《Research Note》

In vivo Administration of β_2 -agonist Clenbuterol and Subsequent Increase in Skeletal Muscle Mass in Neonatal Chicks

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In this study, the effect of β_2 -agonist clenbuterol administration was investigated in chicks (*Gallus gallus domesticus*) at 6 days of age. Chicks were given single subcutaneous administration of clenbuterol dissolved in saline at a concentration of 1 mg/kg body weight. Twenty-four hours after clenbuterol administration, the ratio of skeletal muscle weight to body weight was increased in chicks treated with clenbuterol, while their body and heart weight were not affected. Although expressions of IGF-I and myostatin mRNA were not changed, the expression of atrogin-1/MAFbx mRNA was significantly decreased by clenbuterol administration. This observation suggested that administration of clenbuterol to neonatal chicks might increase the proportion of muscle mass to body mass. And the decreased expression of atrogin-1/MAFbx might be involved in the growth of skeletal muscle of chicks treated with clenbuterol.

Key words: atrogin-1/MAFbx, β_2 -adrenergic agonist, neonatal chicks, skeletal muscle growth

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Introduction

Adrenaline is secreted directly into the blood from the adrenal medulla and functions in several tissues via adrenergic receptors, which are classified into three major groups (α_1 , α_2 , and β). It has been well known that β -adrenergic receptor signaling regulates several physiological events of skeletal muscles (Sato *et al.*, 2011). β_2 -adrenergic agonists (e.g. clenbuterol) induce skeletal muscle hypertrophy in mammals (Choo *et al.*, 1990; Kim and Sainz, 1992; Hinkle *et al.*, 2002), because among the β -adrenergic receptors (β_1 , β_2 , and β_3), β_2 -adrenergic receptor is involved in skeletal muscle hypertrophy (Hinkle *et al.*, 2002). For instance, water supplementation with clenbuterol resulted in the increase in skeletal muscle mass 3 days after administration in rats (Awede *et al.*, 2002). In addition, rats received subcutaneous injections of clenbuterol at a concentration of 1 mg/kg body weight for 14 days showed skeletal muscle hypertrophy (Wineski *et al.*, 2002). Also in birds, dietary supplementation with clenbuterol (1 mg/kg diet) for 3 weeks (between 28 to 49 days of age) improves growth and skeletal muscle characteristics in broiler chickens, i.e., both body and

skeletal muscle weight were increased (Rehfeldt *et al.*, 1997). Although administration of clenbuterol might be effective in improving skeletal muscle growth of chickens, the molecular mechanisms underlie the hypertrophic effect of clenbuterol on skeletal muscle of birds remains unclear.

Insulin-like growth factor-I (IGF-I) acts either in an autocrine or in a paracrine manner to induce skeletal muscle hypertrophy (Adams, 1998), meanwhile, myostatin is also known to act as a negative regulator of skeletal muscle growth (McPherron *et al.*, 1997; Joulia *et al.*, 2003). Over the past decade, it has been elucidated that either IGF-I or myostatin is involved in the clenbuterol-induced hypertrophy of skeletal muscles in mammals (Awede *et al.*, 2002; Matsumoto *et al.*, 2006; Abo *et al.*, 2012). IGF-I and myostatin gene are widely conserved among mammals and birds (McPherron and Lee, 1997; Wood *et al.*, 2005), raising the possibility that these two genes may involved in the hypertrophic effect of clenbuterol on skeletal muscle of birds.

In this study, we examined two hypotheses that 1) subcutaneous administration of clenbuterol to neonatal chicks increases their skeletal muscle mass, and 2) either IGF-I or myostatin are related to the clenbuterol-induced increase in skeletal muscle mass of neonatal chicks. In addition, Navegantes *et al.* (2001) reported that clenbuterol treatment decreases proteolysis in the skeletal muscle of rats. In chicken, gene expression of atrogin-1/MAFbx positively

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correlates with skeletal muscle proteolysis (Ohtsuka *et al.*, 2011). Therefore, we also examined gene expression of atrogin-1/MAF box as a representative of proteolysis related proteins in skeletal muscle of chicks.

Materials and Methods

Animals

All experimental protocols and procedures were reviewed and approved by the Animal Care and Use Committee of the University of Tsukuba (Tsukuba, Ibaraki, Japan). Fertilized eggs from genetically similar Rhode Island Red chicks (*Gallus gallus domesticus*) were a gift from the Ibaraki Prefectural Livestock Research Center (Ishioka, Ibaraki, Japan). Chicks were hatched and bred at the Agricultural and Forestry Research Center, University of Tsukuba. Breeding methods were in accordance with the University of Tsukuba guidelines. All chicks were kept at a thermoneutral temperature (30°C and 60% relative humidity) and were provided food and water *ad libitum* with constant 24 hours lighting.

Experimental Design

Neonatal chicks are highly sensitive to various stimulations, e.g., 6 days-old chicks of Rhode Island Red respond to cold conditions by increasing their muscle mass within 24 hours (Ijiri *et al.*, 2009), therefore, we used Rhode Island Red chicks at 6 days of age. Twelve chicks were randomly divided into two groups (i.e. clenbuterol and control). The clenbuterol groups of chicks were given single subcutaneous injection of clenbuterol dissolved in saline at a concentration of 1 mg/kg body weight. Control chicks were injected subcutaneously with saline alone. At 24 hours after administration of saline or clenbuterol, all chicks were weighed, and then killed by cervical dislocation under ether anesthesia, and the tissues (i.e., sartorius muscles, pectoral muscles, and heart) were collected and weighed. Sartorius muscles collected from both legs were immediately frozen in liquid nitrogen, and stored at -80°C until use for mRNA expression.

RNA Extraction and Quantitative Real-time PCR.

Total RNA was purified using ISOGEN reagent (NIPPON GENE, Tokyo, Japan) according to the manufacturer's instructions. Real-time PCR was performed as described

previously (Kamizono *et al.*, 2010). In brief, cDNA was synthesized at 40 ng RNA per 10 μL of reaction solution with PrimeScript RT reagent Kit (Takara, Shiga, Japan), which was set at reverse transcription 37°C for 15 min, inactivation of reverse transcriptase 85°C for 5 s, and refrigeration 4°C for 5 min using PC-320 (ASTEC, Fukuoka, Japan). The primers used in this study are listed in Table 1. Quantitative real-time PCR was performed in the Applied Biosystems 7300 Real-time PCR system (Applied Biosystems, Foster City, CA) using SYBR Premix ExTagTM (Takara, Shiga, Japan) according to the manufacturer's protocols. The thermal cycle was as follows: 1 cycle at 95°C for 30 s, and 60 cycles at 95°C for 5 s, 60°C for 30 s, and 80°C for 31 s. Expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA was used as an internal standard and was not significantly different between the cold and control groups. Gene expression results are shown as a percentage of the control value.

Statistical Analysis

Results are presented as mean $\pm$ SEM throughout. Where applicable, data were analyzed using Fisher's exact probability test, Student's t-test, or Welch's t-test, taking $P < 0.05$ as the level of significance.

Results

Table 2 shows the body and tissue weight of chicks 24 hours after clenbuterol administration. Although no statistical significance was observed between treatments, the weights of sartorius muscle tended to increase ($P = 0.07$) in chicks treated with clenbuterol. The skeletal muscle yield (ratio of skeletal muscle weight to body weight) was shown in the Table 3. The yields of sartorius muscle and pectoral muscle in chicks treated with clenbuterol at a concentration of 1 mg/kg body weight were significantly higher than those of the control chicks, respectively.

The mRNA expression levels of IGF-I, myostatin, and atrogin-1/MAFbx in the sartorius muscle of chicks 24 hours after clenbuterol administration are shown in Fig 1. The expressions of IGF-I and myostatin mRNA were not different between treatments, while the expression of atrogin-1/MAFbx mRNA in chicks injected with clenbuterol was significantly lower than that in the control chicks 24 hours

Table 1. List of primers sequence used for quantitative real time polymerase chain reaction

Gene		Sequence (5'-3')	Size (BP)	Accession No.
IGF-I ¹	Forward	CTT CAG TTC GTA TGT GGA GAC A	167	NM_001004384.2
	Reverse	GAT TTA GGT GGC TTT ATT GGA G		
myostatin	Forward	TCA CAT TCC CAG GAC CGG GTG AAG A	151	NM_001001461.1
	Reverse	TCC ACT GTC AGCGGG TAG CGA CA		
atrogin-1/MAFbx ²	Forward	CCA ACA ACC CAG AGA CCT GT	174	NM_001030956
	Reverse	GGA GCT TCA CAC GAA CAT GA		
GAPDH ²	Forward	CCT CTC TGG CAA AGT CCA AG	200	NM_204305.1
	Reverse	CAT CTG CCC ATT TGA TGT TG		

¹ Penha *et al.* (2011). ² Nakashima *et al.* (2009). IGF-I, insulin-like growth factor-I; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

Table 2. **Body and tissue weights in chicks injected with clenbuterol**

	control	clenbuterol
body weight (g)	61.20±2.92	56.65±2.54
sartorius muscle weight (mg)	133.25±4.90	147.08±4.61
pectoral muscle weight (mg)	803.42±112.12	931.17±71.75
heart weight (mg)	410.00±15.82	382.67±18.59

Values are means±SEM for 6 control and 6 clenbuterol-treated chicks.

Table 3. **Comparison of tissue yields among the different groups**

	control	clenbuterol
sartorius muscle yield (mg/g body weight)	2.20±0.11	2.61±0.10*
pectoral muscle yield (mg/g body weight)	12.87±1.19	16.36±0.87*

Values are means±SEM for 6 control and 6 clenbuterol-treated chicks. * $P<0.05$ (vs.control).

Fig. 1. **Quantitative real-time PCR analysis of gene expression in sartorius muscle of chicks 24 hours after subcutaneous administration of clenbuterol (1 mg/kg body weight).** (A) Expression of IGF-I mRNA in sartorius muscle of chicks. (B) Expression of myostatin mRNA in sartorius muscle of chicks. (C) Expression of atrogen-1/MAFbx mRNA in sartorius muscle of chicks. Results are expressed as % of respective control value, and values are expressed as means±SE ($n=6$). * $P<0.05$ (vs. control). IGF-I, insulin-like growth factor-I.

after clenbuterol administration.

Discussion

In mammal, it has been described skeletal muscle hypertrophy was induced by clenbuterol administration (Choo *et al.*, 1990; Kim and Sainz, 1992; Hinkle *et al.*, 2002). However, there have been very few reports on the effect of clenbuterol on the skeletal muscle of birds. The data obtained in this study demonstrates that a single administration of clenbuterol to 6 days-old chicks induces increase in the skeletal muscle mass 24 hours after administration. This is in agreement with several other authors' work on the effect of clenbuterol on the skeletal muscle of birds, e.g., the weight

of pectoral muscle and thigh muscles in broiler chickens were increased by dietary supplementation with clenbuterol for 3 weeks (Rehfeldt *et al.*, 1997). Therefore, clenbuterol might have hypertrophic effects on skeletal muscle of birds as well as mammals.

Abo and co-workers (2012) suggested that IGF-I works as a positive regulator in the earlier stages and myostatin functions as a negative regulator in the later stages of the clenbuterol-induced hypertrophy of skeletal muscle in rats, respectively, i.e., increase in the weight of skeletal muscle accompanied by the phosphorylation of IGF-I protein was observed in rats treated with clenbuterol 3 days after administration. However, in this study, mRNA expression

level of IGF-I was not changed in the skeletal muscle of chicks 24 hours after clenbuterol administration. This is in agreement with the earlier studies on the clenbuterol-induced hypertrophy in skeletal muscle; e.g. although 3 days of clenbuterol administration induced increase in IGF-I mRNA expression, the IGF-I mRNA expression was not increased 24 hours after clenbuterol administration in skeletal muscle of rats (Awede *et al.*, 2002). Therefore, it is suggested that IGF-I might not be involved in the initial stage of clenbuterol administration in neonatal chicks. In addition, in our previous study, neonatal chicks exposed to cold for 24 hours showed the cold-induced muscle hypertrophy accompanied by decreased expression of myostatin mRNA, suggesting that this gene could be involved in the initial stage of muscle growth in neonatal chicks (Ijiri *et al.*, 2009). Therefore, in this study, we examined myostatin mRNA expression in the skeletal muscle of neonatal chicks treated with clenbuterol, however, myostatin mRNA expression was not influenced by clenbuterol administration.

As little information is available for clenbuterol-induced hypertrophy in birds, the reason why the weight of skeletal muscle of chicks was increased by clenbuterol administration remains unclear. One possible explanation is inhibition of ubiquitin-dependent proteolysis by decreasing muscle specific ubiquitin ligase, atrogin-1/MAFbx. In this study, we demonstrated that the expression of atrogin-1/MAFbx mRNA was significantly decreased 24 hours after clenbuterol administration. *In ex vivo* cultures, decreased proteolysis was observed in normal rat skeletal muscle within 2 hours after clenbuterol treatment (Navegantes *et al.*, 2001). In denervated rat skeletal muscle, clenbuterol suppresses proteasomal and lysosomal proteolysis and transcription of atrogin-1/MAFbx (Gonçalves *et al.*, 2011). These observations suggested that clenbuterol might affect either proteolysis in skeletal muscle or expression of atrogin-1/MAFbx. In addition, Ohtsuka *et al.* (2011) reported that gene expression of atrogin-1/MAFbx positively correlates with skeletal muscle proteolysis in chickens, hence it is possible that a single administration of clenbuterol to neonatal chicks resulted in the increase in the weight of skeletal muscle by decrease in atrogin-1 mRNA expression. Further studies are needed to gain more information about the molecular mechanisms underlie that hypertrophic effects of clenbuterol on skeletal muscle of neonatal chicks.

In conclusion, *in vivo* administration of clenbuterol at a concentration of 1 mg/kg body weight to neonatal chicks at 6 days of age may alter the proportion of skeletal muscle mass. Neither IGF-I nor myostatin expression was affected by clenbuterol administration; however decreased in atrogin-1/MAFbx expression was observed in the skeletal muscle of chicks treated with clenbuterol, suggesting the possibility that this gene could be involved in the clenbuterol-induced skeletal muscle growth in chicks.

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