may respond only weakly to fasting and refeeding, suggesting that atrogin-1/MAFbx plays a key role in the initiation and development of atrophy in both the cardiac muscle as well as skeletal muscle of chickens.

The present study shows that atrogin-1/MAFbx expression in cardiac muscle is selectively and coordinately upregulated during fasting. A specific transcriptional program that favors muscle atrophy is likely to be triggered upon food deprivation depending on the metabolic and endocrine status of the chicken. Plasma insulin and insulin like growth factor (IGF) -I are widely believed to be major regulators in driving nutrients toward anabolism. In addition, plasma insulin concentrations increase rapidly with refeeding (Yoshizawa *et al.*, 1997). Insulin was shown to reduce muscle protein degradation by lowering the expression of atrogin-1/MAFbx via mechanism involving the ATP-ubiquitin-dependent pathway in skeletal (Rome *et al.*, 2003) and cardiac (Skurk *et al.*, 2005; Paula-Gomes *et al.*, 2013) muscle. On the other hand, glucocorticoids are known to regulate protein turnover in skeletal muscle (Seene, 1994). Glucocorticoids stimulate muscle protein degradation by increasing the expression of atrogin-1/MAFbx (Sandri *et al.*, 2004; Stitt *et al.*, 2004). In this study, we found that expression of atrogin-1/MAFbx was increased by fasting and decreased by subsequent refeeding in chicken cardiac muscle (Fig. 1). Insulin and IGF-I stimulate phosphoinositide 3-kinase and its downstream effector, Akt, which then phosphorylates FOXO proteins in skeletal (Sandri *et al.*, 2004; Stitt *et al.*, 2004) and cardiac (Skurk *et al.*, 2005; Paula-Gomes *et al.*, 2013) muscle. Phosphorylated FOXO proteins are unable to translocate to the nucleus, where they promote the transcription of the atrogin-1/MAFbx gene in skeletal (Sandri *et al.*, 2004; Stitt *et al.*, 2004) and cardiac (Skurk *et al.*, 2005; Paula-Gomes *et al.*, 2013) muscles. Glucocorticoids and conditions that interfere with IGF-I signaling decrease the phosphorylation of FOXO and increase the expression of atrogin-1/MAFbx in skeletal muscle (Stitt *et al.*, 2004; Sackey *et al.*, 2004). In this context, the nature and effect of other factors, such as peripheral hormones and transcriptional proteins, which may regulate atrogin-1/MAFbx gene expression in chicken cardiac muscle, require further investigation.

In conclusion, we demonstrated that muscle-specific ubiquitin ligase atrogin-1/MAFbx gene expression in chicken cardiac muscle is affected by food deprivation and nutritional supply.

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