早稲田大学審査学位論文
博士（人間科学）

Muscle Plasticity and β_2-Adrenergic Receptor Expression

骨格筋の可塑性とβ_2−アドレナリン受容体発現

2012 年 1 月

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PREFACE

The studies presented in this thesis have been carried out under the direction of Prof. Kazuhiko Imaizumi at the Laboratory of Physiological Sciences, Faculty of Human Sciences, Waseda University during 2009-2011.

The thesis consists of three parts of a study; 1) the effects of pharmacological β_2-adrenergic receptor stimulation on the expression of β_2-adrenergic receptor in rat skeletal muscle, 2) the effects of pharmacological glucocorticoid receptor stimulation on the expression of β_2-adrenergic receptor in rat skeletal muscle, and 3) the effects of immobilization-induced muscle disuse on the expression of β_2-adrenergic receptor in rat skeletal muscle.

I wish to express my grateful gratitude to Prof. Kazuhiko Imaizumi. His kind guidance, precious comments, continuous encouragement and valuable discussion are deeply appreciated. I also wish to express my sincere thanks to Prof. Kaoru Tachiyashiki (Department of Natural and Living Sciences, Graduate School of Education, Joetsu University of Education) and Dr. Ken Shirato (Faculty of Human Sciences, Waseda University) for their pertinent guidance, many suggestions and valuable discussion. I also appreciate Prof. Hideki Suzuki (Department of Health and Physical Education, Aichi University of Education), Prof. Hisaya Tsujimoto (Institute of Health and Sports Science, Kurume University), Dr. Sachiko Nomura (Graduate School of Medicine, Osaka City University) and Dr. Jun Tanihata (National Center of Neurology and Psychiatry) for their technical support and kind help.

Finally, I thank my family for their affectionate encouragements throughout this study.

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October 19, 2011

CONTENTS

1.1. Introduction

The skeletal muscle is the most abundant tissue in the human body comprising 40–50% of body mass. Skeletal muscle protein undergoes rapid turnover, which is regulated by the balance between the rates of protein synthesis and degradation. Physical activity (exercise training), and anabolic hormones and drugs (sports doping) increase muscle protein content. However, sarcopenia and muscle disuse (due to unloading, microgravity, or inactivity) and diseases decrease muscle protein content. The rate of protein synthesis is at least in part mediated by β_2-adrenergic receptors (β_2-ARs) in skeletal muscles in both anabolic and catabolic conditions.

ARs belong to the guanine nucleotide-binding G protein-coupled receptor (GPCR) family. Skeletal muscle contains a significant proportion of β-ARs. The β_2 subtype is the most abundant, while ~7–10% of ARs are the β_1 subtype [1, 2]. Furthermore, β_2-AR is more dense in slow-twitch muscles than in fast-twitch muscles [3, 4]. However, the magnitude of anabolic responses to β_2-adrenergic agonists is greater in fast-twitch muscles than in slow-twitch muscles [5-8].

The family of β-ARs was originally believed to signal predominantly via coupling with a stimulatory guanine nucleotide-binding protein, $G\alpha_s$; however, recent studies revealed that both β_2- and β_3-ARs in skeletal muscle are also capable of coupling to an inhibitory guanine nucleotide-binding protein, $G\alpha_i$ [9]. β_2-AR activates the $G\alpha_s$/adenylyl cyclase (AC)/cyclic adenosine monophosphate (cAMP)/cAMP-dependent protein kinase A (PKA) signaling pathway. The signaling pathway is at least in part responsible for the anabolic response of skeletal muscle to β_2-AR stimulation. Further, in addition to the well-documented inhibition of AC activity [10], β_2-AR coupling to $G\alpha_i$ activates $G\alpha_s$-independent pathways [11].

β_2-AR has 7 transmembrane α helices forming 3 extracellular loops, including an NH_2 terminus and 3 intracellular loops that include a COOH terminus [12]. β_2-AR contains phosphorylation sites in the third intracellular loop and proximal cytoplasmic tail. Phosphorylation of these sites triggers the agonist-promoted desensitization, internalization, and degradation of the receptor [13]. These regulatory mechanisms contribute to maintaining agonist-induced β_2-AR

responsiveness in various conditions. In addition to the post-translational process, β_2-AR synthesis, including transcription and subsequent translation, is required to restore transmembrane receptor density.

Under several physiological and pharmacological conditions, changes in β_2-AR density would likely reflect responsibility to circulating catecholamine or injected agonist, and subsequent rate of muscle protein synthesis or degradation. Therefore, the transcriptional and translational responses of β_2-AR are widely thought to play an important role in plastic regulation of muscle protein synthesis and degradation. Furthermore, understanding the correlation between changes in muscle mass and β_2-AR expression in several anabolic or catabolic conditions present scientific evidence to eradicate sports doping and identify novel approaches for attenuating muscle atrophy concomitant with disuse and various diseases. This thesis summarizes the effects of (1) β_2-agonist clenbuterol [CHAPTER 1 and CHAPTER 2], (2) synthesized glucocorticoid dexamethasone [CHAPTER 3], and (3) casted-immobilization [CHAPTER 4] on β_2-AR expression in rat skeletal muscle. The thesis also outlines the functional roles of β_2-adrenergic receptors in skeletal muscle hypertrophy and atrophy as well as the adaptive responses of β_2-adrenergic receptor expression to anabolic and catabolic conditions [CHAPTER 5].

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2. CHAPTER 1

Effects of the β_2-agonist clenbuterol on β_1- and β_2-adrenocepter mRNA expressions of rat skeletal and left ventricle muscle [Sato S, Nomura S, Kawano F, Tanihata J, Tachiyashiki K, Imaizumi K (2008) J Pharmacol Sci 107:393-400]

2.1. Introduction

Doping drugs are categorized into stimulants, analgesics, anabolic agents, diuretics, masking agents, peptide hormones and their homologues, and anti-estrogen agents [1-3]. Athletes are prohibited from using these drugs to improve athletic performance. However, many types of doping drugs have been used by athletes despite many suppressive and side reactions [1, 3]. Furthermore, many substances, including steroids, β_2-agonists, erythropoietins, growth hormones, transforming growth factor-α, β, fibroblast growth factors, mechano-growth factors, and insulin-like growth factor-1 have been used as doping drugs for the improvement of athletic performance such as increasing muscle strength, muscle power, and endurance capacity [1-3]. Especially, anabolic androgenic steroids (i.e., methandienone, nandrolone, 19-norandrogen, stanozolol, and 19-norandrostendion) and β_2-agonists (i.e., clenbuterol, salbutamol, metaproterenol, fenoterol and clorpreneline) have been known to heighten muscle strength and muscle power [1-9].

The β_2-agonist clenbuterol [4-amino-α (*t*-butyl-amino) methyl-3,5-dichlorobenzyl alcohol] has been used as a non-steroidal anabolic drug for sports doping and, as a consequence, focused on since Sydney Olympic Games in 2000. According to the recent World Anti-Doping Agency documents [10], the use of clenbuterol was the fifth most common case in the number of anabolic drugs-used contravention in 2006 (53 cases). Although clenbuterol is known to heighten muscle power and increase muscle mass, the precise mechanism of these responses is still unknown [4-9].

The pharmacokinetics and dynamics of clenbuterol are unique. Clenbuterol showed high affinity toward both β_1-adrenoceptor (β_1-AR) and β_2-adrenoceptor (β_2-AR) and selectivity toward the β_2-AR, relative to other β-agonists [11]. Equilibrium dissociation constants of clenbuterol were

38 and 6.3 nM for β_1- and β_2-ARs, respectively [11]. The high affinity of clenbuterol toward β_1- and β_2-ARs was coupled to a low relative efficacy of clenbuterol to activate either β_1- or β_2-ARs [11]. Most β_2-agonists such as isoproterenol or salbutamol require approximately 1%-3% β-AR occupation for 40%-50% relaxation of the jugular vein, whereas clenbuterol required approximately 100% β-AR occupation for a similar response [11]. Moreover, clenbuterol has a relative longer half-life of about 26 h [12].

Clenbuterol is also mainly used for a therapeutic agent of asthma as a bronchodilator having relaxing actions on bronchial smooth muscles [13]. In addition to bronchodilatation, clenbuterol have many physiological actions such as lipolysis, glycolysis, glycogenolysis, vasodilatation, and cardiac actions [4, 5, 13, 14]. Many of these clenbuterol-induced physiological and pharmacological effects are also shown to be mediated through β_2-AR [3-5, 13-16]. It is well known that β_2-adrenergic stimulation activates a guanine nucleotide-binding protein (G-protein), leading to activation of adenylyl cyclase and production of cyclic adenosine monophosphate (cAMP), which is largely responsible for β_2-AR-mediated intracellular effects [4, 15, 16]. However, long-term β-adrenergic stimulations are known to promote the desensitization and down-regulation of β-ARs [7, 16-19]. Down-regulation is defined as a decline in the total number of receptors caused by prolonged exposures [16]. On the one hand, β-AR binding to agonists is known to be desensitized because of the phosphorylation at the C-terminal tail of receptors by G-protein-coupling receptor kinase and/or at the third intracellular loop of receptors by cAMP-dependent protein kinase A (PKA) [16, 17]. The post processes, internalization-endosome formation-degradation or resensitization, depend on the magnitude, kinetics, and duration of stimulations [16]. On the other hand, activation of cAMP response element binding protein (CREB) by PKA is related with β-AR mRNA transcription [18, 20, 21]. Furthermore, transcribed β-AR mRNA is eliminated by spontaneous and/or β-adrenergic receptor binding protein (β ARBP)–mediated degradation [22-24]. From these view points, the down-regulation of β-AR is associated with both pre-translational and post-translational processes. Although there have been many reports about the post-translational process of β-AR in skeletal muscles, respiratory tissues, and cardiac muscle, little is known about the

transcriptional control and mRNA stability.

It is well known that skeletal muscles are composed of slow-twitch and fast-twitch muscle fibers [15]. The proportion of these muscle fibers is different in each skeletal muscle. Extensor digitorum longus (EDL) and soleus (SOL) muscles are known as a typical fast-twitch fiber-rich and a slow-twitch fiber-rich muscle, respectively, in rats [7, 25]. These muscle fibers are different in the velocity of contraction, metabolic properties, and β-AR distributions [4, 7, 14, 15, 25]. Jensen et al. [26, 27] reported that the β_2-AR subtype was contained in about 80%-95% of total β-ARs in skeletal muscles and relatively higher in the slow-twitch fiber-rich SOL muscle than in the fast-twitch fiber-rich EDL muscle. The effects of β-adrenergic stimulations on β_1- and β_2-AR mRNA expressions in muscle fibers, however, are not still elucidated. Furthermore, β-agonists also are known to induce inotropic and chronotropic actions via β_1- and β_2 ARs of cardiac muscle [16, 28, 29]. The β_1-AR subtype of cardiac muscle is known to be the predominant receptor and contained in about 70%-80% of total β-ARs in human ventricle (ranging from 60%-80% in ventricles of various mammals including human, rat, canine, and feline) [28, 29]. Although β-agonists have been used as therapeutic agents for several types of heart diseases, the effects of β_2-agonists on β-AR mRNA expressions are still unclear [16, 29]. In the present study, therefore, the effects of the administration of the β_2-agonist clenbuterol for 10 days on β_1- and β_2-AR mRNA expressions of EDL, SOL, and cardiac (left ventricle: LV) muscles were studied in adult male rats. The effects of clenbuterol on RNA concentration of these muscles were also examined.

2.2 Materials and methods

2.2.1. Experimental procedures

The present study was carried out according to the protocol shown in Fig. 1. During the experimental period, clenbuterol was administered to rats for 10 days (dose= 1.0 mg/ kg body weight/ day). EDL, SOL and LV muscles were isolated and weighed on the next day after the final

day of the administration of clenbuterol to clarify the effects of β_2-agonist, clenbuterol on β_1 and β_2 AR mRNA expressions of EDL, SOL, and LV muscles.

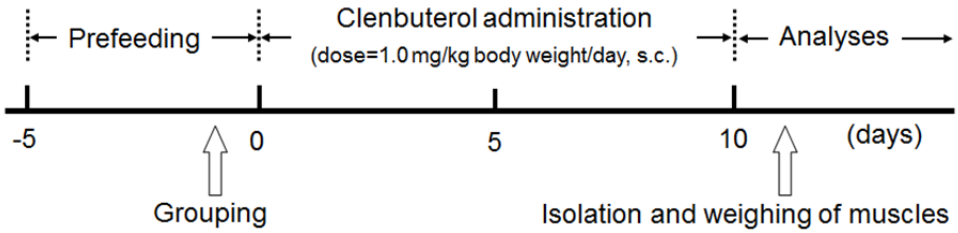

Fig. 1. Experimental protocol used in the study.
Clenbuterol (dose=1.0mg/kg body weight/day) was administered from the cervical portion of the back by a subcutaneous (s.c.) injection (8:00-8:30) for 10 days. In the control group rats, an equivalent volume of 0.9% NaCl solution was administered in the same manner. Muscles were isolated and weighed on the next day after the final day of the administration of clenbuterol.

2.2.2. Animal care

Male 7-week-old Sprague Dawley rats (CLEA Japan, Tokyo) were pre-fed for 5 days to allow adaptation to their new environment [30, 31]. Rats were maintained at a controlled temperature (23°C- 25°C) and a relative humidity (50%- 60%), with fixed light-dark cycles [8:00- 20:00 (light) and 20:00- 8:00 (dark)] [30, 31]. Animal foods (CE-2 cubic type; CLEA Japan) were given to each rat under diet-restricted feeding (feeding dosage= 30 g/ day) and distilled water was given ad libitum [30]. All rats were weighed daily during the experimental period. After the adaptation period, the rats were randomly divided into two groups, the clenbuterol-administered (n= 10, the initial body weight= 279± 2 g, means± S.E.M.) group and the control (n= 9, the initial body weight=279± 2 g, means± S.E.M.) group.

All experimental and animal care procedures were approved by the Committee on Animal Care Use at Waseda University and followed the Guiding Principles for the Care and Use of Animals in the Field of Physiological Sciences established by the Physiological Society of Japan and the American Physiological Society Animal Care Guidelines. We performed procedures with the least possible pain or discomfort to the rats.

2.2.3. Administration of clenbuterol to rats

Clenbuterol hydrochloride (Sigma, St. Louis, MO, USA) was dissolved in 0.9% NaCl solution as a vehicle to obtain a clenbuterol concentration of 0.1%. In the clenbuterol-administered group, clenbuterol (dose= 1.0 mg/ kg body weight/ day) was administered from cervical portion of the back via a subcutaneous (s.c.) injection (8:00- 8:30) for 10 days. In the control group, an equivalent volume of 0.9% NaCl solution was administered in the same manner [30].

2.2.4. Sample storage

Isolated and weighed skeletal muscle was cut the both ends and preserved in RNA*later* solution (Ambion, Austin, TX, USA) to stabilize RNA. In addition, residual blood in the isolated heart was removed by washing with by autoclaved 0.9% NaCl solution, and then the heart was separated into LV and the other sections. Then the LV was weighed and preserved in RNA*later* solution. The samples preserved in RNA*later* solution were stored at -20°C after the incubation at 4°C overnight.

2.2.5. Analyses of mRNA expressions by real-time quantitative reverse transcription-polymerase chain reaction (RT-PCR)

Real-time quantitative RT-PCR was used to quantify β_1- and β_2-AR mRNA expression levels. Stored muscle samples were homogenized using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. RNeasy Mini Spin Column, an adjunct with RNeasy Fibrous Tissue Mini Kit (QIAGEN, Hilden, Germany), was used for the purification of RNA. The RNA concentration was determined by measuring absorbance at 260 and 280 nm (U-3310 Spectrophotometer; Hitachi, Tokyo) according to our routine method [32].

The extracted RNA was subjected to single-stranded cDNA synthesis using a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Faser City, CA, USA) according to the manufacturer's protocol. In quantitative RT-PCR, synthesized cDNA was added to a SYBR Premix Ex Taq reaction mixture (TaKaRa Bio Inc., Shiga) containing 200 nM PCR primer

(forward and reverse) [32]. Primer sequences for real-time RT-PCR were: β_1-AR: 5'-CTG CTA CAA CGA CCC CAA GTG-3' (forward), 5'-AAC ACC CGG AGG TAC ACG AA-3' (reverse) and β_2-AR: 5'-GAG CCA CAC GGG AAT GAC A-3' (forward), 5'-CCA GGA CGA TAA CCG ACA TGA-3' (reverse). TATA-box binding protein (TBP) was used as the reference gene [33-35]. Amplification was performed using an ABI PRISM 7000 Sepuence Detection System (Applied Biosystems) [32]. β_1- and β_2-AR mRNA levels were normalized using the Threshold Cycle (C_t) method in accordance with the manufacturer's protocol and were expressed as ratios relative to TBP mRNA levels [32].

2.2.6. Statistical analyses

Experimental data are presented as the means± S.E.M. The differences between two groups were tested by a Student *t*- test and considered significant when P was < 0.05.

2.3 Results

2.3.1. Effects of clenbuterol on the body weight and the weights, RNA concentration, and total RNA content of muscles

We investigated the effects of clenbuterol on the body weight and the weights, RNA concentration, and total RNA content of EDL, SOL, and LV muscles. As shown in Table 1, there were no significant differences of the body weight between both groups. The weight of EDL muscle was 1.14 times ($P< 0.001$) higher in the clenbuterol-administered group than that in the control group. Furthermore, RNA concentration and total RNA content of EDL muscle were 1.29 ($P< 0.001$) and 1.48 times ($P< 0.001$) higher, respectively, in the clenbuterol-administered group than those in the control group. However, the weight, RNA concentration, and total RNA content of SOL and LV muscles in the clenbuterol-administered group were comparable with those in the control group. These results showed that the effects of clenbuterol on muscle hypertrophy were

markedly higher in fast-twitch fiber-rich EDL muscle than in slow-twitch fiber-rich SOL muscle.

Table 1 Effects of clenbuterol on the body weight and the weight, RNA concentration, and total RNA content of EDL, SOL, and LV muscles.

Parameters	Group		clenbuterol / control
	control	clenbuterol	
Body weight (g)	360 ± 3	364 ± 5	1.01
EDL			
Weight (mg)	170 ± 3	194 ± 4 ***	1.14
RNA concentration (μg/g)	677 ± 23	873 ± 33 ***	1.29
Total RNA content (μg)	115 ± 4	170 ± 9 ***	1.48
SOL			
Weight (mg)	133 ± 3	137 ± 2	1.03
RNA concentration (μg/g)	1216 ± 59	1271 ± 54	1.05
Total RNA content (μg)	163 ± 10	175 ± 10	1.08
LV			
Weight (mg)	796 ± 13	815 ± 25	1.02
RNA concentration (μg/g)	1021 ± 60	1070 ± 39	1.05
Total RNA content (μg)	838 ± 54	855 ± 33	1.02

The values are each the means±S.E.M. (n=8-10/group). control: control group, clenbuterol: clenbuterol-administered group, clenbuterol/control: the relative ratio of the clenbuterol-administered group to the control group. EDL: extensor digitorum longus, SOL: soleus, LV: left ventricle muscles. Statistics: ***p<0.001 (*vs.* the control group).

2.3.2. Effects of clenbuterol on β_1-AR mRNA expression of muscles

Figure 2 showed the effects of clenbuterol on β_1-AR mRNA expression of EDL, SOL and LV muscles. There were no significant differences of β_1-AR mRNA expression of EDL (Fig. 2A) and SOL (Fig. 2B) muscles between both groups. However, β_1-AR mRNA expression of LV muscle was 0.84 times (P< 0.05) lower in the clenbuterol-administered group than in the control group (Fig. 2C).

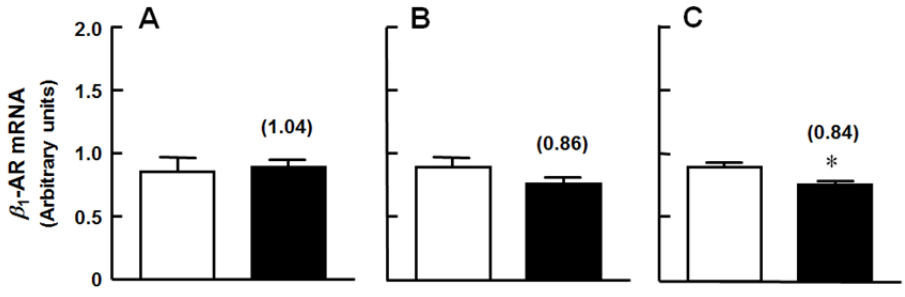

Fig. 2. Effects of clenbuterol on β_1-AR mRNA expression of EDL (A), SOL (B), and LV (C) muscles.
The values are each the mean±S.E.M. of 8-10 rats/group. Open bar: control group and closed bar: clenbuterol administered group. Values in parentheses are shown as the relative ratio of the clenbuterol-administered group to the control group. Statistics: *P<0.05 (vs. the control group).

2.3.3. Effects of clenbuterol on β_2-AR mRNA expression of muscles

As shown in Fig. 3, β_2-AR mRNA expression of EDL was 0.84 times ($P < 0.05$) lower in the clenbuterol-administered group than in the control group (Fig. 3A). However, there were no significant differences of β_2-AR mRNA expression of SOL muscle between both groups (Fig. 3B). Furthermore, β_2-AR mRNA expression of LV muscle was 0.94 times ($P < 0.05$) lower in the clenbuterol-administered group than in the control group (Fig. 3C).

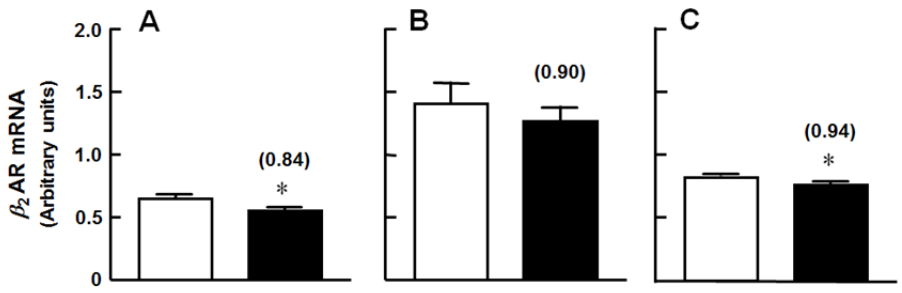

Fig. 3. Effects of clenbuterol on β_2-AR mRNA expression of EDL (A), SOL (B), and LV (C) muscles.
The values are each the mean±S.E.M. of 8-10 rats/group. Open bar: control group and closed bar: clenbuterol administered group. Values in parentheses are shown as the relative ratio of the clenbuterol-administered group to the control group. Statistics: *P<0.05 (vs. the control group).

2.4. Discussion

The purpose of the present study was to elucidate the effects of β_2-agonist clenbuterol (dose= 1.0 mg/ kg body weight/ day, s.c. for 10 days) on β_1- and β_2-AR mRNA expressions of EDL, SOL, and LV muscles in adult male rats. The present results are summarized in Table 2. The main findings of the present study are also summarized as follows: 1) Clenbuterol significantly increased the weight, RNA concentration, and total RNA content of EDL muscle without changing those of SOL and LV muscles (Table 1). 2) Clenbuterol significantly decreased β_1-AR mRNA expression of LV muscle without changing that of EDL and SOL muscles (Fig. 2). 3) Clenbuterol significantly decreased β_2-AR mRNA expression of EDL and LV muscles without changing that of SOL muscle (Fig. 3). These results suggest that the effects of clenbuterol on β_1- and β_2-AR mRNA

expressions and muscle hypertrophy depend on muscle fiber types.

Table 2. Summary of effects of clenbuterol on the weights, RNA concentration, total RNA content, and β_1- and β_2-AR mRNA expressions of EDL, SOL and LV muscles.

Muscles	Weight	RNA concentration	Total RNA content	β_1-AR mRNA expression	β_2-AR mRNA expression
EDL	⇧ (1.14 times)	⇧ (1.29 times)	⇧ (1.48 times)	n.s.	⇩ (0.84 times)
SOL	n.s.	n.s.	n.s.	n.s.	n.s.
LV	n.s.	n.s.	n.s.	⇩ (0.84 times)	⇩ (0.94 times)

Up arrow: significantly higher in the clenbuterol-administered group than in the control group, down arrow: significantly lower in the clenbuterol-administered group than in the control group, n.s.: not significant between both groups. EDL: extensor digitorum longus, SOL: soleus, LV: left ventricle muscles. Values in parentheses are shown as the relative ratio of the clenbuterol-administered group to the control group.

2.4.1. Effects of clenbuterol on skeletal muscle weights

The present study clearly showed that the administration of clenbuterol for 10 days increased the weight of EDL muscle without changing the weight of SOL and LV muscles (Table 1). These results agreed with our previous findings [6]. The present results also demonstrated that clenbuterol significantly increased RNA concentration and total RNA content of fast-twitch fiber-rich EDL muscle (Table 1). These results strongly suggest that clenbuterol increases the synthesis rate of muscle protein in fast-twitch fibers in order to increase muscle mass and protein accretion.

It is well known that β_2-agonists increase the cross-sectional area of fast-twitch fibers [4, 6, 7, 25]. In addition, the effects of β_2-agonists on the functional properties of skeletal muscles may be associated with the metabolic responses [7, 14, 25, 36]. Kitaura et al. [25] reported that clenbuterol induced a transition from slow to fast myosin heavy chain (MHC) phenotypes of SOL muscle, but no

significant changes in MHCs of EDL muscle. Furthermore, they also reported that clenbuterol decreased monocarboxylate transporter 1 content in EDL and SOL muscles, increased the lactate dehydrogenase (LDH) total activity in EDL muscle, and increased LDH-specific activity and the ratio of the muscle-type isozyme of LDH (anaerobic activity) to the heart-type (aerobic activity) in SOL muscle [25]. Additionally, clenbuterol-induced shift of metabolic property from oxidative to glycolytic in SOL muscle is demonstrated by the increase in phosphofructokinase activity and decrease in citrate synthase activity [36]. Hunt et al. [14] reported that clenbuterol prevented adrenalin from antagonizing insulin-stimulated muscle glucose uptake in fast-twitch epitrochlearis and slow-twitch SOL muscles. Ryall et al. [7] also reported that treatment with the β_2-agonist fenoterol caused a small increase in fatiguability due to a decrease in succinate dehydrogenase activity in both EDL and SOL muscles. These findings suggest that the administration of the β_2-agonist clenbuterol shifts the MHC phenotype from slow to fast and the metabolic property from oxidative to glycolytic in slow-twitch fiber-rich SOL muscles and changes the predominant activity to the glycolytic system in all skeletal muscles.

2.4.2. Effects of clenbuterol on β_2-AR mRNA expression in skeletal muscles

The present study clearly demonstrated that clenbuterol decreased β_2-AR mRNA expression of EDL muscle (Fig. 3A). However, no significant effect of clenbuterol on β_2-AR mRNA expression of SOL muscle was observed (Fig. 3B) regardless of higher β_2-AR density. The present results suggest that the different effects of clenbuterol on β_2-AR mRNA expression between fast-twitch fiber-rich EDL and slow-twitch fiber-rich SOL muscles are associated with the intracellular factors without any association with the number and distribution of β_2-AR molecules. In contrast, it was reported that the decrease of β-AR densities on the cell surface was dependent on the receptor density in each muscle fiber, although the details are unclear [27].

Sillence et al. [37] reported that β_2-AR density was decreased in hind-limb muscle harvested from 7-day clenbuterol-administered (dose=0.2 mg/kg body weight) female rats. Similarly, Rothwell et al. [19] reported that β_2-AR density in muscle membranes was decreased in

rat hind-limb muscle injected with clenbuterol (dose=2.0 mg/kg body weight) for 18 days. Although the differences of the experimental protocols may influence the magnitude and kinetics of reduction levels of β_2-AR density, these findings clearly showed that the decrease of β_2-AR density was confirmed under the different protocols such as the dose and duration of the administration of clenbuterol. Although β_2-AR density and mRNA expression should not be considered to be the same subjects, gene expression dynamics and the gene product, protein expression, are deeply associated. In the present study, therefore, the experimental protocol was determined by the protocol of these previous studies regarding β_2-AR density. More elaborated studies focused on the dose- and duration-responses of clenbuterol are required.

It is well known that the genetic information from DNA is mediated via the transcription-translation process of mRNA to synthesize protein. Therefore, the decrease of mRNA expression levels is associated with the accelerated degradation of mRNA and the decreased transcription rate [7, 15]. Mak et al. [18] analyzed the activation of the transcriptional factor of the β-AR gene CREB and showed the decrease of β_1-AR density, mRNA expression, and transcription rate in rat lung after the long-term treatment with the β-agonist isoproterenol, suggesting that the reduction of transcription rate is responsible for the β-agonist-induced decrease of β_1-AR mRNA expression [18]. However, they also showed the decrease of β_2-AR density and mRNA expression without any detectable decline in the transcription rate, indicating that β-agonist destabilizes β_2-AR mRNA [18]. These findings suggest that the decrease of β_2-AR mRNA expression of EDL muscle induced by clenbuterol is not associated with the decline in the transcription rate.

On the other hand, there are some reports describing post-transcriptional regulation such as destabilization of receptor mRNA [22-24]. Hadcock et al. [22] reported that β-adrenergic stimulation reduced the half-life of β-AR mRNA, and this is responsible for the short-term agonist-induced decrease of β-AR mRNA expression without affecting the transcription rate. Furthermore, Port et al. [23] and Pende et al. [24] showed that β-AR mRNA-binding protein (β-ARBP or A+U-rich element RNA- binding/degradation factor 1) played an important role for the β_2-agonist-induced decrease of β-AR mRNA expression. In fact, β-agonist decreased β-AR mRNA

expression [22] and increased the amount of β-ARBP [23], although glucocorticoids, which increased β-AR mRNA expression [22], decreased the amount of β-ARBP [23]. Insulin, which activates its receptor-intrinsic tyrosine kinase activity, decreased the amount of β-ARBP, much like glucocorticoids [23]. These results show that β-AR mRNA expression levels are inversely related to the amount of β-ARBP. Furthermore, these reports indicate that the decrease of β_2-AR mRNA expression of EDL muscle obtained from the present study (Fig. 3A) is related to the amount of β-ARBP. The different effects of clenbuterol on β_2-AR mRNA expression between fast- and slow-twitch fibers may be connected with β-ARBP expression and activation in each muscle fiber. Further studies are needed to clarify the possible mechanisms of clenbuterol-induced suppression of β_2-AR mRNA expression of EDL muscle.

2.4.3. Effects of clenbuterol on β_1- and β_2-AR mRNA expressions of LV muscle

The present study clearly showed that clenbuterol significantly decreased β_1- and β_2-AR mRNA expressions of LV muscle (Figs. 2C and 3C). These results suggest that the decrease of β_2-AR mRNA expression is associated with the direct stimulation of clenbuterol to the receptor and the decrease of β_1-AR mRNA expression is associated with the direct and indirect stimulation of clenbuterol to the receptor.

Barbier et al. [38] showed that endurance training decreased β_1-AR density without changing β_2-AR density in cardiac muscle. Although endurance training-induced sympathetic hyperactivity is known to promote the secretion of catecholamines, the plasma levels of noradrenalin increase more than those of adrenalin in response to endurance training [39]. As β_1-AR is more sensitive to noradrenalin than adrenalin, this subtype is selectively down-regulated in cardiac muscle after the long-term exercise [40]. On the other hand, Cohen et al. [11] reported that clenbuterol showed high affinity towards both β_1- and β_2-ARs and not high selectivity toward β_1-AR, suggesting that clenbuterol nonselectively binds and down-regulates β_1-AR of LV muscle. It is possible that β_1-AR mRNA expression of EDL and SOL muscles was not affected because of the low number of β_1-AR.

Ventricular fibrillation [41], heart failure [42], and diabetes-induced heart diseases [43] dramatically change β-AR density and mRNA expression in heart. In these heart problems, the decrease of myocardial β_1-AR density and mRNA expression without changing myocardial β_2-AR density and mRNA expression is observed [41, 42]. These phenomena resemble the present results with respect to the β-AR mRNA expressions, suggesting that the overload of cardiac functions by clenbuterol-induced increase of plasma volume is associated with β-AR mRNA expressions.

The present study showed that the administration of clenbuterol decreased β_2-AR mRNA expression of fast-twitch fiber-rich EDL muscle without changing that of slow-twitch fiber-rich SOL muscle (Fig. 3: A and B). The administration of clenbuterol also decreased β_1- and β_2-AR mRNA expressions of LV muscle (Figs. 2C and 3C). Further studies are needed to clarify the mechanism of the clenbuterol-induced changes in β_2-AR mRNA expression in fast- and slow-twitch fibers and in cardiac muscle.

2.5. Acknowledgments

We are grateful to Dr. Ken Shirato (School of Medicine, Kyorin University, Mitaka) and Dr. Takashi Ichinose (Faculty of Human Sciences, Waseda University, Tokorozawa) for their helpful support and useful advice. The present study was partly supported by a Grant-in-Aid for the Academic Frontier Project (Waseda University, 2005-2010) of the Ministry of Education, Culture, Sports, Science, and Technology, Japan and a Grant of Establishment of Consolidated Research Institute for Advanced Science and Medical Care, Waseda University.

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3. CHAPTER 2

Adaptive effects of β_2-agonist, clenbuterol on the expressions of β_2-adrenoceptor mRNA in rat fast-twitch fiber-rich muscles [Sato S, Nomura S, Kawano F, Tanihata J, Tachiyashiki K, Imaizumi K (2010) J Physiol Sci 60:119-127]

3.1. Introduction

The β_2-agonist, clenbuterol (4-amino-α(t-butyl-amino)-methyl-3,5-dichlorobenzyl alcohol) has been used as a non-steroidal anabolic drug for sports doping. It has been reported that administration of clenbuterol induces skeletal muscle hypertrophy and inhibits skeletal muscle atrophy [1-8]. These clenbuterol-induced phenomena are caused by the increased rate of protein synthesis and/or reduced rate of proteolysis via the β_2-adrenoceptor (AR) [5, 9-12]. These findings show that the β_2-AR is responsible for both the skeletal muscle hypertrophy and anti-atrophy effects of clenbuterol. However, it has been reported that chronic administration of β-agonists down-regulate the density and/or mRNA expression of β_2-AR [1, 13]. Recently, we have reported that clenbuterol reduced the expression of β_2-AR mRNA in fast-twitch fiber-rich muscle, extensor digitorum longus (EDL) muscle, without changing that in slow-twitch fiber-rich muscle, soleus (SOL) muscle, suggesting that these effects depend on muscle fiber types [1]. However, the mechanisms of this fiber type-dependent decrease are still unknown.

Some reports have showed that cAMP response element binding protein (CREB) and the glucocorticoid receptor (GR) regulate the expression level of β_2-AR mRNA as transcriptional regulatory factors [14-17]. First, it is well known that positive autoregulation of the β_2-AR gene occurs through receptor-mediated elevation of the concentration of cyclic adenosine monophosphate (cAMP), followed by the phosphorylation and activation of CREB [14, 16]. Second, the steroid hormone-GR complex also binds to the β_2-AR gene, and activates transcription, showing that GR modulates the expression of β_2-AR mRNA [16, 17].

On the other hand, Hadcock et al. [18] showed that one mechanism for down-regulation of

β_2-AR mRNA is destabilization of β_2-AR mRNA. It is well known that β_2-AR mRNA contains an AU-rich element (ARE) within the 3'-untranslated region (3'-UTR) that can be recognized by several mRNA binding proteins, including Hu antigen R (HuR), AU-rich element binding/degradation factor1 (AUF1) and heterogenous nuclear ribonucleoprotein A1 (hnRNP A1) [19-22]. These proteins are known to play an important role in the regulation of β_2-AR mRNA stability [19-30].

These findings mentioned above support the hypothesis that clenbuterol-reduced expression of β_2-AR mRNA may be related to expression of transcriptional and post-transcriptional regulatory factors for β_2-AR mRNA levels in skeletal muscles. In this study, therefore, we examined the effects of clenbuterol on expression of CREB, GR, HuR, AUF1 and hnRNP A1 mRNAs in fast-twitch fiber-rich (EDL and plantaris: PLA) and slow-twitch fiber-rich (SOL) muscles in rats.

3.2. Materials and methods

3.2.1. Experimental procedures and animal care

The experimental procedure used in this study is shown in Fig. 1. Briefly, clenbuterol (dose= 1.0mg/kg body weight/day) was administered to rats for ten consecutive days during the experimental period. The EDL, PLA, and SOL muscles were isolated and weighed on the day after the final day of clenbuterol administration [1].

Male 7-week-old Sprague Dawley rats (CLEA Japan, Tokyo) were pre-fed for 5 days to allow adaptation to their new environment [1, 31]. Rats were maintained at a controlled temperature (23-25°C) and relative humidity (50-60%), with fixed light-dark cycles (8:00–20:00 (light) and 20:00–8:00 (dark)) [1, 31, 32]. Animal foods (CE-2 cubic type; CLEA Japan) was given to each rat under diet-restricted feeding (feeding chow= 30g/day) and distilled water was given ad libitum [1]. All rats were weighed daily during the experimental period. After the adaptation

period, the rats were randomly divided into two groups, the clenbuterol-administered (n= 10, the initial body weight= 279± 2g, mean± standard error of the mean (SEM)) and the control (n= 10, the initial body weight=278± 2g, mean± S EM) group.

All experimental and animal care procedures were approved by the Committee on Animal Care Use at Waseda University and followed the Guiding Principles for the Care and Use of Animals in the Field of Physiological Sciences established by the Physiological Society of Japan [1, 31-35] and also American Physiological Society Animal Care Guidelines. We performed procedures with the least possible pain or discomfort to the rats [1, 31-34].

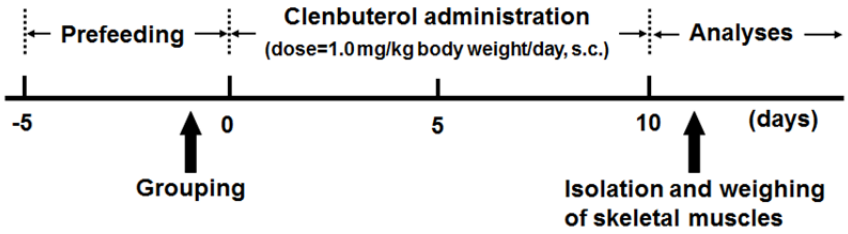

Fig. 1 Experimental procedure used in this study.
After a 5-day-adaptation period, the rats were randomly divided into the clenbuterol-administered group and the control group. Clenbuterol (dose=1.0 mg/kg body weight/day) was administered to the cervical portion of the back by subcutaneous (s.c.) injection (8:00-8:30) for ten consecutive days. In the control group rats, an equivalent volume of 0.9% NaCl solution was administered in the same manner. Skeletal muscles were isolated and weighed on the day after the final day of clenbuterol administration.

3.2.2. Administration of clenbuterol to rats

Clenbuterol hydrochloride (Sigma, St. Louis, MO, USA) was dissolved in 0.9% NaCl solution as a vehicle to obtain a clenbuterol concentration of 0.1% [1, 31]. In the clenbuterol-administered group, clenbuterol (dose= 1.0mg/kg body weight/day) was administered to cervical portion of the back via a subcutaneous (s.c.) injection (8:00- 8:30) for ten consecutive days [1]. In the control group, an equivalent volume of 0.9% NaCl solution was administered in the same manner [1, 31].

3.2.3. Sample storage

Isolated and weighed skeletal muscles were cut at both ends and preserved in RNA*later*

solution (Ambion, Austin, TX, USA) to stabilize RNA [1]. The samples were stored at -20°C after the incubation at 4°C overnight until they could be used for RNA extraction [1].

3.2.4. Analysis of mRNA expressions by real-time quantitative reverse transcription-polymerase chain reaction

RNA was extracted from stored muscle samples by use of TRIzol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol [1, 32]. RNA concentration was determined by measuring absorbance at 260 nm (U-3310 Spectrophotometer; Hitachi, Tokyo, Japan) [1, 32, 34]. The extracted RNA was subjected to single-stranded cDNA synthesis using a high-capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's protocol [1, 32, 34]. In the real-time quantitative polymerase chain reaction (PCR), synthesized cDNA was added to a Power SYBR Green PCR Master Mix (Applied Biosystems) containing 200 nM PCR primer (forward and reverse) [1]. The relative amount of each mRNA was calculated and normalized by the value of 18S rRNA gene. The oligonucleotide sequences for the primers are shown in Table 1. Amplification was performed using an ABI Prism 7000 sepuence detection system (Applied Biosystems).

Table 1 Oligonucleotide sequences used for PCR.

Gene	Direction of oligonucleotide	Sequences
β_1-AR	forward	5'-CTG CTA CAA CGA CCC CAA GTG-3'
	reverse	5'-AAC ACC CGG AGG TAC ACG AA-3'
β_2-AR	forward	5'-GAG CCA CAC GGG AAT GAC A-3'
	reverse	5'-CCA GGA CGA TAA CCG ACA TGA-3'
β_3-AR	forward	5'-TCT GTG TAA CTG CCA GCA TCG A-3'
	reverse	5'-TGG TAA CCA GCG TGC CGT AA-3'
CREB	forward	5'-CTA GTG CCC AGC AAC CAA GT-3'
	reverse	5'-GGA GGA CGC CAT AAC AAC TC-3'
GR	forward	5'-TAC CAC AGC TCA CCC CTA CC-3'
	reverse	5'-AGC AGG GTC ATT TGG TCA TC-3'
HuR	forward	5'-AGG TTT GTC CAG AGG GGT TG-3'
	reverse	5'-TTT GTT CTG GTT GGG ATT GG-3'
AUF1	forward	5'-GGG CCA AAG CCA TGA AAA C-3'
	reverse	5'-CAA CCT CAC CAAAAC CAC CA-3'
hnRNP A1	forward	5'-CTT TGC TAA ACC ACG AAA CCA AG-3'
	reverse	5'-CAC TTC TCT GGC TCT CCT CTC C-3'
18S rRNA	forward	5'-GTG CAT GGC CGT TCT TAG TTG-3'
	reverse	5'-AGC ATG CCG AGA GTC TCG TT-3'

3.2.5. Statistical analysis

Experimental data were presented as the mean± SEM. The differences between two groups were tested by a Student *t*- test and considered significant when *p* value was <0.05.

3.3. Results

3.3.1. Effects of clenbuterol on the weight, RNA concentration in, and RNA content of PLA muscle

No significant effects of clenbuterol on body weight were observed as reported previously [1]. As shown in Fig. 2, the weight (0.40±0.01g) of, RNA concentration (1.19±0.06mg/g) in, and RNA content (0.48±0.03mg) of PLA muscle in the clenbuterol-administered group were 1.18 (p<0.001), 1.31 (p<0.05) and 1.55 (p<0.01) times higher than those (0.34±0.01g, 0.91±0.01mg/g and 0.31±0.04mg, respectively) in the control group. These results were qualitatively similar to our previous findings in EDL muscle [1] (Table 2), and clearly showed that the effects of clenbuterol on muscle hypertrophy were greater in fast-twitch fiber-rich muscle than in slow-twitch fiber-rich muscle.

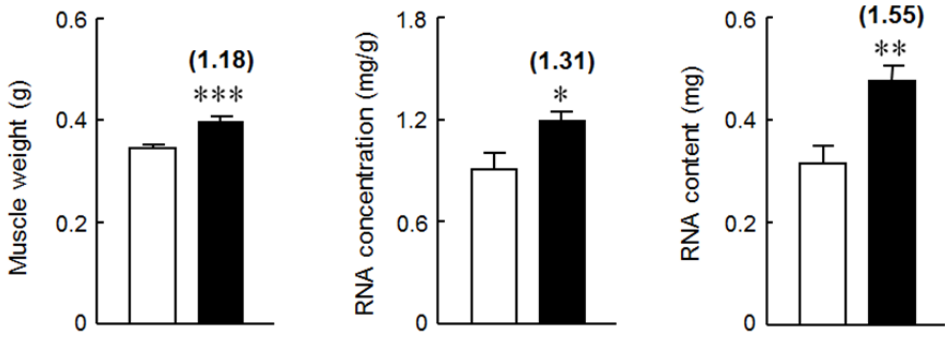

Fig. 2. Effects of clenbuterol on the weight of, RNA concentration in, and RNA content of PLA muscle.
The values are shown as mean±SEM (n=10/group). Open bars, control group; closed bars, clenbuterol-administered group. Values in parentheses are values for the clenbuterol-administered group relative to the control group. Statistics: *p<0.05, **p<0.01, and ***p<0.001 (vs. the control group).

3.3.2. Effects of clenbuterol on expression of β_1, β_2 and β_3-AR mRNAs in skeletal muscles

Figure 3 showed the effects of clenbuterol on expressions of β_1, β_2 and β_3-AR mRNAs in EDL, PLA, and SOL muscles. Expression of β_2-AR mRNA in EDL and PLA muscles was 0.69 (p<0.01) and 0.67 (p<0.01) times lower in the clenbuterol-administered group than in the control group, respectively (Fig. 3). The smaller effects of clenbuterol on expression of β_2-AR mRNA in PLA muscle were comparable with those in EDL muscle (Fig. 3). In contrast, there were no significant differences of expression of β_2-AR mRNA in SOL muscles between both groups (Fig. 3). These findings support the previous suggestion that the effects of clenbuterol on expression of β_2-AR mRNA depend on muscle fiber types [1]. However, there were no significant differences of expression of β_1 and β_3-AR mRNAs in these muscles between both groups (Fig. 3).

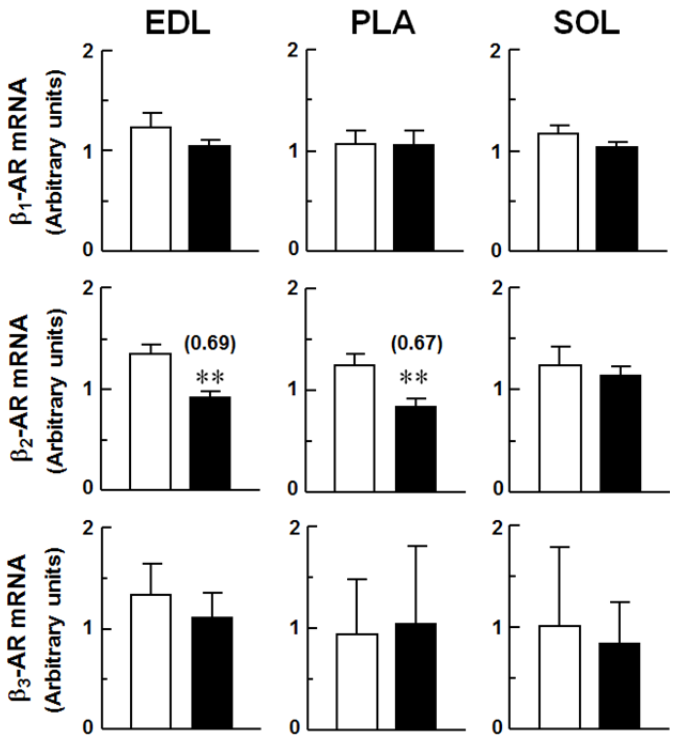

Fig. 3 Effects of clenbuterol on expression of β_1, β_2, and β_3-AR mRNAs in EDL, PLA, and SOL muscles.
The values are shown as mean±SEM (n = 10/group). The relative amount of each mRNA was calculated and normalized by the values of 18S rRNA gene. Open bars, control group; closed bars, clenbuterol-administered group. Values in parentheses are values for the clenbuterol-administered group relative to the control group. Statistics: **p<0.01 (vs. the control group).

3.3.3. Effects of clenbuterol on expression of transcriptional factor mRNAs in skeletal muscles

The effects of clenbuterol on the expression of CREB and GR mRNAs in EDL, PLA and SOL muscles were shown in Fig. 4. There were no significant differences of the expression of CREB mRNA in these skeletal muscles between both groups (Fig. 4). Expression of GR mRNA in EDL and PLA muscles was 0.70 (p<0.01) and 0.80 (p<0.05) times lower in the clenbuterol-administered group than in the control group, respectively (Fig. 4). However, no significant differences of expression of GR mRNA in SOL muscle were observed between both groups (Fig. 4). These results clearly show that the effects of clenbuterol on expression of GR mRNA depend on muscle fiber types.

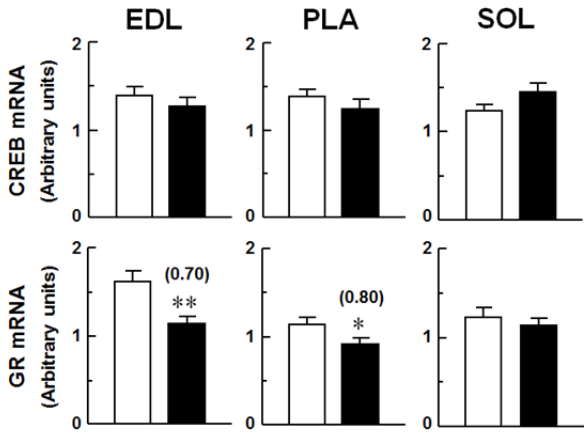

Fig. 4 Effects of clenbuterol on expression of CREB and GR mRNAs in EDL, PLA, and SOL muscles.
The values are shown as the mean±SEM (n=10/group). The amount of each mRNA was calculated and normalized by the value for the 18S rRNA gene. Open bars, control group; closed bars, clenbuterol-administered group. Values in parentheses are values for the clenbuterol-administered group relative to the control group. Statistics: *p<0.05 and **p<0.01 (vs. the control group).

3.3.4. Effects of clenbuterol on expression of post-transcriptional regulatory factor mRNAs in skeletal muscles

Figure 5 shows the effects of clenbuterol on expression of HuR, AUF1, and hnRNP A1 mRNAs in EDL, PLA, and SOL muscles. Expression of HuR mRNA in EDL and PLA muscles was 0.79 (p<0.01) and 0.82 (p<0.05) times lower, respectively, in the clenbuterol-administered group than in the control group (Fig. 5). However, no significant effect of clenbuterol on expression of

HuR mRNA in SOL muscle was observed (Fig. 5). Expression of AUF1 mRNA in EDL and PLA muscles was 0.73 (p<0.001) and 0.76 (p<0.01) times lower, respectively, in the clenbuterol-administered group than in the control group (Fig. 5). However, there was no significant difference between expression of AUF1 mRNA in SOL muscle in these groups (Fig. 5). Expression of hnRNP A1 mRNA in EDL and PLA muscles was 0.66 (p<0.01) and 0.70 (p<0.001) times lower, respectively, in the clenbuterol-administered group than in the control group (Fig. 5). However, there was no significant difference between expression of hnRNP A1 mRNA in SOL muscle in these groups (Fig. 5). Thus, clenbuterol significantly reduced expression of HuR, AUF1 and hnRNP A1 mRNAs in EDL and PLA muscles without changing those in SOL muscle, showing that the effects of clenbuterol on expression of these post-transcriptional regulatory factor mRNAs are specific to fast-twitch fiber-rich muscles such as EDL and PLA muscles.

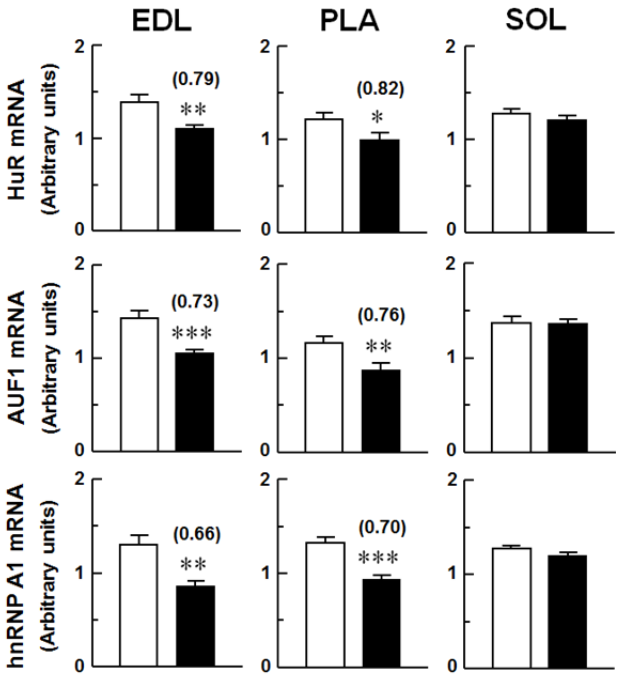

Fig. 5 Effects of clenbuterol on expression of CREB and GR mRNAs in EDL, PLA, and SOL muscles.
The values are shown as the mean±SEM (n=10/group). The amount of each mRNA was calculated and normalized by the value for the 18S rRNA gene. Open bars, control group; closed bars, clenbuterol-administered group. Values in parentheses are values for the clenbuterol-administered group relative to the control group. Statistics: *p<0.05, **p<0.01 and ***p<0.001 (vs. the control group).

3.4. Discussion

The purpose of this study is to elucidate the effects of the β_2-agonist, clenbuterol (dose= 1.0mg/kg body weight/ day for 10 days, s.c.) on mRNA expression of transcriptional (CREB and GR) and post-transcriptional (HuR, AUF1 and hnRNP A1) regulatory factors for β_2-AR mRNA levels in fast-twitch fiber-rich (EDL and PLA) and slow-twitch fiber-rich (SOL) muscles in rats. The results are summarized in Table 2 and suggest that muscle fiber type-dependent effects of clenbuterol on expression of β_2-AR mRNA are closely related to the decrease of mRNA expression of transcriptional and post-transcriptional regulatory factors for β_2-AR mRNA levels.

Table 2. Summary of results.

Parameters	Muscles		
	EDL	PLA	SOL
Weight	⇧ [#]	⇧ (1.18)	n.s. [#]
RNA concentration	⇧ [#]	⇧ (1.31)	n.s. [#]
RNA content	⇧ [#]	⇧ (1.55)	n.s. [#]
β_1-AR mRNA	n.s.	n.s.	n.s.
β_2-AR mRNA	⇩ (0.69)	⇩ (0.67)	n.s.
β_3-AR mRNA	n.s.	n.s.	n.s.
CREB mRNA	n.s.	n.s.	n.s.
GR mRNA	⇩ (0.70)	⇩ (0.80)	n.s.
HuR mRNA	⇩ (0.79)	⇩ (0.82)	n.s.
AUF1 mRNA	⇩ (0.73)	⇩ (0.76)	n.s.
hnRNP A1 mRNA	⇩ (0.66)	⇩ (0.70)	n.s.

Up arrow: significantly higher in the clenbuterol-administered group than in the control group. Down arrow: significantly lower in the clenbuterol-administered group than in the control group. n.s.: not significant between both groups. Values in parentheses are shown as the relative value of the clenbuterol-administered group to the control group. [#]: our previous date [1].

Our previous study showed that clenbuterol increased the weight of, RNA concentration in, and RNA content of EDL muscle without changing those in SOL muscle [1]. This study also demonstrated that clenbuterol increased the weight of, RNA concentration in, and RNA content of PLA muscle (Fig. 2). These results clearly suggest that the effects of clenbuterol on the synthesis

rate of muscle protein are greater in fast-twitch fiber than in slow-twitch fiber, and there are no differences in the effects of clenbuterol on the weight of and RNA concentration in fast-twitch fiber-rich muscles between extensor (EDL) and flexor (PLA) muscles. This study also showed that clenbuterol reduced the expression of β_2-AR mRNA in EDL and PLA muscles without changing that in SOL muscle (Fig. 3), clearly supporting our previous suggestion that the effects of clenbuterol on expression of β_2-AR mRNA depend on muscle fiber type [1].

It is well known that β_2-AR regulates expression of several genes including β_2-AR itself through the signaling pathway [14, 15, 36-38]. The transcriptional responses of several genes including β_2-AR to cAMP are localized to the cAMP response element, which is constituted by the palindromic sequence TGACGTCA in the 5'-flanking region and recognized by CREB [14, 15, 39, 40]. This study clearly shows that clenbuterol did not change the expression of CREB mRNA in skeletal muscles (Fig. 4). Mak et al. [13] reported that β-agonist, isoproterenol reduced the density of β_2-AR and expression of β_2-AR mRNA without any detectable decline in the rate of transcription. These findings suggest that the decrease of expression of β_2-AR mRNA induced by clenbuterol is not associated with the decline in the abundance of CREB or even in the rate of transcription in fast-twitch fiber-rich muscles. Furthermore, in this study, statistical regression analyses showed that expression of β_2-AR mRNA was not strongly correlated with expression of CREB mRNA in EDL, PLA and SOL muscles (data not shown).

On the other hand, glucocorticoid is associated with the transcription of the β_2-AR gene [17]. The GR-ligand complex undergoes a conformational change resulting in dissociation of heat shock protein 90 and unmasking of a nuclear localization signal into the nucleus, where it binds directly to glucocorticoid response element constituted by the consensus sequence AGAACAnnnTGTTCT in the 5'-flanking region, and activates gene transcription including β_2-AR [17, 41, 42]. Our study clearly shows that clenbuterol reduced expression of GR mRNA in EDL and PLA muscles (Fig. 4). Recently, we also showed that synthesized glucocorticoid, dexamethasone-induced down-regulation of expression of β_2-AR mRNA in SOL muscle may be related to the relatively much larger reduction in the expression of GR mRNA in SOL muscle than in

EDL muscle [32]. Furthermore, in the current study, the statistical regression analyses showed that the positive correlation between expression of β_2-AR mRNA and expression of GR mRNA in fast-twitch fiber-rich, EDL (r=0.59) and PLA (r=0.67) muscles was stronger than that in slow-twitch fiber-rich, SOL muscle (r=0.41) (data not shown). These findings suggest that the decrease of expression of β_2-AR mRNA induced by clenbuterol is closely associated with the decline in expression of GR mRNA in fast-twitch fiber-rich muscles. These findings also indicate that the GR has an adaptable role in regulation of expression of β_2-AR mRNA in various situations caused by exposure to internal and external stimuli.

Hadcock et al. [18] showed that one mechanism for down-regulation of β_2-AR mRNA is destabilization of β_2-AR mRNA rather than decline in the rate of transcription. The regulation of stability and turnover of β_2-AR mRNA has been associated with the interaction with mRNA binding proteins, including HuR, AUF1 and hnRNP A1 that often bind to AREs commonly located within their 3'-UTR [19-22]. HuR is a ubiquitously expressed and a member of the embryonic lethal abnormal vision family of RNA-binding proteins [43, 44]. Overexpression of HuR leads to stabilization [24, 25], and inverse reduction of levels of HuR induces the decline in half-life [27, 28] of mRNAs carrying AREs in their 3'-UTR. These findings suggest that HuR stabilizes mRNAs containing AREs within their 3'-UTR, including β_2-AR mRNA. Our current study showed that clenbuterol reduced expression of HuR mRNA in EDL and PLA muscles (Fig. 5), strongly suggesting that clenbuterol-induced down-regulation of expression of HuR mRNA reduces the stability of β_2-AR mRNA, and consequently, reduces expression of β_2-AR mRNA in fast-twitch fiber-rich muscles.

On the other hand, overexpression of AUF1 leads to degradation of mRNAs carrying AREs within their 3'-UTR, suggesting that AUF1 is involved mostly in degradation of β_2-AR mRNA and competes against the role of HuR [26]. Our current study, however, showed that clenbuterol reduced the expression of AUF1 mRNA in EDL and PLA muscles (Fig. 5), suggesting that clenbuterol-reduced expression of AUF1 mRNA heightens the stability of β_2-AR mRNA in fast-twitch fiber-rich muscles. Although the cause of these disagreements is uncertain, it is possible

that the decrease in expression of AUF1 mRNA may be associated with the response to maintain the balance of the stability of several intravital mRNAs containing AREs other than β_2-AR mRNA, because the action of AUF1 competes against that of HuR in respect of the stability of mRNAs containing AREs.

According to results of Dreyfuss et al. [45], hnRNP A1 is associated with the pre-mRNA, small nuclear ribonucleoprotein complex where they facilitate the processing of nascent transcripts into mRNA, for example by modulating mRNA splicing. However, more recent evidence that hnRNP A1 can shuttle from the nucleus to the cytoplasm has led to the speculation that hnRNP A1 has an additional role in affecting the stability of mRNAs containing AREs within their 3'-UTR such as β_2-AR mRNA [23]. Our current study showed that clenbuterol reduced the expression of hnRNP A1 mRNA in EDL and PLA muscles (Fig. 5), suggesting that clenbuterol-induced down-regulation of the expression of hnRNP A1 mRNA reduces the rate of modulation of β_2-AR mRNA splicing and perhaps the stability of β_2-AR mRNA and, consequently, expression of β_2-AR mRNA in fast-twitch fiber-rich muscles. Furthermore, in this study, the positive correlation between expression of β_2-AR mRNA and expression of HuR ($r=0.79$ and $r=0.58$, respectively), AUF1 ($r=0.74$ and $r=0.78$, respectively), and hnRNP A1 ($r=0.63$ and $r=0.74$, respectively) mRNAs in EDL and PLA muscles was stronger than those (HuR: $r=0.58$, AUF1: $r=0.34$ and hnRNP A1: $r=0.48$, respectively) in SOL muscle (data not shown).

In conclusion, this study showed that clenbuterol reduced mRNA expression of transcriptional and post-transcriptional regulatory factors for β_2-AR mRNA levels in fast-twitch fiber-rich muscles, suggesting that these phenomena are related to expression pattern of β_2-AR mRNA in skeletal muscles. These clenbuterol-induced responses of expression of β_2-AR mRNA in skeletal muscles may play an important role in the regulation of β_2-AR-mediated hypertrophy.

3.5. Acknowledgments

We are grateful to Dr. Ken Shirato (Department of Disease Glycomics, Institute of Scientific and Industrial Research, Osaka University, Ibaraki, Osaka) for his helpful support and useful advice. This study was partly supported by a Grant-in-Aid for the Academic Frontier Project (Waseda University, 2005-2009:KI), a Grant of Establishment of Consolidated Research Institute for Advanced Science and Medical Care, Waseda University (2004-2008:KI), and a Grant-in-Aid of the global center of excellence (COE) program, Graduate School of Sports Sciences, Waseda University (2009-2013:KI) of the Ministry of Education, Culture, Sports, Science, and Technology, Japan.

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4. CHAPTER 3

Synthesized glucocorticoid, dexamethasone regulates the expressions of β_2-adrenoceptor and glucocorticoid receptor mRNAs but not proteins in slow-twitch soleus muscle of rats [Sato S, Shirato K, Tachiyashiki K, Imaizumi K (2011) J Toxicol Sci 36:479-486]

4.1. Introduction

It is generally accepted that prolonged hypokinesia (i.e. reduction in limb movement) and/or hypodynamia (i.e. reduction in muscle loading) resulted from prolonged bed rest, life in a wheel chair, restricted movement, limited muscular function and microgravity environment activate sympatho-adrenal axis associated with increases in circulating levels of noradrenalin and adrenalin from adrenal medulla [1]. Similarly, activation of hypothalamo-hypophyseal-adrenocortical axis is associated with releases into peripheral blood of corticotropin releasing factor (CRF) from hypothalamus, adrenocorticotropic hormone (ACTH) from pituitary anterior, and glucocorticoids from adrenal cortex [2, 3]. These endocrine factors play an important functional role in the regulation of the rate of protein synthesis and degradation in skeletal muscles [4, 5]. Administration of high doses of glucocorticoids increased the rate of muscle protein degradation and decreased the rate of muscle protein synthesis, leading to muscle atrophy in humans [5]. Glucocorticoids also increased the expression and activity of the ubiquitin-proteasome pathway that play an important role in the major proteolytic mechanism of muscle atrophy [6, 7]. Some reports showed that the degree of synthesized glucocorticoid, dexamethasone (DEX)-induced decrease in muscle weight was higher in fast-twitch fiber-rich (extensor digitorum longus (EDL)) muscle than in slow-twitch fiber-rich (soleus (SOL)) muscle [8, 9].

On the other hand, β_2-adrenoceptor (AR) in skeletal muscles plays an important physiological role in muscle plasticity and equilibrium between muscle protein synthesis and degradation [4, 10, 11]. We reported that administration of β_2-agonist, clenbuterol (CLE: 4-amino-α(t-butyl-amino)methyl-3,5-dichlorobenzyl alcohol), one of doping drugs, increased the weight of

EDL muscle without changing that of SOL muscle [12]. Further, administration of CLE decreased the density of β_2-AR in fast-twitch muscles [13] and decreased the expression of β_2-AR mRNA in EDL muscle without changing that in SOL muscle [12, 14]. On the contrary, Cornett *et al*. [15] reported that glucocorticoids increased the transcription of β_2-AR by acting toward glucocorticoid response elements (GREs) on β_2-AR gene via glucocorticoid receptor (GR) using HepG2 cells in vitro. However, our recent report demonstrated that administration of DEX decreased the expression of β_2-AR mRNA in SOL muscle without changing that in EDL muscle [8]. Huang *et al*. [13, 16] also reported that corticosterone failed to increase β_2-AR density in fast-twitch muscle, whereas concurrent treatment of CLE with DEX prevented CLE-induced down-regulation of β_2-AR density in the same muscle. Thus, DEX-induced changes in the expression of β_2-AR in skeletal muscles are not fully clear, especially in respects of transcription and post-transcription regulations.

In addition, Collins *et al*. [17] demonstrated that β_2-AR gene is positively autoregulated through receptor-mediated elevation of cAMP concentration, followed by phosphorylation and activation of cAMP response element binding protein (CREB). β_2-AR mRNA is also shown to contain an AU-rich element (ARE) within the 3'-untranslated region (3'-UTR) that can be recognized by several mRNA binding proteins, including Hu antigen R (HuR) and AU-rich element binding/degradation factor1 (AUF1) [18], which proteins play an important role in the regulation of β_2-AR mRNA stability [19-22]. However, the mechanisms for muscle fiber type-dependent down-regulation of the expression of β_2-AR mRNA induced by DEX are still unknown. In the present study, therefore, we examined the effects of DEX on mRNA and protein expressions of β_2-AR and GR, and mRNA expressions of CREB, HuR and AUF1 in EDL and SOL muscles of rats.

4.2. Materials and methods

4.2.1. Animal care and experimental protocol

All experimental procedures and animal care were approved by the Committee on the

Animal Care, Ethics and Use, Waseda University, and followed to the Guiding Principles for the Care and Use of Animals in the Field of Physiological Sciences, the Physiological Society of Japan [23].

Male 7-week-old Sprague Dawley rats (CLEA Japan, Tokyo, Japan) were pre-fed for 5 days to allow adaptation to their new environment. The rats were housed two to a cage in a temperature (23–25°C) and humidity (50–60%)-controlled room with a 12:12 hr light-dark cycle. Animal foods (CE-2 cubic type; CLEA Japan) were given to each rat under pair feeding, and distilled water was given *ad libitum*. After the adaptation period for 5 days, the rats were randomly divided into DEX-administered group (n=9, the initial body weight=262±2 g, mean±S.E.) and control group (n=10, the initial body weight=260±2 g, mean±S.E.).

Dexamethasone 21-phosphate (Sigma, St. Louis, MO, USA) was dissolved in a 0.9% NaCl as a vehicle to obtain 0.1% DEX [8]. In DEX-adminsitered group, DEX (dose=1.0 mg/kg body weight/day) was administered to rats from the cervical portion of the back via a subcutaneous injection for 10 days. In control group, an equivalent volume of 0.9% NaCl was administered to the rats in the same manner. On the next day after the final administration of DEX, the rats were sacrificed by decapitation, and then EDL and SOL muscles were isolated and rapidly frozen in liquid nitrogen. The muscle samples were stored at -80°C until they could be used for RNA and protein extractions.

4.2.2. RNA extraction and real-time quantitative RT-PCR

Total RNA was extracted using a TRIzol reagent (Invitrogen, Carlsbad, CA, USA). Total RNA content was determined by measuring absorbance at 260 nm. The extracted total RNA was subjected to single-stranded cDNA synthesis using a high-capacity cDNA reverse transcription (RT) kit (Applied Biosystems, Foster City, CA, USA) which is contained random primer for RT. In real-time quantitative PCR, synthesized cDNA was added to a power SYBR green PCR master mix (Applied Biosystems) containing 200 nM PCR primer (forward and reverse). The primer oligonucleotide sequences used for real-time quantitative PCR are described previously [14].

Amplification was performed using an ABI PRISM 7000 Sepuence Detection System (Applied Biosystems). PCR amplification program consisted of an initial denaturation step for 10 sec at 95^{o}C, followed by the shuttle PCR standard protocol of 40 cycles. Each mRNA level was determined using the threshold cycle (Ct) method in accordance with the manufacturer's protocol. The relative amount of each mRNA was normalized by the value of reference gene, 18S rRNA.

4.2.3. Protein extraction and western blotting

For analysis of the expression of β_2-AR, according to the methods of Dohm *et al*. [24] and Kern *et al*. [25], whole muscle cell organelles were separated into cytosol and membrane-rich fractions, in order to investigate DEX-induced trafficking of β_2-AR between cytosol and cell membrane. Frozen muscles were homogenized in an ice-cold homogenized buffer (0.3 M KCl, 0.1 M KH_2PO_4, 50 mM K_2HPO_4 and 10 mM EDTA; pH 6.53, 1:20, w/v) containing protease inhibitor cocktail (Thermo Fisher Scientific, Rockford, IL, USA) by Polytoron homogenizer (IKA, Baden-Wurttemberg, Germany). After the incubation on ice for 15 min, the homogenate was centrifuged at 100,000 g for 60 min at 4^{o}C. The resultant supernatant was collected and used as cytosol fraction. The resultant pellet was slowly homogenized in an ice-cold solubilized buffer (0.3 M KCl, 0.1 M KH_2PO_4, 50 mM K_2HPO_4, 10 mM EDTA and 1% Triton X-100, 1:20, w/v) containing protease inhibitor cocktail (Thermo Fisher Scientific). The homogenate was incubated on ice for 120 min and vortexed once a quarter. After the incubation, the homogenate was centrifuged at 100,000 g for 60 min at 4^{o}C. The resultant supernatant was collected and used as membrane-rich fraction. Protein samples were frozen at -80^{o}C until used for western blot analysis.

For analysis of the expression of GR protein, cytosol and nuclear-mixed fraction were extracted from whole muscle cell organelles because GR plays a role as transcription factor. Total protein of cytosol/nuclear fraction was extracted by Tissue Protein Extraction Reagent (T-PER, Thermo Fisher Scientific) according to the manufactured protocol. Briefly, frozen muscles were homogenized in an ice-cold T-PER (1:20, w/v) containing protease inhibitor cocktail (Thermo Fisher Scientific) by Polytoron homogenizer (IKA). The homogenate was centrifuged at 10,000 g for 10

min at 4°C. The resultant supernatant was collected as cytosol/nuclear fraction and stored at -80°C until used for western blot analysis.

Protein concentration was determined by BCA protein assay kit (Thermo Fisher Scientific). There was no significant difference in protein contents per tissue between two groups in each fraction (data not shown) of EDL and SOL muscles. Twenty five micrograms of protein were incubated (5 min, 100°C) with an equal volume of reducing sample buffer (0.5 M Tris-HCl, 10% SDS, 3% β-mercaptoethanol, 50% glycerol, 0.02% bromophenol blue), and then subjected to 4-12% SDS-polyacrylamide gel (Invitrogen). After electrophoresis, the proteins were transferred onto a PVDF membrane (Invitrogen) at 20 V for 120 min. The PVDF membrane was first incubated for 60 min in a washing buffer (PBS + 0.1% Tween 20) containing 1% skim milk. After being washed, the membrane was incubated with a primary antibody in the washing buffer over night. The following antibodies were used at a 1:500 dilution: β_2-AR (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and 1:2,000 dilution: GR (abcam, Cambridge, UK). After being washed, the membrane was incubated for 90 min with donkey anti-rabbit immunoglobulin G horseradish peroxidase-linked secondary antibody (1:10,000 dilution). The membrane was washed, and the immunoreactive bands were visualized using ECL (GE healthcare, Buckinghamshire, UK). The signal was quantified with a Lumino-Image Analyzer LAS-3000 System (Fuji Photo Film, Tokyo, Japan).

4.2.4. Statistical analyses

Experimental data were presented as mean±S.E. The differences between two groups were tested by a Student's t-test and considered to be significant when p value was < 0.05.

4.3. Results

4.3.1. Expressions of β_2-AR mRNA and protein

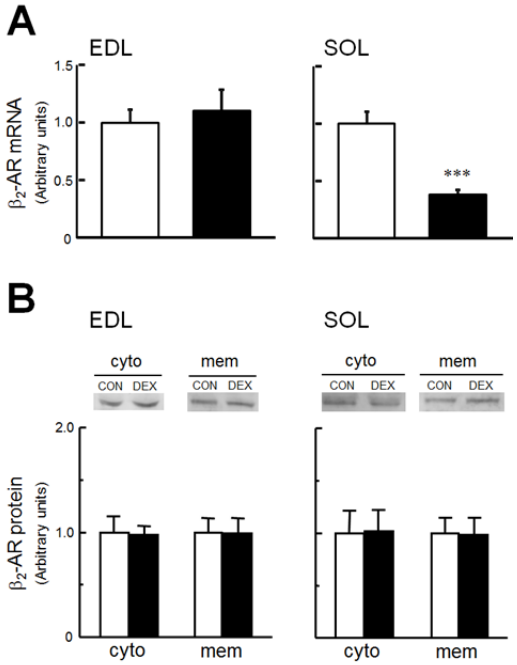

Fig. 1. Expressions of β_2-AR mRNA (A) and protein (B) in EDL and SOL muscles.
A: Expression levels of β_2-AR mRNA in the EDL and soleus muscles. Total RNA was extracted from isolated skeletal muscles of control and DEX-administered rats and subjected to real-time PCR analysis. The expression level of β_2-AR mRNA was normalized to those of 18S rRNA gene. The values shown in the bar graph are related to the optical density of the control group (set = 1). Open bar, control group (n=9) and filled bar, DEX-administered group (n=8). EDL, extensor digitarum longus and SOL, soleus. Values are shown as means±S.E. Statistics: ***$p < 0.001$ (vs. control group). **B** Expression levels of β_2-AR protein in the cytosol and membrane-rich fractions of EDL and soleus muscles. Total protein of cytosol and membrane-rich fractions was extracted from isolated skeletal muscles of control and DEX-administered rats and subjected to western blot analysis. The values shown by the bar graphs are related to the optical density of control group (set = 1). Open bar, control group (CON: n=6) and filled bar, DEX- administered group (DEX: n=6). cyto, cytosol fraction and mem, membrane-rich fraction. EDL, extensor digitarum longus and SOL, soleus. Values are shown as means±S.E.

Figure 1A shows the effects of DEX on the expressions of β_2-AR mRNA in EDL and SOL muscles. No significant change in the expression of β_2-AR mRNA in EDL muscle between both groups was found (Fig. 1A). On the contrary, DEX decreased the expression of β_2-AR mRNA in SOL muscle by 62% ($p < 0.001$) (Fig. 1A). These findings clearly support our previous suggestion that the effects of DEX on the expression of β_2-AR mRNA depend on muscle fiber types [8]. In addition, no significant changes in the expressions of β_1- and β_3-AR mRNAs in EDL and SOL muscles between two groups were found (data not shown).

We next investigated the effects of DEX on the expression of β_2-AR protein in skeletal

muscles. As shown in Fig. 1B, the immunoblot data clearly showed the existence of β_2-AR protein in cytosol and membrane-rich fractions of rat skeletal muscles. No significant change in the expression of β_2-AR protein was observed in cytosol and membrane-rich fractions of EDL muscle between both groups (Fig. 1B). DEX also did not change the expression of β_2-AR protein in cytosol and membrane-rich fractions of SOL muscle (Fig. 1B).

4.3.2. Expressions of GR mRNA and protein

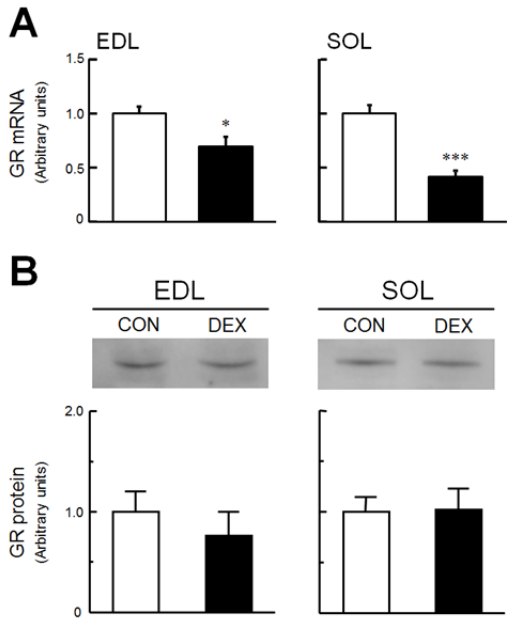

Fig. 2. Expressions of GR mRNA (A) and protein (B) in EDL and SOL muscles.
A: Expression levels of GR mRNA in EDL and SOL muscles. Total RNA was extracted from isolated skeletal muscles of control and DEX-administered rats and subjected to real-time PCR analysis. The expression level of GR mRNA were normalized to those of 18S rRNA gene. The values shown in the bar graph are related to the optical density of the control group (set = 1). Open bar, control group (n=9) and filled bar, DEX-administered group (n=8). EDL, extensor digitarum longus and SOL, soleus. Values are shown as means±S.E. Statistics: $*p < 0.05$ and $***p < 0.001$ (vs. control group). **B:** Expression levels of GR protein in the cytosol/nuclear fraction of EDL and soleus muscles. Total protein of cytosol/nuclear fraction was extracted from isolated skeletal muscles of control and DEX-administered rats and subjected to western blot analysis. The values shown by the bar graphs are related to the optical density of control group (set = 1). Open bar, control group (CON: n=6) and filled bar, DEX- administered group (DEX: n=6). EDL, extensor digitarum longus and SOL, soleus. Values are shown as means±S.E.

Figure 2A shows the effects of DEX on the expression of GR mRNA in EDL and SOL muscles. DEX decreased the expression of GR mRNA in EDL and SOL muscles by 31% ($p <$

0.05) and 58% ($p < 0.001$), respectively (Fig. 2A). The degree of DEX-induced decline in the expression of GR mRNA was relatively much higher in slow-twitch than in fast-twitch fiber-rich muscle, which agreed with our previous results [8].

Next, we examined the effects of DEX on the expression of GR protein in skeletal muscles. Figure 2B shows the immunoblot data of GR protein in cytosol/nuclear fraction of rat skeletal muscles. As shown in Fig. 2B, DEX did not change in the expression of GR protein in EDL and SOL muscles. Thus, DEX did not alter the expressions of β_2-AR and GR proteins in EDL and SOL muscles, whereas decreased the expressions of β_2-AR and GR mRNAs in SOL muscle.

4.3.3. mRNA expressions of transcription and post-transcription factors

Finally, we determined whether decreased expression of β_2-AR mRNA in SOL muscle is associated with transcriptional and post-transcriptional regulations. Therefore, we investigated typical transcription (CREB) and post-transcription (HuR and AUF1) factors of β_2-AR mRNA. As shown in Fig. 3, DEX tended to increase the expression of CREB mRNA by 128% ($p = 0.06$) in EDL muscle. On the other hand, DEX decreased the expression of CREB mRNA in SOL muscle by 34% ($p < 0.01$) (Fig. 3). Thus, the effects of DEX on the expression of CREB mRNA clearly differ from muscle fiber types.

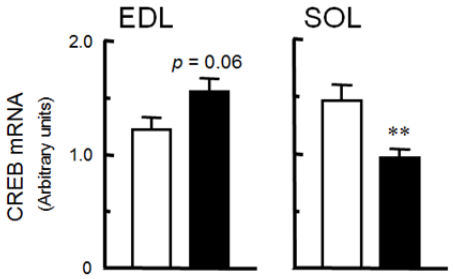

Fig. 3. Effects of DEX on the expression of CREB mRNA in EDL and SOL muscles. Total RNA was extracted from isolated skeletal muscles of control and DEX-administered rats and subjected to real-time PCR analysis. The expression level of CREB mRNA were normalized to those of 18S rRNA gene. Open bar, control group (n=9) and filled bar, DEX-administered group (n=8). EDL, extensor digitarum longus and SOL, soleus. Values are shown as means±S.E. Statistics: $^{**}p < 0.01$ (vs. control group).

Figure 4 shows the effects of DEX on the expressions of HuR and AUF1 mRNAs in skeletal muscles. Although the expression of HuR mRNA in EDL muscle tended to increase to 125% (p = 0.08) by DEX (Fig. 4), no significant change in the expression of HuR mRNA in SOL muscle was observed between both groups (Fig. 4). DEX also increased the expression of AUF1 mRNA in EDL muscle by 130% ($p < 0.01$) (Fig. 4). However, DEX did not change the expression of AUF1 mRNA in SOL muscle (Fig. 4).

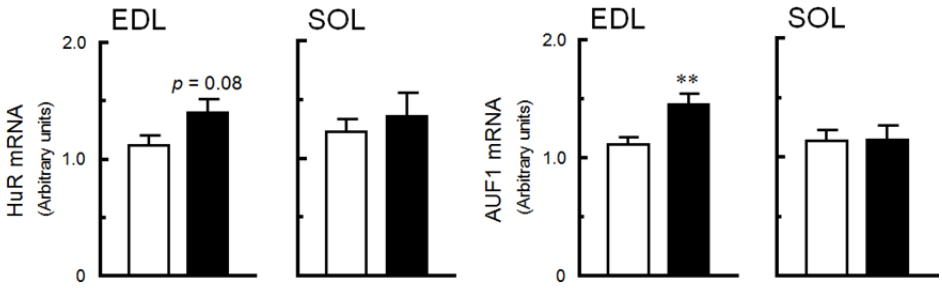

Fig. 4. Effects of DEX on the expressions of HuR and AUF1 mRNAs in EDL and SOL muscles.
Total RNA was extracted from isolated skeletal muscles of control and DEX-administered rats and subjected to real-time PCR analysis. The expression levels of HuR and AUF1 mRNAs were normalized to those of 18S rRNA gene. Open bar, control group (n=9) and filled bar, DEX-administered group (n=8). EDL, extensor digitarum longus and SOL, soleus. Values are shown as means±S.E. Statistics: $**p < 0.01$ (vs. control group).

4.4. Discussion

The present study demonstrated that the expression of β_2-AR protein in cytosol and membrane-rich fractions of EDL and SOL muscles was not affected by DEX (Fig. 1B). Huang *et al*. [13, 16] reported that corticosterone and DEX did not change the density of β_2-AR in fast-twitch muscle, but concurrent treatment of DEX with CLE prevented CLE-induced down-regulation of β_2-AR density in fast-twitch muscle. Thus, DEX and/or corticosterone did not up-regulate the expression of β_2-AR protein in skeletal muscles, expect for preventive effects on down-regulation. However, this study (Fig. 1A) and our previous study [8] also demonstrated that

DEX decreased the expression of β_2-AR mRNA in SOL muscle without changing that in EDL muscle. These findings indicate that different effects of DEX on the expression of β_2-AR between mRNA and protein levels in SOL muscle are closely related with transcription and post-transcription regulations of β_2-AR mRNA.

It is generally accepted that GR-ligand complex undergoes a conformational change resulting in dissociation of heat shock protein 90 (HSP 90) and unmasking of a nuclear localization signal into the nucleus, where it binds directly to GREs constituted by the consensus sequence AGAACAnnnTGTTCT in the 5'-flanking region, and activates gene transcription including β_2-AR in vitro [15, 26]. However, the present study clearly showed that DEX decreased the expression of GR mRNA in EDL and SOL muscles, although the degree of DEX-induced decline was relatively higher in SOL muscle than in EDL muscle (Fig. 2A). These findings suggest that DEX-induced decline in the abundance of GR mRNA is closely associated with the decrease in the expression of β_2-AR mRNA. As already described, however, DEX did not alter the expressions of β_2-AR and GR proteins (Figs. 1B and 2B), suggesting that 1) the magnitude of GR-ligand complex-induced transcriptional promotion of β_2-AR gene is relatively smaller in vivo, 2) it takes more time for the protein level to reduce after the detection of a reduced mRNA level, and 3) the decline in the expressions of β_2-AR and GR mRNAs is associated with the acceleration of translation rate. Further studies are obviously necessary to clarify the correlated regulation of the expressions between β_2-AR and GR in vivo.

Since the expression of β_2-AR was regulated at mRNA level but not protein levels, we next studied the effects of DEX on the expression of transcription and post-transcription factors of β_2-AR mRNA. The present study showed that DEX tended to increase the expression of CREB mRNA in EDL muscle, and contrastingly decreased that in SOL muscle (Fig. 3). Transcriptional responses of β_2-AR gene to cAMP are known to be localized to cAMP response element (CRE), which is constituted by the palindromic sequence TGACGTCA in the 5'-flanking region on β_2-AR gene and recognized by CREB [17]. These findings suggest that DEX-induced down-regulation of CREB mRNA in SOL muscle (Fig. 3) may be associated with the decrease in the expression of

β_2-AR mRNA. On the other hand, DEX-induced up-regulation of CREB mRNA in EDL muscle (Fig. 3) may be associated with the conservation of the expression of β_2-AR mRNA.

The present study also showed that DEX tended to increase the expressions of HuR and AUF1 mRNAs in EDL muscle without changing those in SOL muscle (Fig. 4). It is widely accepted that overexpression of HuR leads to stabilization [19, 21], and inversely reduction of HuR levels decreases half-life [22] of mRNAs carrying AREs in their 3'-UTR, suggesting that HuR stabilizes mRNAs containing AREs within their 3'-UTR, including β_2-AR mRNA. In contrast, Loflin et al. [20] reported that overexpression of AUF1 leads to degradation of mRNAs carrying AREs within their 3'-UTR, suggesting that AUF1 involves mostly in degradation of β_2-AR mRNA and competes against the role of HuR. These findings indicate that DEX-induced decrease in the expression of β_2-AR mRNA is not correlated with mRNA levels of these factors. However, parallel pattern of the expressions of HuR and AUF1 mRNAs in EDL muscle (Fig. 4) may be related to the conservation of stability of mRNAs containing AREs within their 3'-UTR such as β_2-AR mRNA. Further studies are needed to clarify detailed mechanism for the effects of DEX on post-transcriptional regulation of β_2-AR mRNA in skeletal muscles.

In conclusion, this study demonstrated that the expressions of β_2-AR and GR are regulated at mRNA levels but not protein levels by DEX. The decreased actions on the expressions of β_2-AR and GR mRNAs are clearly dependent on muscle fiber type. Further, it is generally accepted that the content of β_2-AR in slow-twitch fibers is much greater than in fast-twitch fibers, and the content of GR in slow-twitch fibers is lower than in fast-twitch fibers, which hypothesizes that the difference of the content of β_2-AR and GR between fast- and slow-twitch fibers may be related to fiber type dependent decrease in the expressions of β_2-AR and GR mRNAs. Further, the present results also suggest that DEX-induced decrease in the expression of β_2-AR mRNA in slow-twitch fiber-rich SOL muscle is associated with the transcriptional regulations.

4.5. Acknowledgments

The present study was partly supported by Research Fellowships of the Japan Society for Promotion of Science for Young Scientists (2010-2011:S. S.), a Grant-in-Aid for the Academic Frontier Project, Faculty of Human Sciences, Waseda University (2005-2009:K. I.) of the Ministry of Education, Culture, Sports, Science, and Technology, Japan, and a Grant-in-Aid of global center of excellence (COE) program, Graduate School of Sport Sciences, Waseda University (2009-2013:K. I.) of the Ministry of Education, Culture, Sports, Science, and Technology, Japan.

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5. CHAPTER 4

Casted-immobilization downregulates glucocorticoid receptor expression in rat slow-twitch soleus muscle [Sato S, Suzuki H, Tsujimoto H, Shirato K, Tachiyashiki K, Imaizumi K (2011) Life Sci 89:962-967]

5.1. Introduction

Muscle disuse results in increased myofibrillar protein breakdown, causing a progressive decrease in muscle strength that is associated with decrease in the cross-sectional area of muscle fibers. It is commonly seen in various catabolic conditions, including hypokinesia (reduction in limb movement) and hypodynamia (reduction in muscle loading). Prolonged hypokinesia and/or hypodynamia have been shown to increase the secretion of glucocorticoids, which contribute to increased catabolism of muscle proteins via the ubiquitin-proteasome pathway [1-3].

Glucocorticoids bind to the glucocorticoid receptor (GR), which regulates transcription of a variety of target genes through interaction with their promoter regions. Glucocorticoid responsiveness is partly dependent upon the expression level of GR [4]. Chronic glucocorticoid treatment typically leads to downregulation of GR expression, both in cell culture and intact tissue [5]. This downregulation of the receptor reflects glucocorticoid effects on both GR gene transcription [6] and protein turnover [7]. Our group also reported that chronic administration of a synthesized glucocorticoid, dexamethasone, decreased the expression of GR mRNA in skeletal muscle [8-10].

Prolonged hypokinesia and/or hypodynamia have been shown to increase the secretion of catecholamines (adrenaline and noradrenaline), which selectively bind to the β_2-adrenergic receptor (β_2-AR). β_2-AR in skeletal muscle plays an important physiological role in muscle plasticity via maintenance of muscle protein synthesis and degradation [11-14]. Furthermore, several studies have demonstrated that glucocorticoids and the GR complex activate the transcription of the β_2-AR gene through interaction with glucocorticoid response elements (GREs) in its promoter region [15],

leading to an upregulation of β_2-AR expression [16, 17].

The positive correlation between the expression of GR and β_2-AR likely play a physiological role in maintaining the balance between muscle protein catabolism and anabolism. However, the correlation between disuse-induced muscle atrophy and the expression of GR and β_2-AR remains unknown. In this study, we examined the effects of casted-immobilization (knee and foot arthrodesis), a model for muscle disuse, for 10 days on the expression of GR and β_2-AR in fast-twitch (extensor digitorum longus: EDL) and slow-twitch (soleus: SOL) rat muscles.

5.2. Materials and methods

5.2.1. Animal care and experimental protocol

Experimental procedures and animal care were approved by the Committee on Animal Care, Ethics and Use, Waseda University, and conducted according to the Guiding Principles for the Care and Use of Animals in the Field of Physiological Sciences, the Physiological Society of Japan [18].

Male 7-week-old Sprague Dawley rats (CLEA Japan, Tokyo, Japan) were allowed to acclimatize to their new environment for 5 days. The rats were housed two to a cage in a temperature (23–25^{o}C) and humidity (50–60%)-controlled room with a 12:12 h light-dark cycle. Animal food (CE-2 cubic type; CLEA Japan) was given to each rat by pair feeding, and once-boiled tap water was given ad libitum. After the 5-day adaptation period, the rats were randomly divided into casted-immobilization (n = 7, initial body weight = 263 ± 2 g, mean ± SEM) and sedentary control (n = 8, initial body weight = 261 ± 1 g, mean ± SEM) groups.

According to the method of Booth and Kelso [19], the casted-immobilization group rats were immobilized by arthrodesis for 10 days. Briefly, knee and ankle joints were fixed in the neutral position with a scotch cast (3-J; 3M health care, Tokyo, Japan) under sodium pentobarbital (dose = 45 mg/kg body weight) anesthesia. Blood samples were collected in heparinized

microcapillary tubes at day 0 (baseline, before cast operation), and days 1, 4, and 9 of the experimental period. After casted-immobilization for 10 days, rats were sacrificed by decapitation, and then the EDL and SOL muscles were isolated and immediately frozen in liquid nitrogen. Muscle samples were stored at -80 ^{o}C until use.

5.2.2. Blood analysis

Plasma growth hormone (GH), catecholamine (adrenaline and noradrenaline), and corticosterone concentrations were measured by the Rat GH ELISA kit (AKRGH-010; Shibayagi, Gunma, Japan), 2-CAT (A-N) Research ELISA kit (Labor Diagnostika Nord GmbH & Co. KG, Nordhorn, Germany), and YK240 Corticosterone EIA kit (Yanaihara Institute Inc., Shizuoka, Japan), respectively. Plasma creatine kinase (CK) activity was measured with a DRI-CHEM 7000 (FUJIFILM Medical Co., Tokyo, Japan), using the corresponding slide (CPK-PIII, FUJIFILM Medical Co.).

5.2.3. Real-time quantitative RT-PCR

Total RNA extracted from skeletal muscle was reverse-transcribed using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA) with random primers. Synthesized cDNA was added to a Power SYBR Green PCR Master Mix (Applied Biosystems) with 200 nM PCR primers (forward and reverse). The primer oligonucleotide sequences used for real-time quantitative PCR were as follows [9,14]: GR, 5′-TAC CAC AGC TCA CCC CTA CC-3′ (forward), 5′-AGC AGG GTC ATT TGG TCA TC-3′ (reverse); β_2-AR, 5′-GAG CCA CAC GGG AAT GAC A-3′ (forward), 5′-CCA GGA CGA TAA CCG ACA TGA-3′ (reverse); 18S rRNA, 5′-GTG CAT GGC CGT TCT TAG TTG-3′ (forward), 5′-AGC ATG CCG AGA GTC TCG TT-3′ (reverse). Amplification was performed using an ABI PRISM 7000 Sequence Detection System (Applied Biosystems). The relative amount of each mRNA was normalized to the value of the 18S rRNA gene.

5.2.4. Protein extraction

For analysis of the GR protein, total protein from the cytosol/nuclear fraction was extracted using the Tissue Protein Extraction Reagent (T-PER; Thermo Fisher Scientific, Rockford, IL) according to the manufacturer's protocol. Briefly, frozen muscles were homogenized in ice-cold T-PER (1:20, w/v) containing a protease inhibitor cocktail (Thermo Fisher Scientific) with a polytron homogenizer (IKA, Baden-Wurttemberg, Germany). The homogenate was centrifuged at 10,000 g for 10 min at 4 ^{o}C. The resultant supernatant was collected as the cytosol/nuclear fraction.

For analysis of the β_2-AR protein, whole muscle cell organelles were separated into cytosol and membrane-rich fractions. Frozen muscles were homogenized in ice-cold homogenization buffer (0.3 M KCl, 0.1 M KH_2PO_4, 50 mM K_2HPO_4 and 10 mM EDTA; pH 6.53, 1:20, w/v) containing a protease inhibitor cocktail (Thermo Fisher Scientific) with a polytron homogenizer (IKA). After a 15-min incubation on ice, the homogenate was centrifuged at 100,000 g for 60 min at 4 ^{o}C. The resultant supernatant was collected as the cytosol fraction. The pellet was homogenized in an ice-cold solubilization buffer (0.3 M KCl, 0.1 M KH_2PO_4, 50 mM K_2HPO_4, 10 mM EDTA and 1% Triton X-100; pH 6.53, 1:20, w/v) containing a protease inhibitor cocktail (Thermo Fisher Scientific). The homogenate was incubated on ice for 120 min and vortexed once every 15 min. After the incubation, the homogenate was centrifuged at 100,000 g for 60 min at 4 ^{o}C. The resultant supernatant was collected as the membrane-rich fraction. Total proteins extracted from each fraction were stored at -80 ^{o}C until use.

5.2.5. Western blotting

The protein concentration of each separated fraction was determined using a BCA Protein Assay kit (Thermo Fisher Scientific). Thirty (cytosol/nuclear fraction) or twenty (cytosol and membrane-rich fractions) micrograms of protein were incubated (5 min, 100 ^{o}C) with an equal volume of reducing sample buffer (0.5 M Tris-HCl, 10% SDS, 3% β-mercaptoethanol, 50% glycerol, 0.02% bromophenol blue), and then subjected to 4–12% SDS-polyacrylamide gel electrophoresis (Invitrogen, Carlsbad, CA). After electrophoresis, the proteins were transferred to a PVDF

membrane (Invitrogen). The membrane was incubated for 60 min in PBS-T (1×PBS + 0.1% Tween-20) containing 1% skim milk (w/v). After washing with PBS-T, the membrane was incubated with an anti-GR antibody (1:2500 dilution; Abcam, Cambridge, UK) or anti-β_2-AR antibody (1:100 dilution; Santa Cruz Biotechnology, Santa Cruz, CA) overnight. Following overnight incubation, the membrane was washed with PBS-T, and then incubated for 90 min with donkey anti-rabbit immunoglobulin G horseradish peroxidase-linked secondary antibody (1:10,000 dilution; GE healthcare, Buckinghamshire, UK). The membrane was again washed with PBS-T, and the immunoreactive bands were visualized using ECL (GE healthcare). The signal was quantified with a Lumino-Image Analyzer LAS-3000 System (Fuji Photo Film, Tokyo, Japan).

5.2.6. Statistical analysis

Experimental data were presented as mean ± SEM. The differences between two groups were tested by a Student's t-test. Subsequent post hoc analyses to determine significant differences from baseline in both groups were performed by Dunnett's test. The differences were considered significant when the P value was <0.05.

5.3. Results

5.3.1. Muscle atrophy

Body weight was 9% ($P < 0.01$) lower in casted-immobilized rats (279 ± 6 g) than in sedentary control rats (306 ± 3 g), suggesting that casted-immobilization slows down body growth. As shown in Table 1, although the relative weight of EDL muscle per body weight was unchanged by casted-immobilization for 10 days, the relative weight of SOL muscle per body weight was reduced by 47% ($P < 0.001$) after casted-immobilization. RNA and protein concentrations in the EDL muscle were not changed by casted-immobilization. In contrast, casted-immobilization decreased RNA and protein concentrations in the SOL muscle by 20% ($P < 0.01$) and 16% ($P < 0.05$),

respectively, as compared with sedentary control rats. These results suggest that casted-immobilization suppresses muscle protein synthesis and/or promotes muscle protein degradation, depending on muscle fiber type.

Table 1
Changes in weight, and RNA and protein concentrations in skeletal muscles from rats of the sedentary control and cast-immobilization groups.

Parameter	Group		cast/con
	con	cast	
EDL			
Weight (mg)	158 ± 5	136 ± 2 **	0.86
Weight (mg)/body weight (g)	0.52 ± 0.02	0.49 ± 0.01	0.94
RNA concentration (mg/g tissue)	1.10 ± 0.03	1.13 ± 0.33	1.03
Protein concentration (mg/g tissue)	246 ± 6	233 ± 5	0.95
Soleus			
Weight (mg)	108 ± 2	52 ± 2 ***	0.48
Weight (mg)/body weight (g)	0.35 ± 0.01	0.19 ± 0.01 ***	0.53
RNA concentration (mg/g tissue)	1.44 ± 0.67	1.15 ± 0.46 **	0.80
Protein concentration (mg/g tissue)	209 ± 5	175 ± 10 *	0.84

The values are mean ± SEM (n = 7–8 per group). Data for protein concentration are for the cytosol/nuclear fraction for GR analysis. Con, sedentary control group; cast, casted-immobilization group; cast/con, the relative ratio of the casted-immobilization group to the sedentary control group. *P < 0.05, **P < 0.01 and ***P < 0.001 (vs. sedentary control group).

5.3.2. Plasma GH, catecholamine, and corticosterone concentrations, and CK activity

We examined the plasma concentration of GH, which promotes cell proliferation and body growth, and plasma CK activity, which is considered an indicator of muscle protein degradation. There was no change in plasma GH concentration during the experimental period (Fig. 1A). Plasma CK activity at day 4 of the experimental period was relatively (P = 0.07) higher in the casted-immobilization group than in the sedentary control group (Fig. 1B).

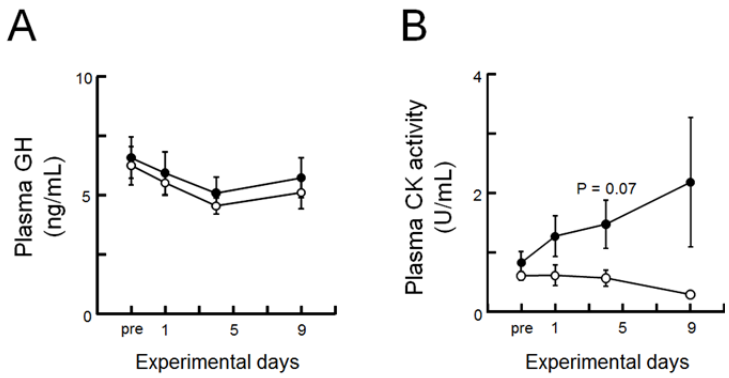

Fig. 1 Changes in plasma GH concentration (A) and CK activity (B).
Open circle, sedentary control group and filled circle, casted-immobilization group. Values: mean ± SEM (n = 7 -8/group).

Plasma concentrations of stress-responsive hormones during the experimental period were also measured. There were no differences in plasma catecholamine (adrenaline and noradrenaline) concentrations between the two groups during the experimental period (Figs. 2A and 2B). Plasma corticosterone concentration at day 1 of the experimental period was relatively (P = 0.08) higher in the casted-immobilization group than in the sedentary control group (Fig. 2C).

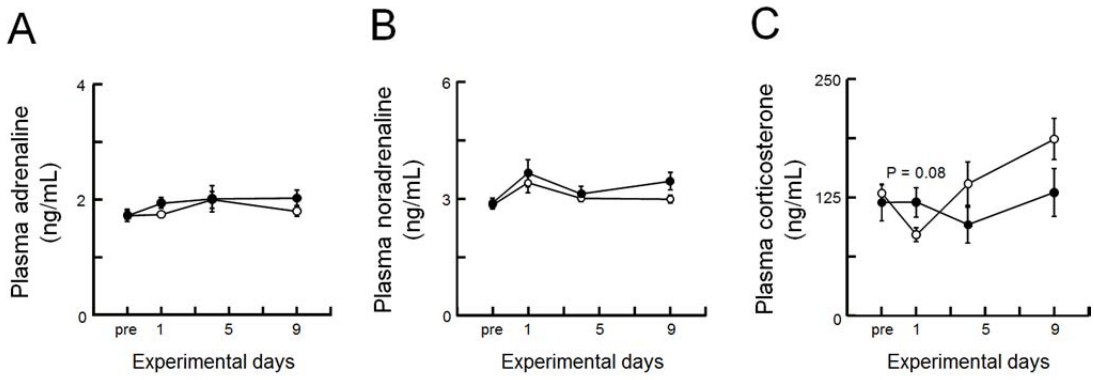

Fig. 2 Changes in plasma concentrations of adrenaline (A), noradrenaline (B) and corticosterone (C).
Open circle, sedentary control group and filled circle, casted-immobilization group. Values: mean ± SEM (n = 5 -8/group).

5.3.3. GR and β_2-AR expression

We next investigated the effects of casted-immobilization on the expression of GR and β_2-AR in skeletal muscle. As shown in Fig. 3A, casted-immobilization decreased the expression of GR mRNA in the SOL muscle by 36% ($P < 0.05$) without changing that in the EDL muscle. Furthermore, casted-immobilization decreased the expression of GR protein in the cytosol/nuclear fraction in the SOL muscle by 63% ($P < 0.001$), without changing that in the EDL muscle (Fig. 3B). These results clearly demonstrate that casted-immobilization specifically downregulates GR expression in slow-twitch SOL muscle.

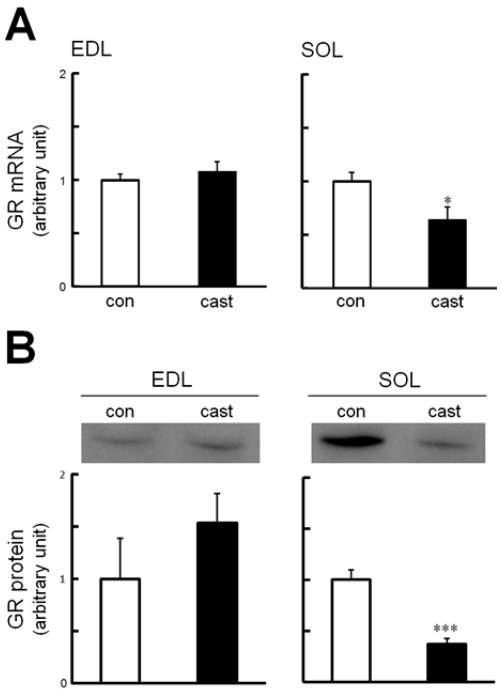

Fig. 3 Expression of GR mRNA (A) and protein (B) in EDL and SOL muscles.
A Expression of GR mRNA in EDL and SOL muscles. The expression level of GR mRNA was normalized to that of the 18S rRNA gene. The values shown in the bar graph are relative to the optical density of the sedentary control group (set = 1). Open bar, sedentary control group (con) and filled bar, casted-immobilization group (cast). Values: mean ± SEM (n = 7–8/group). *P < 0.05 (vs. sedentary control group). **B** Expression of GR protein in the cytosol/nuclear fraction from EDL and SOL muscles. Total protein from the cytosol/nuclear fraction were subjected to western blot analysis. The values shown in the bar graphs are relative to the optical density of the sedentary control group (set = 1). Open bar, sedentary control group (con) and filled bar, casted-immobilization group (cast). Values: mean ± SEM (n = 6/group). ***P < 0.001 (vs. sedentary control group).

As shown in Fig. 4A, casted-immobilization decreased the expression of β_2-AR mRNA in the SOL muscle by 45% ($P < 0.05$), and also relatively ($P = 0.06$) decreased that in the EDL muscle. These results suggest that casted-immobilization suppresses the transcription of the β_2-AR gene, especially in slow-twitch SOL muscle. However, there was no difference in β_2-AR protein expression in the cytosol and membrane-rich fractions in the EDL and SOL muscles in both groups (Fig. 4B).

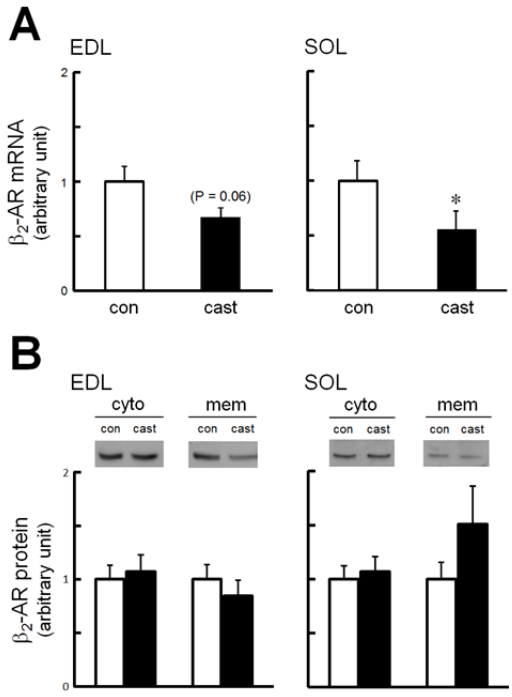

Fig. 4 Expression of β_2-AR mRNA (A) and protein (B) in EDL and SOL muscles.
A Expression of β_2-AR mRNA in EDL and soleus muscles. The expression level of β_2-AR mRNA was normalized to that of the 18S rRNA gene. The values shown in the bar graph are relative to the optical density of the sedentary control group (set = 1). Open bar, sedentary control group (con) and filled bar, casted-immobilization group (cast). Values: mean ± SEM (n = 7–8/group). *P < 0.05 (vs. sedentary control group). **B** Expression of β_2-AR protein in the cytosol and membrane-rich fractions from EDL and SOL muscles. Total protein from the cytosol and membrane-rich fractions were subjected to western blot analysis. The values shown in the bar graphs are relative to the optical density of the sedentary control group (set = 1). Open bar, sedentary control group (con) and filled bar, casted-immobilization group (cast). Cyto, cytosol fraction and mem, membrane-rich fraction. Values: mean ± SEM (n = 6/group).

5.4. Discussion

The present study clearly demonstrated that casted-immobilization induced muscle atrophy, which was greater in slow-twitch SOL muscle than in fast-twitch EDL muscle (Table 1). Furthermore, decreased RNA and protein concentrations following casted-immobilization were also observed in the SOL muscle but not in the EDL muscle (Table 1). These results are consistent with findings from hindlimb suspension models [20-24], suggesting that there is a significant reduction in transcriptional and translational capacities in slow-twitch muscle after casted-immobilization, as well as hindlimb suspension. In addition, the acceleration of protein degradation was seen in atrophied SOL muscle after hindlimb suspension [24]. The present study also demonstrated that casted-immobilization relatively increased plasma CK activity (Fig. 1B) without changing the plasma GH concentration (Fig. 1A). Thus, casted-immobilization-induced muscle atrophy in slow-twitch muscle is due, in part, to an increased rate of protein degradation.

Numerous studies have focused on the effects of glucocorticoids and the synthesized glucocorticoid, dexamethasone, on the expression of GR mRNA and protein [8-10, 15, 25, 26]. Treatment of L6 muscle cells with dexamethasone [25] and L6 myotubes and C2C12 myotubes with glucocorticoids [26] have been shown to downregulate GR expression. Our group has previously demonstrated that chronic administration of dexamethasone reduced the expression of GR mRNA in the EDL and SOL muscles [8-10]. Thus, glucocorticoid-dependent downregulation of GR expression has been frequently observed *in vitro* and *in vivo*. In the present study, downregulation of GR expression was observed in the SOL muscle after casted-immobilization for 10 days (Fig. 3), without changing the plasma corticosterone concentration (Fig. 2C). These results suggest that immobilization-induced downregulation of GR expression is mediated by other regulatory factors, such as transcription factors for the GR gene and signaling molecules downstream of GR. Furthermore, the interaction of glucocorticoids with GR is necessary for the activation of the ubiquitin-proteasome pathway and subsequent muscle protein degradation via upregulation of muscle atrophy F-box (MAFbx) and muscle ring-finger 1 (MuRF1) expression, and alteration of

activity in the upstream protein kinase B (Akt)/Forkhead box O (FOXO) pathway [2, 3]. Therefore, casted-immobilization-induced downregulation of GR expression in the SOL muscle may attenuate muscle atrophy and protein degradation.

Our groups and others attempt to clarify the mechanism of immobilization-induced muscle atrophy. Krawiec et al. [22] reported that immobilization-induced atrophy is proteasome dependent but glucocorticoids independent in the predominant fast type gastrocnemius muscle. Our study also showed that casted-immobilization did not alter the expression GR mRNA and protein in fast-twitch EDL muscle (Fig. 3). From these insights, immobilization-induced atrophy in fast-twitch muscles is independent upon the density of GR as well as the downstream signaling. On the other hand, casted-immobilization-induced preferential decrease in the expression of GR mRNA and protein in slow-twitch muscle (Fig. 3) suggests that the mechanism of immobilization-induced atrophy in slow-twitch muscle might be different from that in fast-twitch muscle. Although detailed mechanism of immobilization-induced atrophy was not examined in this study, the atrophy in slow-twitch muscle might be dependent in part upon glucocorticoid signal. Thus, our study regarding the correlation between muscle atrophy and GR expression in fast- and slow-twitch muscle is different insights from previous reports of others such as Krawiec et al. [22] who demonstrated the involvement of glucocorticoid signaling against immobilization-induced muscle atrophy, and is the first report in the fields of muscle atrophy, muscle remodeling and muscle physiology. The insights of this study should contribute at least in part toward the clarification of the mechanism of immobilization-induced muscle atrophy.

β_2-AR-mediated functions, such as muscle hypertrophy, are partly regulated by receptor density, which is associated with synthesis of the receptor and downregulation of the receptor. Furthermore, glucocorticoids and the GR complex activate the transcription of the β_2-AR gene through interaction with GREs, a consensus *cis*-acting DNA sequence AGA ACA nnn TGT TCT in its promoter regions [15], and upregulation of β_2-AR expression [16, 17]. These findings support the present results that casted-immobilization reduced the expression of β_2-AR mRNA in the SOL muscle (Fig. 4A), where GR expression was also downregulated (Fig. 3). These results suggest

that casted-immobilization suppresses transcription of the β_2-AR gene by downregulation of GR expression in the SOL muscle, which may attenuate β_2-AR-mediated muscle protein anabolism. Nevertheless, casted-immobilization for 10 days did not change the expression of β_2-AR protein in each fraction (Fig. 4B). It may take more time for β_2-AR protein expression to be reduced after the reduction in mRNA expression, provided that the turnover of β_2-AR protein is relatively slow.

5.5. Conclusions

Casted-immobilization, a model for muscle disuse, downregulates GR expression in slow-twitch muscle, suggesting that muscle disuse suppresses glucocorticoid signals, such as muscle protein degradation and transcription of the β_2-AR gene, via downregulation of GR expression in slow-twitch muscles. These findings may prove useful for identifying new therapeutic targets and novel approaches to attenuate muscle atrophy that is concomitant with physiological and pathological catabolic conditions.

5.6. Acknowledgments

The present study was supported in part by Research Fellowships from the Japan Society for Promotion of Science for Young Scientists (2010-2011: S. Sato), a Grant-in-Aid of Scientific research C (2010-2012: H. Suzuki) from the Ministry of Education, Culture, Sports, Science and Technology, Japan, and a Grant-in-Aid from global center of excellence (COE) program, Graduate School of Sport Sciences, Waseda University (2009-2013: K. Imaizumi) of the Ministry of Education, Culture, Sports, Science and Technology, Japan.

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6. CHAPTER 5

Muscle plasticity and β_2-adrenergic receptors: Adaptive responses of β_2-adrenergic receptor expression to muscle hypertrophy and atrophy [Sato S, Shirato K, Tachiyashiki K, Imaizumi K (2011) J Biomed Biotechnol 2011:1-10]

6.1. Introduction

The skeletal muscle is the most abundant tissue in the human body comprising 40–50% of body mass. Skeletal muscle protein undergoes rapid turnover, which is regulated by the balance between the rates of protein synthesis and degradation. Physical activity (exercise training), and anabolic hormones and drugs (sports doping) increase muscle protein content. However, sarcopenia and muscle disuse (due to unloading, microgravity, or inactivity) and diseases decrease muscle protein content. The rate of protein synthesis is at least in part mediated by β_2-adrenergic receptors (β_2-ARs) in skeletal muscles in both anabolic and catabolic conditions.

ARs belong to the guanine nucleotide-binding G protein-coupled receptor (GPCR) family. Skeletal muscle contains a significant proportion of β-ARs. The β_2 subtype is the most abundant, while ~7–10% of ARs are the β_1 subtype [1, 2]. Furthermore, β_2-AR is more dense in slow-twitch muscles than in fast-twitch muscles [3, 4]. However, the magnitude of anabolic responses to β_2-adrenergic agonists is greater in fast-twitch muscles than in slow-twitch muscles [5-8].

The family of β-ARs was originally believed to signal predominantly via coupling with a stimulatory guanine nucleotide-binding protein, $G\alpha_s$; however, recent studies revealed that both β_2- and β_3-ARs in skeletal muscle are also capable of coupling to an inhibitory guanine nucleotide-binding protein, $G\alpha_i$ [9]. β_2-AR activates the $G\alpha_s$/adenylyl cyclase (AC)/cyclic adenosine monophosphate (cAMP)/cAMP-dependent protein kinase A (PKA) signaling pathway. The signaling pathway is at least in part responsible for the anabolic response of skeletal muscle to β_2-AR stimulation. Further, in addition to the well-documented inhibition of AC activity [10], β_2-AR coupling to $G\alpha_i$ activates $G\alpha_s$-independent pathways [11].

β_2-AR has 7 transmembrane α helices forming 3 extracellular loops, including an NH_2 terminus and 3 intracellular loops that include a COOH terminus [12]. β_2-AR contains phosphorylation sites in the third intracellular loop and proximal cytoplasmic tail. Phosphorylation of these sites triggers the agonist-promoted desensitization, internalization, and degradation of the receptor [13]. These regulatory mechanisms contribute to maintaining agonist-induced β_2-AR responsiveness in various conditions.

The adaptive responses of β_2-AR expression to anabolic and catabolic conditions in skeletal muscles are shown in Figure 1. Understanding the correlation between changes in muscle mass and β_2-AR expression in several anabolic or catabolic conditions present scientific evidence to eradicate sports doping and identify novel approaches for attenuating muscle atrophy concomitant with disuse and various diseases. This review will discuss the effects of (1) pharmacological β_2-AR stimulation (sports doping), (2) muscle hypertrophy (exercise training), and (3) muscle atrophy (catabolic conditions and hormones) on β_2-AR expression in skeletal muscles.

6.2. Pharmacological stimulation of β_2-AR

6.2.1. Muscle hypertrophy and β_2-AR

A β_2-adrenergic agonist, clenbuterol [1-(4-amino-3,5-dichlorobenzyl)-2-(tert-butylamino) ethanol] is used as a non-steroidal anabolic drug for sports doping. According to the recent World Anti-Doping Agency (WADA) documents, clenbuterol was the seventh most commonly used anabolic agent in 2009 (67 cases; 2.0% of all anabolic agents used).

Numerous studies have shown that the administration of β_2-adrenergic agonists induces muscle hypertrophy in many species [23-25]. Experiments using mice lacking β_1-AR, β_2-AR, or both demonstrate that β_2-adrenergic agonist-induced functions such as muscle hypertrophy are mediated by β_2-AR [26]. β_2-Adrenergic agonists promote muscle growth by increasing the rate of protein synthesis and/or decreasing protein degradation [23-25]. Furthermore, β_2-adrenergic

agonists induce slow-to-fast [myosin heavy chain (MHC)I/β → MHCIIa → MHCIId/x → MHCIIb] transformation of muscle fibers.

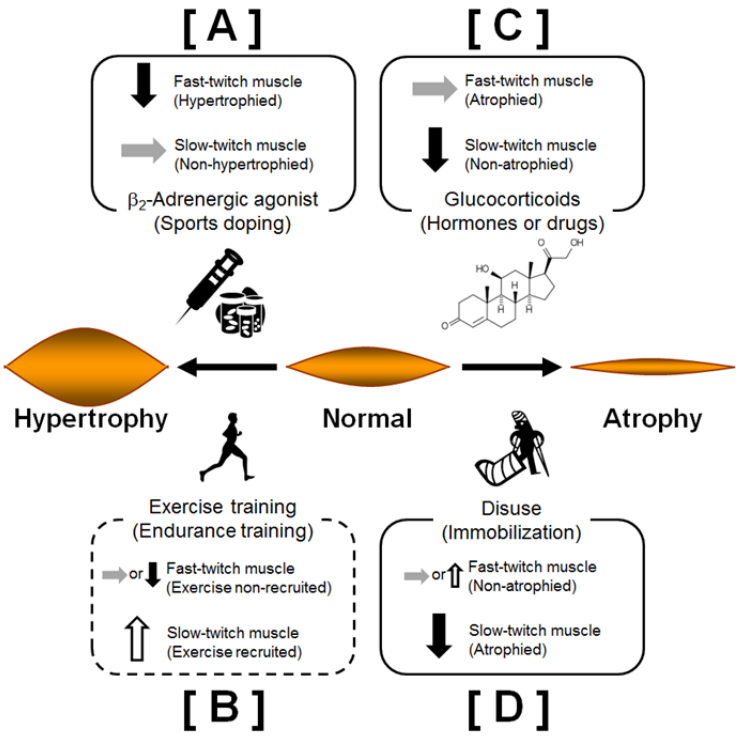

FIGURE 1: Changes in β_2-AR expression in hypertrophied and atrophied skeletal muscles.
[A] β_2-AR stimulation using anabolic drugs downregulates β_2-AR expression in hypertrophied fast-twitch muscles but not in slow-twitch muscles [4, 7, 8, 14-17]. **[B]** Exercise training such as endurance training upregulates β_2-AR expression in exercise-recruited slow-twitch muscles, whereas no changes or downregulation are observed in fast-twitch muscles [18, 19], although muscle mass is not altered. However, although exercise training such as isometric strength training induces muscle hypertrophy, there is no insight regarding the effects of such exercise on β_2-AR expression. The differential effects of types of exercise training on physiological responses such as β_2-AR expression and muscle hypertrophy should be clarified in more detailed and are currently being investigated by our group. **[C]** Catabolic hormones or drugs such as glucocorticoids downregulate β_2-AR expression in non-atrophied slow-twitch muscles but not fast-twitch muscles [16, 20, 21]. **[D]** Muscle disuse downregulates β_2-AR expression in atrophied slow-twitch muscle, whereas no changes or upregulation of receptor expression are observed in fast-twitch muscles [14, 22]. Up arrow (open arrow): upregulation of β_2-AR expression; down arrow (filled arrow): downregulation of β_2-AR expression; lateral arrow (shade arrow): no change.

The β_2-AR signaling pathway involves the agonist-dependent activation of $G\alpha_s$, which in turn activates AC, resulting in increased cAMP production. Cyclic AMP-activated PKA initiates the transcription of many target genes via the phosphorylation of cAMP-response-element- (CRE-) binding protein (CREB) or adaptor proteins such as CREB-binding protein (CBP) and p300, subsequently promoting protein synthesis [23]. While β_2-AR-mediated signaling was traditionally

believed to involve selective coupling to $G\alpha_s$, recent studies revealed that β_2-AR exhibits dual coupling to both $G\alpha_s$ and $G\alpha_i$ in skeletal muscles [9, 23]. In addition to $G\alpha_s$, $G\alpha_i$-linked $G\beta\gamma$ subunits play an active role in various cell signaling processes such as the phosphoinositol 3-kinase (PI3K)/protein kinase B (Akt)/mammalian target of rapamycin (mTOR)/p70S6K and PI3K/Akt/forkhead box-O (FOXO) pathways. These signaling pathways play important roles in β_2-adrenergic agonist-induced hypertrophy in skeletal muscles [23].

In addition to promoting protein synthesis, the hypertrophic response of skeletal muscles following β_2-adrenergic agonist administration is associated with decreased protein degradation. β_2-Adrenergic agonists attenuate protein degradation predominantly via Ca^{2+}-dependent proteolysis and the ATP/ubiquitin-dependent pathway [27-31]. However, there is little knowledge regarding the preventive effects of β_2-adrenergic agonists on the proteolysis system compared with the protein synthesis system.

The hypertrophic responses to β_2-adrenergic agonists are observed much frequently in fast-twitch muscle than in slow-twitch muscle. Our group previously demonstrated that clenbuterol administration (1.0 $mg \cdot kg^{-1} \cdot day^{-1}$) to rats for 10 days increases the mass of fast-twitch EDL muscle without altering in slow-twitch soleus muscle [7, 8]; other groups also observed the same tendency [5, 6, 32-35]. However, the mechanisms of the fiber type-dependent effects of β_2-adrenergic agonists on muscle hypertrophy remain unclear.

Pearen et al. [36, 37] and Kawasaki et al. [38] identified that β_2-AR activation increases the expression of the orphan nuclear receptor, NOR-1 (NR4A3), a negative regulatory factor of myostatin (a member of the transforming growth factor-β superfamily and a potent negative regulator of muscle mass), in fast-twitch muscles without altering that in slow-twitch muscles. Furthermore, Shi et al. [32] demonstrate the possibility that β_2-adrenergic agonist-induced fiber type-dependent hypertrophy is in part due to the extracellular signal-regulated kinase (ERK)/mitogen activated protein kinase (MAPK) pathway. Moreover, the pharmacological inhibition of the PI3K/Akt/mTOR signaling pathway revealed that the attenuation of the anabolic response to clenbuterol is greater in fast-twitch muscles than in slow-twitch muscles [30]. In addition to the

protein synthesis system, Yimlamai et al. [35] found that clenbuterol inhibits ubiquitination more strongly in fast-twitch muscles than in slow-twitch muscles. Thus, β_2-AR-mediated signaling pathways tend to promote muscle hypertrophy to a greater extent in fast-twitch muscle than in slow-twitch muscle.

6.2.2. Post-translational regulation of β_2-AR

As shown in Table 1, some reports focus on the responses of β_2-AR expression to β_2-AR stimulation in skeletal muscles [4, 7, 8, 14-17]. This is because β_2-AR functions such as muscle hypertrophy are maintained via receptor density, including synthesis and downregulation as well as receptor sensitivity, which includes receptor sensitization, desensitization, phosphorylation, and internalization [13, 39, 40].

The desensitization of β_2-AR is associated with receptor phosphorylation. McCormick et al. [41] demonstrate that fast-twitch fibers mainly express non-phosphorylated β_2-AR, whereas slow-twitch fibers predominantly express phosphorylated β_2-AR. Furthermore, treating muscle fibers with β_2-adrenergic agonists (e.g., clenbuterol, formoterol, and salbutamol) increases the phosphorylation of β_2-AR in slow-twitch fibers but not in fast-twitch fibers [41]. On the other hand, the receptor phosphorylation occurs via the actions of protein kinases (such as PKA) and/or GPCR kinase (GRK). Rat skeletal muscles contain predominantly GRK2 and GRK5; GRK protein is expressed more in fast-twitch muscles than in slow-twitch muscles. These expression levels in each type of muscle fiber are not altered by β_2-adrenergic agonist administration [42]. Thus, there is a negative correlation between the level of phosphorylated β_2-AR and receptor kinase. Therefore, further investigation is needed to reveal the detailed mechanism of β_2-AR phosphorylation.

Following β_2-AR phosphorylation, the receptor is internalized into the cytosol. The internalized β_2-AR is then degraded or dephosphorylated and subsequently recycled to the membrane [13, 43-45]. Prolonged administration of β_2-adrenergic agonists leads to the downregulation of β_2-AR density in skeletal muscles [15-17]. These post-translational regulations are advantageous for maintaining the rate of muscle protein synthesis and/or degradation.

Table 1
Responses of β_2-AR expression in skeletal muscle to anabolic and catabolic conditions.

Conditions	Species	β_2-AR		Other findings	References
		protein	mRNA		
β_2-AR stimulation					
Fenoterol (1.4 mg·kg^{-1}·day^{-1}, 4 weeks)	Rat	↓ (FT) →(ST)	n.d.		4
Clenbuterol (1.0 mg·kg^{-1}·day^{-1}, 10 days)	Rat	n.d.	↓ (FT) →(ST)	β_1-AR mRNA ↓ (LV) β_2-AR mRNA ↓ (LV)	7
Clenbuterol (1.0 mg·kg^{-1}·day^{-1}, 10 days)	Rat	n.d.	↓ (FT) →(ST)	GR mRNA ↓ (FT) HuR mRNA ↓ (FT) AUF1 mRNA ↓ (FT) hnRNP A1 mRNA ↓ (FT)	8
Fenoterol (1.4 mg·kg^{-1}·day^{-1}, 2–7 days)	Rat	→(FT, ST)	↓ (FT, ST)	$G\alpha_s$ content→(FT, ST) AC activity→(FT, ST)	14
Clenbuterol (2.0 mg·kg^{-1}·day^{-1}, 18 days)	Rat	↓ (FT+ST)	n.d.		15
Clenbuterol (4.0 mg·kg^{-1} of feed, 10 days)	Rat	↓ (FT)	n.d.	β_2-AR affinity→(FT)	16
Clenbuterol (0.2 mg·kg^{-1}·day^{-1}, 7 days)	Rat	↓ (FT+ST)	n.d.		17
Clenbuterol (50 μM) Formoterol (100 μM) Salbutamol (500 μM)	Mouse (ex vivo)	Phosphorylated β_2-AR ↑ (ST), →(FT)	n.d.	cAMP concentration ↑ (FT, ST)	41
Endurance training					
Treadmill (12 weeks)	Rat	↓ (FT)	n.d.	β_2-AR affinity→ AC activity ↓ $G\alpha_s$ content ↓	18
Treadmill (18 weeks)	Rat	→(FT) ↑ (ST)	n.d.	AC activity ↑ (FT, ST) β_2-AR density→(acute)	19
Catabolic conditions					
Dexamethasone (1.0 mg·kg^{-1}·day^{-1}, 10 days)	Rat	→(FT, ST)	→(FT) ↓ (ST)	GR mRNA ↓ (FT, ST) CREB mRNA ↓ (ST) AUF1 mRNA ↑ (FT)	20
Dexamethasone (1.0 mg·kg^{-1}·day^{-1}, 10 days)	Rat	n.d.	→(FT) ↓ (ST)	GR mRNA ↓ (FT, ST) β_1-AR mRNA ↑ (LV)	21
Dexamethasone (0.2 mg·kg^{-1}·day^{-1}, 10 days)	Rat	→(FT)	n.d.	β_2-AR affinity→(FT)	16
Casted-immobilization (10 days)	Rat	→(FT, ST)	→(FT) ↓ (ST)	GR mRNA ↓ (ST) GR protein ↓ (ST)	22
Aging	Rat	→(FT, ST)	n.d.		4
Injury (bupivacaine injection)	Rat	↑ (FT) ↓ (ST)	↑ (FT) ↓ (ST)	$G\alpha_s$ content ↑ (FT), ↓ (ST) AC activity ↑ (FT, ST)	14

FT, fast-twitch muscle; ST, slow-twitch muscle; LV, left ventricle muscle. Up arrow, increase; down arrow, decrease; lateral arrow, no change. n.d., no data.

6.2.3. Short-term and chronic transcriptional regulation of β_2-AR

β_2-AR synthesis, including transcription and subsequent translation, is required to restore transmembrane receptor density. The process of β_2-AR synthesis can be separated into 2 pathways: (1) the positive autoregulation of β_2-AR gene transcription via receptor-mediated elevation of cAMP concentration followed by the phosphorylation and activation of CREB [46, 47], and (2) the transactivation of the β_2-AR gene via interaction between hormones and the nuclear receptor complex and response elements on the β_2-AR promoter region [48]. In particular, the transcription of the β_2-AR gene and the subsequent mRNA expression via cAMP-mediated CRE activation increased in response to short-term β_2-adrenergic agonist exposure [46, 47]. Moreover, treatment with glucocorticoids or thyroid hormone transactivates the β_2-AR gene both in vitro and in vivo [48-51].

Our previous reports demonstrate that clenbuterol administration (1.0 $mg \cdot kg^{-1} \cdot day^{-1}$) for 10 days to rats decreases β_2-AR mRNA expression in the fast-twitch EDL muscle without altering that in the slow-twitch soleus muscle [7, 8]. Furthermore, the mRNA expression of GR was also decreases with clenbuterol treatment in the EDL muscle but not in the soleus muscle [8]. Glucocorticoids and the GR complex activate the transcription of the β_2-AR gene via interaction with glucocorticoid response elements (GREs), consensus *cis*-acting DNA sequences (i.e., AGA ACA nnn TGT TCT) on its promoter regions [48], thus upregulating β_2-AR expression [16, 50, 51]. These findings corroborate our results that there is a positive correlation between the expression levels of β_2-AR and GR in skeletal muscles. Beitzel et al. [14] also report that administrating the β-adrenergic agonist, fenoterol (1.4 $mg \cdot kg^{-1} \cdot day^{-1}$, i.p.), for 5 days decreases β_2-AR mRNA expression in the EDL and soleus muscles. Thus, in contrast to the transactivation of the β_2-AR gene and increase in the mRNA level in response to short-term agonist exposure, chronic β_2-adrenergic stimulation inhibits β_2-AR synthesis in skeletal muscles.

6.2.4. Post-transcriptional regulation of β_2-AR

In addition to post-translational and transcriptional regulation, several groups focus on the

post-transcriptional regulation of β_2-AR mRNA. β_2-AR mRNA contains an AU-rich element (ARE) within the 3′-untranslated region (3′-UTR) that can be recognized by several mRNA-binding proteins, including HuR, AUF1, and hnRNP A1 [52-55]. These factors play a role in the regulation of β_2-AR mRNA stability [52-55]. Our study demonstrates that clenbuterol-induced stimulation of β_2-AR decreases the mRNA expressions of these factors in the EDL but not in the soleus muscle [8], suggesting that the post-transcriptional process of β_2-AR synthesis requires the stability of its mRNA to be regulated.

6.3. Exercise training and β_2-AR

Strength resistance training increases muscle mass [56], fiber cross-sectional area [57], protein and RNA contents [58], and the capacity to generate force [59]. In contrast to strength training, endurance training is characterized by increased mitochondrial mass [60], increased oxidative enzymes [61], decreased glycolytic enzymes [62], increased slow contractile and regulatory proteins [62], and decreased fast-fiber area [63]. These findings suggest that the functional roles of β_2-AR in skeletal muscles differ with the type of exercise training.

6.3.1. Strength exercise training and β_2-AR

Mounier et al. [64] investigated the changes in the weight of the EDL muscle induced by clenbuterol administration, strength training, and a combination of both. They found that the effects of strength training and clenbuterol on muscle hypertrophy were not additive in fast-twitch muscles. Their report also demonstrates that the strength training-induced enhancement of lactate dehydrogenase-specific activity is completely inhibited by clenbuterol administration, while the clenbuterol-induced decrease in monocarboxylate transporter1 mRNA expression is completely offset by strength training [64]. Thus, there are no synergetic effects of a combination of strength training and β_2-AR stimulation on muscle mass. Furthermore, strength training counteracts

molecular modifications such as glycolytic control induced by chronic clenbuterol administration in fast-twitch muscles to some extent. However, our evidence regarding the synergistic effects of strength training and β_2-AR stimulation is insufficient because the experimental models of strength-trained animals are not fully established.

6.3.2. Endurance exercise training and β_2-AR

In contrast to strength training, β_2-AR stimulation affects endurance training-induced modulations such as contractile activity [65], muscle fiber type shift [65], metabolic enzyme activity [66], and insulin resistance [67, 68]. Lynch et al. [65] demonstrated that low-intensity endurance training prevents clenbuterol-induced slow-to-fast (type I fiber → type II fiber) fiber type transformation in the EDL and soleus muscles, and thereby offsets the clenbuterol-induced decrease in Ca^{2+} sensitivity in fast-twitch fibers. These results suggest that endurance training-heightened muscle aerobic capacity is attenuated by β_2-AR stimulation-induced muscle fiber type transformations. Furthermore, pharmacological β-AR blockage diminishes the endurance training-induced increase in citrate synthase activity in the fast-twitch plantaris muscle [66]. Moreover, clenbuterol administration prevents the endurance training-induced improvement in insulin-stimulated glucose uptake and attenuates the increase in citrate synthase activity in the skeletal muscles of obese Zucker rats [67, 68]. These findings demonstrate that the endurance training-induced increase in aerobic metabolism in skeletal muscles requires moderate but not excessive stimulation of β_2-AR.

Recently, Miura et al. [69] demonstrated that an increase in peroxisome proliferator-activated receptor-γ coactivatior-1α (PGC-1α) mRNA in response to exercise is mediated by β_2-AR activation. Furthermore, the Ca^{2+}-signaling [70] and p38 MAPK pathways [71], which is downstream of β_2-AR, are activated in skeletal muscles in response to exercise, which regulates PGC-1α expression. Since PGC-1α promotes mitochondrial biogenesis [72], the exercise-induced activation of β_2-AR may in part enhance aerobic capacity by increasing PGC-1α expression. Thus, β_2-AR stimulation is essential for enhancing the effects of exercise training on

muscle functions such as fiber type shift as well as oxidative and anaerobic metabolism.

6.3.3. Response of β_2-AR expression to exercise training

As mentioned above, the functional roles of β_2-AR during exercise training are physiologically important in skeletal muscles. Therefore, changes in the expression and sensitivity of β_2-AR should be important for the metabolic, anabolic, and catabolic adaptations of skeletal muscles during exercise training. Nevertheless, there is little information on the response of β_2-AR expression to exercise training in skeletal muscles. However, many studies demonstrate the effects of exercise training on β_2-AR expression in several tissues and cell types such as myocardia [73, 74], adipocytes [75], and macrophages [76]. Barbier et al. [73] demonstrated that exercise training induces changes in the distribution of β_1-, β_2-, and β_3-AR densities in the rat left ventricle. In adipocytes, the exercise-induced trafficking of β_2-AR into the cell membrane from the cytosol is coupled with adipocytes' function to increase intracellular cAMP production [75]. Kizaki et al. [76] also found a reduction in the expression of β_2-AR mRNA in macrophages and highlight the significance of β_2-AR in the exercise training-induced improvement of macrophages' innate immune function. Thus, changes in β_2-AR expression play a role in physiological adaptations to exercise training in several tissues.

A few studies also report the effects of exercise training on β-AR in skeletal muscles [18, 19, 77, 78] (Table 1). Nieto et al. [18] demonstrate that β-AR density and $G\alpha_s$ content in the fast-twitch gastrocnemius muscle are significantly lower in endurance-exercised rats than in controls. They also reveal that exercise reduces receptor- and non-receptor-mediated (i.e., pharmacological stimulation of AC by forskolin) AC activity in muscles [18]. However, Buckenmeyer et al. [19] report that endurance training increases β-AR density in slow-twitch muscles that are primarily recruited during endurance training, whereas β-AR density is not altered in fast-twitch muscles. Their report also demonstrates that receptor-mediated AC activity in slow-twitch muscles is increased by endurance training, and non-receptor-mediated AC activity is increased by training in both fast- and slow-twitch muscles [19]. In contrast to chronic endurance training, the effects of

acute exercise on β-AR density and AC activity in each type of muscle were not observed [73]. Therefore, endurance exercise training-induced changes in β_2-AR expression and signaling in slow-twitch muscle contributes to the adaptation of metabolic and anabolic capacities during exercise.

6.4. Muscle atrophy and β_2-AR

6.4.1. Preventive roles of β_2-AR in disuse-induced muscle atrophy

Muscle wasting and weakness are common in physiological and pathological conditions, including aging, cancer cachexia, sepsis, other forms of catabolic stress, denervation, disuse (e.g., unloading, inactivity, and microgravity), burns, human immunodeficiency virus (HIV)-acquired immunodeficiency syndrome (AIDS), chronic kidney or heart failure, chronic obstructive pulmonary disease (COPD), and muscular dystrophies. For many of these conditions, the anabolic properties of β_2-adrenergic agonists provide therapeutic potential for attenuating or reversing muscle wasting, muscle fiber atrophy, and muscle weakness. These β_2-adrenergic agonists also have important clinical significance for enhancing muscle repair and restoring muscle function after muscle atrophy.

In particular, muscle disuse, which is mainly reflected by increased myofibrillar protein breakdown, causes a progressive decrease in muscle strength associated with a decreased cross-sectional area of muscle fibers. Therefore, preventing disuse-induced muscle atrophy is a problem requiring urgent attention and highlights β_2-AR as a target of pharmacological stimulation. Since 2000, many groups have focused on the preventive effects of β_2-adrenergic agonist on disuse-induced muscle atrophy [4, 34, 35, 79].

Yimlamai et al. [35] demonstrate that clenbuterol attenuates the hindlimb unweighting-induced atrophy and reduces ubiquitin conjugates only in fast-twitch plantaris and tibialis anterior muscles but not in the slow-twitch soleus muscle; this suggests that clenbuterol alleviates hindlimb unweighting-induced atrophy, particularly, in fast-twitch muscles at least in part

through a muscle-specific inhibition of the ubiquitin-proteasome pathway. However, Stevens et al. [34] report that clenbuterol treatment accelerates hindlimb unweighting-induced slow-to-fast (MHCI/β → MHCIIa → MHCIId/x → MHCIIb) transformation in the soleus muscle. β_2-Adrenergic agonist also reverses muscle wasting and weakness in several conditions such as ageing [4], muscular dystrophy [29], denervation [80], cancer cachexia [28], and myotoxic injury [81].

6.4.2. Preventive roles of β_2-AR in catabolic hormone-induced muscle atrophy

Prolonged muscle disuse and/or unloading increases the secretion of glucocorticoids, which promotes the catabolism of muscle proteins via the ubiquitin-proteasome pathway [82, 83]. Sepsis also elevates plasma glucocorticoids and adrenocorticotropic hormone (ACTH) levels [84]. Therefore, several studies focus on the counteractive effects of β_2-AR stimulation on glucocorticoid-induced muscle atrophy [16, 85]. Huang et al. [16] report that clenbuterol almost prevents the decrease in the weight of gastrocnemius/plantaris muscle bundles induced by dexamethasone, a synthetic glucocorticoid. Pellegrino et al. [85] demonstrate that concurrent treatment of clenbuterol with dexamethasone minimizes MHC transformation induced by clenbuterol (slow-to-fast) or dexamethasone (fast-to-slow) alone. Thus, β_2-AR stimulation plays an inhibitory role in muscle atrophy and weakness induced by catabolic diseases, mechanical unloading, catabolic hormones, and pharmacological agents.

6.4.3. Response of β_2-AR expression to catabolic hormones

Although the effectiveness of β_2-AR stimulation on muscle atrophy is well documented, catabolic condition-induced changes in the expression of β_2-AR in skeletal muscles are not fully understood. Understanding the responses of β_2-AR expression to muscle atrophy is required to establish treatments for muscle atrophy.

Table 1 shows the catabolic condition-induced changes in β_2-AR expression in skeletal muscles. Our group investigated whether catabolic hormones or agents alter β_2-AR expression in

skeletal muscles [20, 21]. Dexamethasone administration (1.0 $mg \cdot kg^{-1} \cdot day^{-1}$) to rats for 10 days decreases the expression of β_2-AR mRNA in the soleus muscle without altering that in the EDL muscle, although the expression of β_2-AR protein in the EDL and soleus muscles is not altered [20, 21]. Dexamethasone also does not alter β_2-AR density in gastrocnemius/plantaris muscle bundles [16]. These phenomena are specifically observed in skeletal muscles; meanwhile, glucocorticoids and the GR complex activate the transcription of β_2-AR gene in the human hepatoma cell line (HepG2) [48], subsequently leading to the upregulation of β_2-AR levels in DDT_1 MF-2 smooth muscle cells [50] and lung tissue [16, 51]. Furthermore, dexamethasone decreases the expression of GR mRNA in the soleus muscle [20, 21]. Dexamethasone also decreases and increases the expression of CREB mRNA, a transcription factor of the β_2-AR gene [46, 47], in the soleus and EDL muscles, respectively [20]. These findings suggest that the dexamethasone-induced decrease in the expression of β_2-AR mRNA in the slow-twitch soleus muscle is associated with transcriptional regulations.

6.4.4. Response of β_2-AR expression to muscle disuse

The effects of physiological and pathological catabolic condition-induced muscle atrophy on β_2-AR expression have also been studied (Table 1) [4, 14, 22]. Our recent investigation demonstrates that casted immobilization (knee and foot arthrodesis) for 10 days markedly induces atrophy in the soleus muscle, whereas it decreased the expression of β_2-AR mRNA [22]. Decreased GR mRNA and protein expression was also detected in the soleus muscle [22]. These results suggest that casted immobilization decreases the expression of β_2-AR mRNA in slow-twitch muscles via the downregulation of GR levels and subsequent glucocorticoid signals. On the other hand, Ryall et al. [4] demonstrate that aging-induced muscle wasting is observed in the EDL and soleus muscles, although there are no age-associated changes in β_2-AR density in these muscles. Furthermore, in the regeneration process from muscle injury induced by bupivacaine injection, β_2-AR density and mRNA expression as well as $G\alpha_s$ content are decreased in the soleus but increased in the EDL muscle [14]. Thus, the effects of catabolic conditions such as disuse, aging,

and injury on β_2-AR expression are different from and/or dependent on the conditions, especially in fast-twitch muscles, whereas decreasing tendencies are observed in slow-twitch muscles.

Both pharmacological and mechanical studies indicate that the preventive effects of β_2-AR stimulation on muscle atrophy and weakness are limited by decreased β_2-AR synthesis and subsequently decreased density. In order to use β_2-adrenergic agonists as a therapeutic agent for muscle wasting, further studies are necessary to obtain detailed evidence regarding the responses of β_2-AR expression and function to muscle atrophy.

6.5. Conclusions

In this review, we discussed adaptive responses of β_2-AR expression in skeletal muscles to β_2-adrenergic agonist treatment, exercise training, muscle disuse, and glucocorticoid treatment. This review also outlined the functional roles of β_2-AR in skeletal muscles. Skeletal muscle partly requires β_2-AR activation for hypertrophy, regeneration, and atrophy prevention; however, its functions and responsiveness must be adaptively regulated by the receptor itself via downregulation, synthesis, and desensitization. New insight in the form of scientific evidence is needed to eradicate sports doping and to identify new therapeutic targets for attenuating muscle atrophy induced by physiological and pathological conditions.

6.6. Acknowledgments

We would like to thank Prof. Hideki Suzuki, Department of Health and Physical Education, Aichi University of Education, and Prof. Hisaya Tsujimoto, Institute of Health and Sports Science, Kurume University, for their valuable advices and suggestions. This work was supported by Research Fellowships of the Japan Society for Promotion of Science for Young Scientists (2010–2011: S. Sato), and a Grant-in-Aid of the Global Center of Excellence (COE) program, Graduate School of Sport Sciences, Waseda University (2009–2013: K. Imaizumi) of the Ministry of

Education, Culture, Sports, Science, and Technology, Japan.

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7. Concluding remarks

This thesis summarized the adaptive responses of β_2-adrenergic receptor (β_2-AR) expression to β_2-AR stimulation-induced muscle hypertrophy (CHAPTER 1 and CHAPTER 2), glucocorticoid receptor stimulation-induced muscle atrophy (CHAPTER 3), and immobilization-induced muscle atrophy (CHAPTER 4). The key findings are summarized as follows:

1. The administration of β_2-agonist clenbuterol (dose=1.0mg/kg body weight/day) to rats for 10 days increased the weight of the fast-twitch muscle without changing that of the slow-twitch muscle. Clenbuterol decreased the expression of β_2-AR mRNA in the fast-twitch muscle but not in the slow-twitch muscle. The mRNA expression of glucocorticoid receptor (GR), one of the transcription factors of β_2-AR gene, was also decreased with clenbuterol treatment in the fast-twitch muscle but not in the slow-twitch muscle. Thus, there is a positive correlation between the expression levels of β_2-AR and GR mRNAs in the fast-twitch muscle, suggesting that decreased rate of transcription of the β_2-AR gene is related to the reduction of GR expression.
2. The administration of synthesized glucocorticoid dexamethasone (dose=1.0mg/kg body weight/day) to rats for 10 days induced muscle atrophy, which was greater in the fast-twitch muscle than in the slow-twitch muscle. Dexamethasone decreased the expression of β_2-AR mRNA in the slow-twitch muscle but not in the fast-twitch muscle. The expression of GR mRNA was also decreased with dexamethasone treatment in both fast- and slow-twitch muscles, although the degree was higher in the slow-twitch muscle than in the fast-twitch muscle. Thus, there is a positive correlation between the expression levels of β_2-AR and GR mRNAs in the slow-twitch muscle. Nevertheless, the expression of β_2-AR and GR proteins was not affected by dexamethasone in both fast- and slow-twitch muscles. It may take more time for β_2-AR and GR protein expressions to be reduced after the reduction in mRNAexpression.
3. Casted-immobilization for 10 days induced muscle atrophy of rats, which was greater in the

slow-twitch muscle than in the fast-twitch muscle. Casted-immobilization decreased the expression of β_2-AR mRNA in the slow-twitch muscle but not in the fast-twitch muscle. However, the expression of β_2-AR protein was not affected by casted-immobilization in both fast- and slow-twitch muscles. The expression of GR mRNA and protein was also downregulated after 10-day casted-immobilization in the slow-twitch muscle without changing that in the fast-twitch muscle. These results suggest that casted-immobilization suppresses glucocorticoid signals, such as muscle protein degradation and transcription of the β_2-AR gene, via downregulation of GR expression in the slow-twitch muscle.

The thesis also integrated and discussed the adaptive responses of β_2-AR expression to anabolic and catabolic conditions (CHAPTER 5). The β_2-AR in the skeletal muscle plays a physiological role in the regulation of muscle plasticity and energy balance. Skeletal muscle partly requires β_2-AR activation for hypertrophy, regeneration, and atrophy prevention; however, its functions and responsiveness must be adaptively regulated by the receptor itself via downregulation, synthesis, and desensitization. This thesis will present scientific evidence to eradicate sports doping and identify novel approaches for attenuating muscle atrophy concomitant with disuse and various diseases.

8. Supplement (Publication list)

I. 学位（修士）論文

1) **Sato S**. Muscle fiber type dependent effects of β_2-agonist, clenbuterol on β_1- and β_2-adrenoceptor mRNA expressions in rats. **早稲田大学大学院人間科学研究科修士論文、pp. 1-28、人間科学研究、22（補遺）: 40 (2009)**

II. 学術論文

a) 骨格筋の可塑性とβ_2-アドレナリン受容体発現応答とその機構（博士論文）

1) **Sato S**, Nomura S, Kawano F, Tanihata J, Tachiyashiki K and Imaizumi K. Effects of the β_2-agonist clenbuterol on β_1- and β_2-adrenoceptor mRNA expressions of rat skeletal and left ventricle muscles. ***Journal of Pharmacological Sciences,* 107 (4) : 393-400 (2008)**（日本薬理学会英文誌・審査付き）
2) 佐藤章悟、谷端　淳、今泉和彦．骨格筋と心筋におけるβ_1-とβ_2-アドレナリン受容体の遺伝子発現とその調節（特集：伝達物質と受容体）．*生体の科学*、**60 (5) : 426-427 (2009)**（(財）金原一郎医学医療振興財団／医学書院・東京・依頼執筆）
3) Kawano F, Tanihata J, **Sato S**, Nomura S, Shiraishi A, Tachiyashiki K and Imaizumi K. Effects of dexamethasone on the expression of β_1-, β_2- and β_3-adrenoceptor mRNAs in skeletal and left ventricle muscles in rats. ***Journal of Physiological Sciences,* 59 (5) : 383-390 (2009)**（日本生理学会英文誌・審査付き）
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6) **Sato S**, Suzuki H, Tsujimoto H, Shirato K, Tachiyashiki K and Imaizumi K. Casted-immobilization downregulates glucocorticoid receptor expression in rat slow-twitch soleus muscle. ***Life Sciences,* 89 (25-26) : 962-967 (2011)**（**Elsevier Inc.**・審査付き）
7) **Sato S**, Shirato K, Tachiyashiki K and Imaizumi K. Muscle plasticity and β_2-adrenergic receptors: Adaptive responses of β_2-adrenergic receptor expression to muscle hypertrophy and

atrophy (Advances in Muscle Physiology and Pathophysiology 2011: invited review). ***Journal of Biomedicine and Biotechnology*, 2011 : 1-10 (2011)**（**Hindawi Publishing Corporation**・審査付き）

b) 各種栄養素摂取等による生体応答とその反応機構（参考論文）

1) Someya Y, Tanihata J, **Sato S**, Kawano F, Shirato K, Sugiyama M, Kawashima Y, Nomura S, Tachiyashiki K and Imaizumi K. Zinc-deficiency induced changes in the distribution of rat white blood cells. ***Journal of Nutritional Science and Vitaminology*, 55 (2) : 162-169 (2009)**（日本栄養・食糧学会－日本ビタミン学会連合英文誌・審査付き）
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III. 学会発表

a) 国際学会・国際シンポジウム

1) **Sato S**, Tanihata J, Kawano F, Tachiyashiki K and Imaizumi K. Effects of β_2-agonist, clenbuterol on the mRNA expression of mRNA binding/degradation factors of β-adrenoceptor in rat muscles. ***The 36-th International Congress of Physiological Sciences (July, Kyoto)***, ***Journal of Physiological Sciences,*** **59 (Supplement 1), 218 (2009)**

2) Kawano F, Tanihata J, **Sato S**, Nomura S, Shiraishi A, Tachiyashiki K and Imaizumi K. Effects of dexamethasone on β_1-, β_2- and β_3-adrenoceptor mRNA expressions of skeletal and left ventricle muscles in rats. ***The 36-th International Congress of Physiological Sciences (July, Kyoto), Journal of Physiological Sciences,*** **59 (Supplement 1): 216 (2009)**

3) Aritoshi S, Tanihata J, **Sato S**, Kawano F, Tachiyashiki K and Imaizumi K. Subacute effects of dihydrocapsaicin and capsaicin on the distribution of white blood cells, and plasma free fatty acid, glycerol and glucose levels in rats. ***The 36-th International Congress of Physiological Sciences (July, Kyoto), Journal of Physiological Sciences,*** **59 (Supplement 1): 163 (2009)**

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7) **Sato S**. Effects of β_2-agonist, clenbuterol on β_2-adrenoceptor mRNA expression and the regulatory factors in rat skeletal and left ventricle muscles. ***International Sports Science Network Forum in Nagano 2009 (August, Karuizawa),* Abstracts, p. 55 (2009)**
8) **Sato S**, Tsujimoto H, Suzuki H, Tachiyashiki K and Imaizumi K. Effects of casted-immobilization on the expressions of β-adrenergic receptors, glucocorticoid receptor and uncoupling proteins in brown and white adipose tissues of rats. ***The 7th Congress of Federations of Asian and Oceanian Physiological Societies (September, Taipei),* Abstract, p. 191 (2011)**
9) **Sato S**, Suzuki H, Tsujimoto H, Tachiyashiki K and Imaizumi K. Casted-immobilization downregulates glucocorticoid receptor level in rat slow-twitch soleus muscle. ***The 7th Congress of Federations of Asian and Oceanian Physiological Societies (September, Taipei),* Abstract, p. 275 (2011)**

b) 国内学会・国内シンポジウム

【招待講演】

1) 佐藤章悟、野村幸子、河野風雲、立屋敷かおる、今泉和彦．骨格筋の可塑性とβ_2-アドレナリン受容体遺伝子発現との関連（シンポジウム 4＜S4＞「運動器（筋・骨を育む栄養素）」）．***第 64 回日本栄養・食糧学会大会（5 月、徳島）*、講演要旨集、p. 49 (2010)**

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6) 佐藤章悟、河野風雲、谷端　淳、今泉和彦、立屋敷かおる．β_2-アゴニスト・クレンブテロールによるラットβ_1-およびβ_2-アドレナリン受容体 mRNA 発現への筋線維タイプ依存的な影響．***第145回日本体力医学会関東地方会（3月、東京）*、体力科学、58 (3) : 412 (2009)**

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