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Research paper

The β_2-adrenergic receptor is involved in differences in the protein degradation level of the pectoral muscle between fast- and slow-growing chicks during the neonatal period

Saki Shimamoto[a,b], Daichi Ijiri[a,b,*], Kazuki Nakashima[c], Mana Kawaguchi[b], Akira Ohtsuka[a,b]

[a] The United Graduate School of Agricultural Sciences, Kagoshima University, 1-21-24 Korimoto, Kagoshima 890-0065, Japan
[b] Department of Biochemical Science and Technology, Kagoshima University, 1-21-24 Korimoto, Kagoshima 890-0065, Japan
[c] Animal Metabolism and Nutirition Division, Institute of Livestock and Grasslan Science, NARO, 2 Ikenodai, Tsukuba 305-0901, Japan

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ABSTRACT

The aim of this study was to investigate whether *β_2-AR* mRNA expression is involved in either *atrogin-1/MAFbx* mRNA expression or protein degradation in chicken skeletal muscle by comparing fast- and slow-growing chicks during the neonatal period. Based on their body weight gain from 1 to 5 days of age, 5-day-old chicks (*Gallus gallus domestics*) were divided into a slow-growing and a fast-growing group, the mean weight gains of which were 6.3 ± 1.3 g/day and 11.3 ± 0.9 g/day, respectively. The ratio of pectoral muscle weight to total body weight was higher in the fast-growing group of chicks than in the slow-growing group. In addition, the plasma 3-methylhistidine concentration, an index of protein degradation in skeletal muscle, was significantly lower in the fast-growing than in the slow-growing chicks. The mRNA expression of *β_2-AR*, which we previously found is involved in decreasing muscle protein degradation by suppression *atrogin-1/MAFbx* mRNA expression, was significantly higher in the pectoral muscle of the fast-growing group compared with that of the slow-growing group. Concordantly, lower mRNA expression of *atrogin-1/MAFbx* was observed in the pectoral muscle of the fast-growing chicks. However, in the sartorius muscle, which is a muscle in the thigh, the ratio of the muscle weight to total body weight was not significantly different between the two groups of chicks at 5 days of age. In addition, there was no significant difference in the mRNA expressions of *β_2-AR* and *atrogin-1/MAFbx* in the sartorius muscle between these two groups. These results suggest that *β_2-AR* expression levels might be physiologically significant in the control of protein degradation in the pectoral muscle of neonatal chicks.

1. Introduction

Skeletal muscle mass is controlled through a delicate balance between protein synthesis and protein degradation (Russell, 2010). Changes in the rate of protein degradation may contribute to either normal muscle growth or muscle atrophy (Goldspink, 1976; Goldspink and Goldspink, 1977). In chickens, it has been considered that the muscle protein degradation rate is one of the major factors that affects muscle growth, because fast-growing strains and/or individuals show a lower protein degradation rate compared with slow-growing counterparts (Hayashi et al., 1985; Maeda et al., 1986; Tomas et al, 1988). However, the molecular mechanisms for regulating protein degradation rates in the skeletal muscle of chickens remain unclear.

The rate of protein degradation is regulated through the ubiquitin (Ub)–proteasome system (Jagoe and Goldberg, 2001; Lecker et al., 2004, 2006). In this system, proteins destined for degradation are covalently linked to a chain of Ub molecules, which marks them for breakdown by the 26S proteasome (Ciechanover, 1998; Hershko and Ciechanover, 1998). Since the expression of Ub ligases clearly correlates with polyubiquitination (Sacheck et al., 2004), they appear to play an important role in the control of polyubiquitination, a rate-limiting step in the Ub–proteasome system (Ciechanover, 1998). In chick skeletal muscle, muscle atrophy F box (atrogin-1/MAFbx) is a muscle-specific Ub ligase, and its mRNA expression level shows a positive linear correlation with muscle free 3-methylhistidine (MeHis) content, which is an index of muscle protein degradation (Ohtsuka et al., 2011),

It is well known that increases in skeletal muscle mass induced by β_2-adrenergic receptor (β_2-AR) agonists (for example, clenbuterol and

Abbreviations: Ub, Ubiquitin; AR, adrenergic receptor; atrogin-1/MAFbx, muscle atrophy F-box; Foxo, forkhead box O; MeHis, 3-methylhistidine; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IGF-I, insulin-like growth factor-I

* Corresponding author at: Department of Biochemical Science and Technology, Kagoshima University, 1-21-24 Korimoto, Kagoshima 890-0065, Japan.
E-mail address: ijiri@chem.agri.kagoshima-u.ac.jp (D. Ijiri).

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formoterol) are associated with decreased rates of Ub–proteasome-dependent protein degradation (Busquets et al., 2004; Yimlamai et al., 2005). We previously reported that a β_2-AR agonist also inhibits protein degradation in the skeletal muscles of neonatal chicks (Ijiri et al., 2013, 2014; Shimamoto et al., 2016). In addition, a β_2-AR agonist decreased *atrogin-1/MAFbx* mRNA expression in chick skeletal muscle via phosphorylation of Akt, a serine/threonine protein kinase, and a transcription factor forkhead box O 1 (Foxo1) (Shimamoto et al., 2016). Thus, in chick skeletal muscle, pharmacologic activation of β_2-AR-Akt-Foxo1 signaling is suggested to control muscle mass via suppressing *atrogin-1/MAFbx* mRNA expression. However, it has been not fully understood that the relationship between β_2-AR and protein degradation level expression in chicken skeletal muscles.

In broiler chicken, there is a positive linear correlation with the body weight at 1st week of age and final body weight before shipment (Poultry World, 2013). Therefore, in this study, we investigated whether *β_2-AR* mRNA expression is involved in either *atrogin-1/MAFbx* mRNA expression or protein degradation in chicken skeletal muscle, by comparing fast- and slow-growing chicks during the neonatal period. In this study, the mRNA expressions of *β_2-AR* and *atrogin-1/MAFbx* were examined in two different skeletal muscles; the pectoral muscle, which is a muscle in the breast, and the sartorius muscle, which is a muscle in the thigh.

2. Materials and methods

2.1. Animals

All experimental protocols and procedures were reviewed and approved by the Animal Care and Use Committee of Kagoshima University (approval number A17006, issued on June 30th 2017). Fifty male chicks (*Gallus gallus domestics,* ROSS308) at 1 day of age were supplied by a commercial hatchery (Kumiai Hina Center, Kagoshima, Japan). They were individually numbered and housed together in an electrically heated battery brooder (1200 mm × 600 mm × 400 mm) and provided with water and a semi-purified diet with no animal protein, as shown in Table 1. Body weight was measured at 1 and 5 days of age to calculate body weight gain from 1 to 5 days of age. At 5 days of age, the chicks were divided into two groups based on their body weight gain, a slow-growing group (6.3 ± 1.3 g/day) and fast-growing group (11.3 ± 0.9 g/day). Six chicks were randomly chosen from each of these groups. These chicks were killed by decapitation under carbon dioxide anesthesia, and dissected to collect the pectoral muscle, the sartorius muscle, and liver. They were measured, and then snap frozen in liquid nitrogen and stored at −80 °C until use. Blood samples were collected in heparinized test tubes, which were promptly centrifuged at 5900*g* for 10 min at 4 °C to separate plasma, and then stored at −30 °C until analysis.

Table 1
Composition of experimental diet.

Ingredients (g/100 g)	
Corn meal	57.90
Soybean meal	34.00
Corn oil	4.30
$CaCO_3$	0.66
$CaHPO_4$	2.00
NaCl	0.50
DL-Methionine	0.14
Mineral and vitamin premix[1]	0.50
Calculated analysis	
Crude protein (%)	20.0
Metabolizable energy (MJ/kg)	13.0

[1] Content per kg of the vitamin and mineral premix: vitamin A 300,000 IU, vitamin D3 40,000 IU, DL-alpha-tocopherol acetate 2000 mg, Menadione sodium bisulfite 368 mg, thiamin nitrate 444 mg, riboflavin 720 mg, calcium d-pantothenate 2174 mg, nicotinamide 7000 mg, pyridoxine hydrochloride 851 mg, biotin 30 mg, folic acid 110 mg, cyanocobalamine 2 mg, calcium iodinate 108 mg, MgO 198,991 mg, $MnSO_4$ 32,985 mg, $ZnSO_4$ 19,753 mg, $FeSO_4$ 43,523 mg, $CuSO_4$ 4019 mg and choline chloride 299,608 mg.

2.2. RNA extraction and quantitative real-time PCR

The pectoral muscle, sartorius muscle, and liver were homogenized in ISOGEN II (Nippon gene, Tokyo, Japan) according to the manufacturer's instructions. Real-time PCR was performed as described previously (Shimamoto et al., 2016). In brief, cDNA was synthesized from 60 ng of RNA per 10 μL of reaction solution using the PrimeScript RT Reagent Kit (RR036A; Takara, Shiga, Japan). Samples were incubated at 37 °C for 15 min, 85 °C for 5 s, and 4 °C for 5 min. Gene expression level was measured by real-time PCR using the 7300 Real-Time PCR system (Applied Biosystems, Foster City, CA, USA) with SYBR Select Master Mix (Applied Biosystems). Thermal cycling conditions were as follows: an initial hold at 50 °C for 2 min, 95 °C for 2 min, and then 45 cycles at 95 °C for 15 s, 55 °C for 15 s, and 72 °C for 1 min. The primers used in this study are listed in Table 2. Each sample was run in duplicate along with no template and negative RT controls in each plate. Efficiencies and R2 were assessed using a five-points cDNA serial dilution. Melting curve analysis was conducted and revealed a single peak for all primer pairs. Coefficients of variations were 7–11%. Amplification, dissociation curves and gene expression analysis were performed using Dissociation Curves software (Applied Biosystems). Because there were no significant differences of cycle threshold values of 18S ribosomal RNA each the groups, the level of *18S ribosomal* RNA was used as an internal standard. The results of gene expression levels are shown as a ratio to the value in slow-growing group chicks.

2.3. Protein extraction for western blot analysis

The pectoral muscle and sartorius muscle of chicks was homogenized in 5 mL of lysis buffer consisting of 50 mM Tris, 150 mM NaCl, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM EDTA, and 1% protease inhibitor cocktail, pH 8.0 to examine the phosphorylation levels of Akt and Foxo1. The lysate was centrifuged at 20,000*g* for 10 min at 4 °C and the supernatant was collected. Total protein concentration was estimated by a protein-dye binding assay (Bradford, 1976) using a commercial kit (500-0116; Bio-Rad, Hercules, CA, USA) with bovine serum albumin as the standard. Aliquots were stored at −80 °C until analyzed by western blotting.

Table 2
List of primer sequences used for quantitative real time polymerase chain reaction.

Gene		Sequence (5′-3′)
Atrogin-1/MAFbx	Forward	CCA ACA ACC CAG AGA CCT GT
	Reverse	GGA GCT TCA CAC GAA CAT GA
β_2-adrenergic receptor	Forward	GAC GCC GGA ACG CTG AG
	Reverse	GAA GAC AGT GAC CAG CAC GA
IGF-I	Forward	CTT CAG TTC GTA TGT GGA GAC A
	Reverse	GAT TTA GGT GGC TTT ATT GGA G
IGF-I receptor	Forward	CTG TGT CCG ACA AAT GGG GA
	Reverse	TGA CGG TCA GTT TCG GGA AG
Insulin receptor	Forward	GAC TCT CCA ACG AAC AGG TG
	Reverse	TCA GCA TCT CAA TGA CCT CAA
18S ribosomal RNA	Forward	AAA CGG CTA CCA CAT CCA AG
	Reverse	CCT CCA ATG GAT CCT CGT TA

atrogin/MAFbx, muscle atrophy F-box; IGF-I, insulin-like growth factor-I.

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