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The effects of intraperitoneal clenbuterol injection on protein degradation and myostatin expression differ between the sartorius and pectoral muscles of neonatal chicks

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ABSTRACT

The purpose of this study was to investigate the effects of injection of the β2-adrenergic receptor agonist clenbuterol on the skeletal muscles of neonatal chicks (*Gallus gallus domesticus*). One-day-old chicks were randomly divided into four groups and given a single intraperitoneal injection of clenbuterol (0.01, 0.1, or 1 mg/kg) or phosphate-buffered saline. Twenty-four hours after the injection, the sartorius muscles (which consist of both slow- and fast-twitch fibers) of chicks that received 0.01 or 0.1 mg/kg clenbuterol were significantly heavier than those of controls, while there were no between-group differences in the weight of the pectoral muscles, which consist of only fast-twitch fibers. Muscle free N^t-methylhistidine, regarded as an index of myofibrillar proteolysis, was decreased in the sartorius muscle of the clenbuterol-injected chicks, while it was not affected in the pectoral muscles. In the sartorius muscle of the clenbuterol-injected chicks, myostatin and atrogin-1/MAFbx mRNA expressions were decreased, while insulin-like growth factor-I was unaffected. These observations suggested, in 1-day-old chicks, clenbuterol might increase mass of the sartorius muscle by decreasing myostatin gene expression and protein degradation.

1. Introduction

Skeletal muscle mass is controlled through the 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 birds, lower level of muscle protein degradation in fast-growing broiler chicken than slow-growing layer chicken contributed to their rapid growth (Hayashi et al., 1984). However, the molecular mechanisms underlying regulation of muscle protein degradation levels in birds have been not fully understood.

It is well known that β_2-adrenergic receptors (AR) agonists (e.g., clenbuterol and formoterol) increase skeletal muscle mass in mammals (Choo et al., 1990; Kim and Sainz, 1992; Hinkle et al., 2002; Joassard et al., 2013). The β_2-AR agonists-induced increase in muscle mass is associated with an increased rate of protein synthesis (Hesketh et al., 1992; Navegantes et al., 2004) and decreased rates of either calcium dependent (Navegantes et al., 2001) or ubiquitin–proteasome system (UPS) dependent protein degradation (Busquets et al., 2004; Yimlamai et al., 2005). In mammalian skeletal muscles, clenbuterol suppresses UPS dependent proteolysis via reducing mRNA expression of atrogin-1/MAFbx, which is a muscle-specific ubiquitin ligase (Gonçalves et al., 2012). In birds, we previously reported that a single injection of clenbuterol into 6-day-old chicks increased skeletal muscle weight accompanied by decreased expression of atrogin-1/MAFbx gene (Ijiri et al., 2013), raising the possibility that clenbuterol may suppress myofibrillar protein degradation via inhibiting atrogin-1/MAFbx gene expression in birds as well as mammals.

In mammalian skeletal muscles, protein synthesis/degradation balance and atrogin-1/MAFbx gene expression are regulated by both myostatin and insulin-like growth factor-I (IGF-I) (Sacheck et al., 2004; Amirouche et al., 2009). Myostatin acts as a negative regulator of skeletal muscle growth (McPherron et al., 1997; McPherron and Lee, 1997), and IGF-I also acts either in an autocrine or paracrine manner to induce skeletal muscle hypertrophy (Adams, 1998).

Abbreviations: MeHis, N^t-methylhistidine; AR, adrenergic receptors; IGF-I, insulin-like growth factor-I; i.p., intraperitoneal; PBS, phosphate buffered saline; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

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The purpose of this study was to examine whether intraperitoneal (i.p.) clenbuterol injection into neonatal chicks affects protein degradation. First, we determined the most effective concentration of clenbuterol for promoting the growth of skeletal muscle in chicks. And then, we examined the amount of N^t-methylhistidine (MeHis), to evaluate the degree of muscle protein degradation rate, in plasma and skeletal muscles. In mammal, the effects of clenbuterol on the skeletal muscle mass differ with muscle fiber types, i.e., clenbuterol increased the weight of fast-twitch fiber-rich extensor digitorum longus, while it did not affect the weight of slow-twitch fiber-rich soleus (Sato et al., 2008). Therefore, in this study, the effect of clenbuterol was examined in two different skeletal muscles; the sartorius muscle which is composed of both fast- and slow-twitch fibers and the pectoral muscle which is composed of only fast-twitch fibers (Nishida et al., 1997). In this study, we found that decreased amount of MeHis was observed in the sartorius muscle (but not in the pectoral muscle) of the clenbuterol-injected chicks. Then, we examined mRNA expressions of myostatin, IGF-I, and atrogin-1/MAFbx to investigate the molecular mechanisms of clenbuterol-induced decrease in protein degradation rate in chickens. In addition, we examined whether pretreatment with β-AR antagonist propranolol counteract the effect of clenbuterol on avian skeletal muscle.

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 (Kagoshima, Japan). Ninety-four male 1-day-old ROSS 308 chicks (*Gallus gallus domesticus*) were supplied by a commercial hatchery (Kumiai Hina Center, Kagoshima, Japan). The chicks were given housed in an electrically heated battery brooder in which the temperature was kept at 35 °C.

2.2. Experiment 1

The purpose of this experiment was to determine the concentration of clenbuterol most effective for promoting the growth of skeletal muscle in chicks. Twenty-four 1-day-old chicks were randomly divided into four groups (one control and three clenbuterol-treated groups). The control group received a single i.p. injection of phosphate-buffered saline (PBS), and the three clenbuterol-treated groups received single i.p. injections of 0.01, 0.1, or 1 mg/kg clenbuterol. Clenbuterol was dissolved in PBS (0.08, 0.8, and 8 μg/μL, respectively), and the injection volume was adjusted 40–60 μL to administer at a dose of 0.01, 0.1, and 1 mg/kg body weight, respectively. The chicks were given free access to water and diet. Twenty-four hours after injection, all chicks were weighed and then killed by cervical dislocation under ether anesthesia. The sartorius muscles and pectoral muscles were collected and weighed. The muscles were snap frozen in liquid nitrogen and stored at −80 °C until use.

2.3. Experiment 2

To examine the effect of clenbuterol injection on protein degradation levels, we examined the amount of free MeHis in plasma, the sartorius muscle, and the pectoral muscle of chicks. Ten 1-day-old chicks were randomly divided into two groups (a control group and a clenbuterol group). Chicks in the clenbuterol group were injected with 0.1 mg/kg clenbuterol i.p., while the control chicks received a single i.p. injection of PBS. Clenbuterol was dissolved in PBS (0.8 μg/μL), and the injection volume was adjusted 40–60 μL to administer at a dose of 0.1 mg/kg body weight. The chicks were given free access to water and a semipurified diet with no animal protein as shown in Table 1. Twenty-four hours after injection, all chicks were weighed and then killed by cervical dislocation under ether anesthesia. Blood samples were collected in heparinized test tubes, which were quickly centrifuged at 5900×*g* for 10 min at 4 °C to separate plasma, and stored at −30 °C until analysis. The sartorius muscles and pectoral muscles were collected and frozen in liquid nitrogen and stored at −80 °C until use.

2.4. Experiment 3

The purpose of this experiment was to investigate whether gene expressions of myostatin, IGF-I, and atrogin-1/MAFbx are related to the greater skeletal muscle weight in the chicks injected with clenbuterol. The expressions of these genes in the sartorius and pectoral muscles were quantified using real-time PCR. Thirty-six 1-day-old chicks were randomly divided into two groups (a control and a clenbuterol group). The clenbuterol injection was performed in the same way as in experiment 2. The chicks were given free access to water and diet. Twelve chicks (six control and six clenbuterol) were weighed and then killed by cervical dislocation under ether anesthesia at 1, 4, and 24 h after clenbuterol injection. The sartorius muscles and pectoral muscles were collected and weighed. The skeletal muscles were snap frozen in liquid nitrogen and stored at −80 °C until use.

2.5. Experiment 4

The purpose of this experiment was to examine whether the effect of clenbuterol on avian skeletal muscle is counteracted by the β-AR antagonist propranolol. Twenty-four 1-day-old chicks were randomly divided into two groups. A group of chicks were received PBS by i.p. injection with or without propranolol pretreatment 30 min before injection (Control treatment and Propranolol treatment). Another group of chicks received clenbuterol by i.p. injection with or without propranolol pretreatment 30 min before injection (Clenbuterol treatment and Propranolol + Clenbuterol treatment). Clenbuterol was prepared as well as experiment 2, and propranolol was dissolved in PBS (8 μg/μL), and the injection volume was adjusted 40–60 μL to administer at a dose of 10.0 mg/kg body weight. The concentration of propranolol was

Table 1
Composition of experimental diets.

	Control
Ingredients (g/100 g)	
Corn meal	47.50
Alfalfa meal	2.50
Soybean meal	40.50
Corn oil	5.73
$CaCO_3$	0.66
$CaHPO_4$	2.00
NaCl	0.50
DL-methionine	0.11
Mineral and vitamin premix[1]	0.50
Calculated analysis	
Crude protein (%)	22.5
Metabolizable energy (MJ/kg)	12.9

[1] Content per kg of the vitamin and mineral premix: vitamin A 90 mg, vitamin D3 1 mg, DL-alpha-tocopherol acetate 2000 mg, vitamin K3 229 mg, thiamin nitrate 444 mg, riboflavin 720 mg, calcium D-pantothenate 2174 mg, nicotinamide 7000 mg, pyridoxine hydrochloride 700 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.