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ORIGINAL PAPER

Adaptive effects of the β_2-agonist clenbuterol on expression of β_2-adrenoceptor mRNA in rat fast-twitch fiber-rich muscles

Shogo Sato · Sachiko Nomura · Fuuun Kawano · Jun Tanihata · Kaoru Tachiyashiki · Kazuhiko Imaizumi

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Abstract Administration of the β_2-agonist clenbuterol has been shown to reduce the expression of β_2-adrenoceptor (AR) mRNA in fast-twitch fiber-rich (extensor digitorum longus, EDL) muscle without changing that in slow-twitch fiber-rich (soleus, SOL) muscle in rats. However, the regulatory mechanism for muscle fiber type-dependent down-regulation of the expression of β_2-AR mRNA induced by clenbuterol is still unclear. Therefore, mRNA expression of transcriptional and post-transcriptional regulatory factors for β_2-AR mRNA levels in fast-twitch fiber-rich (EDL and plantaris, PLA) and slow-twitch fiber-rich (SOL) muscles in clenbuterol-administered (1.0 mg/kg body weight/day for 10 days, subcutaneous) rats was studied by real-time reverse transcription-polymerase chain reaction. Administration of clenbuterol significantly reduced expression of β_2-AR mRNA in EDL and PLA muscles without changing that in SOL muscle. Administration of clenbuterol also significantly reduced the mRNA expression of transcriptional regulatory factor (glucocorticoid receptor) and mRNA stabilizing factor (Hu antigen R) in EDL and PLA muscles without changing those in SOL muscle. These results suggest that muscle fiber type-dependent effects of clenbuterol on expression of β_2-AR mRNA are closely related to the down-regulation of mRNA expression of transcriptional and post-transcriptional regulatory factors for β_2-AR mRNA levels.

Keywords Clenbuterol · β_2-Adrenoceptor · Glucocorticoid receptor · Skeletal muscle · Down-regulation

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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-regulates 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 shown 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 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].

The 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.

Materials and methods

Experimental procedures and animal care

The experimental procedure used in this study is shown in Fig. 1. Briefly, clenbuterol (dose = 1.0 mg/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 food (CE-2 cubic type; CLEA Japan) was given to each rat under diet-restricted feeding (feeding chow = 30 g/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 ± 2 g, mean ± standard error of the mean (SEM)) and the control ($n = 10$, the initial body weight = 278 ± 2 g, mean ± SEM) groups.

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].

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

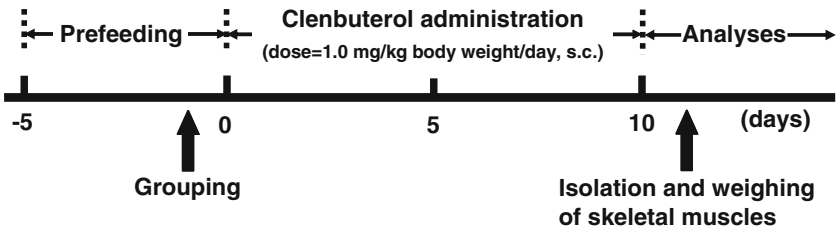

Fig. 1 Experimental procedure used in this study. After a 5-day-adaptation period, the rats were randomly divided into the clenbuterol-administered group and the control group. Clenbuterol (dose = 1.0 mg/kg body weight/day) was administered to the cervical portion of the back by subcutaneous (s.c.) injection (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

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 CAA AAC 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′

clenbuterol-administered group, clenbuterol (dose = 1.0 mg/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].

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 incubation at 4°C overnight until they could be used for RNA extraction [1].

Analysis of mRNA expression by real-time quantitative reverse transcription-polymerase chain reaction

RNA was extracted from stored muscle samples by use of TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's procedure [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 procedure [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 sequence detection system (Applied Biosystems).

Statistical analysis

Experimental data were presented as the mean ± SEM. The differences between two groups were tested by a Student's *t* test and considered significant when p value was <0.05.

Results

Effects of clenbuterol on the weight of, 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,

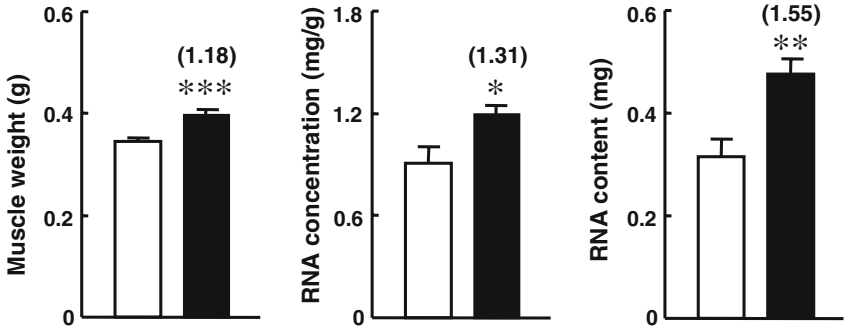

Fig. 2 Effects of clenbuterol on the weight of, RNA concentration in, and RNA content of PLA muscle. The values are shown as mean ± SEM ($n = 10$/group). *Open bars*, control group; *closed bars*, clenbuterol-administered group. Values in parentheses are values for the clenbuterol-administered group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

the weight (0.40 ± 0.01 g) of, RNA concentration (1.19 ± 0.06 mg/g) in, and RNA content (0.48 ± 0.03 mg) 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 ± 0.01 g, 0.91 ± 0.01 mg/g, and 0.31 ± 0.04 mg, respectively) in the control group. These results were qualitatively similar to our previous findings for 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.

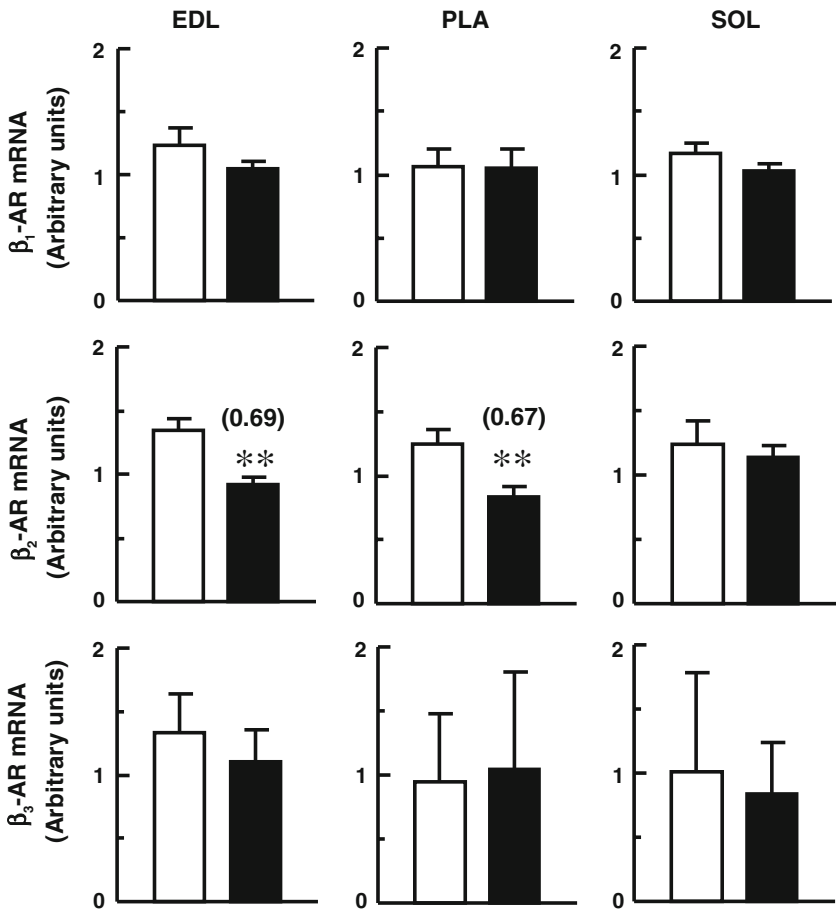

Fig. 3 Effects of clenbuterol on expression of β_1, β_2, and β_3-AR mRNAs in EDL, PLA, and SOL muscles. The values are shown as mean ± SEM ($n = 10$/group). The relative amount of each mRNA was calculated and normalized by the value of 18S rRNA gene. *Open bars*, control group; *closed bars*, clenbuterol-administered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

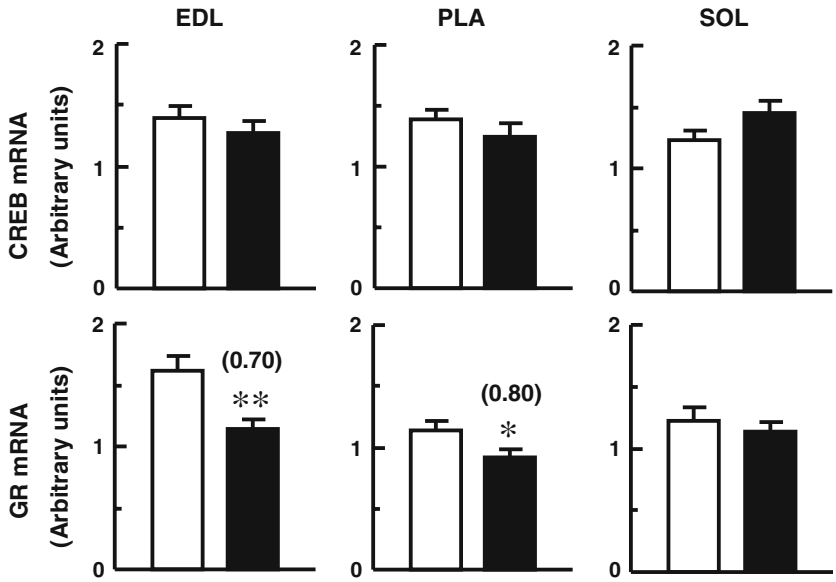

Fig. 4 Effects of clenbuterol on expression of CREB and GR mRNAs in EDL, PLA and SOL muscles. The values are shown as the mean ± SEM ($n = 10$/group). The amount of each mRNA was calculated and normalized by the value for the 18S rRNA gene. *Open bars*, control group; *closed bars*, clenbuterol-administered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

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

Figure 3 shows the effects of clenbuterol on expression 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).

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.

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-

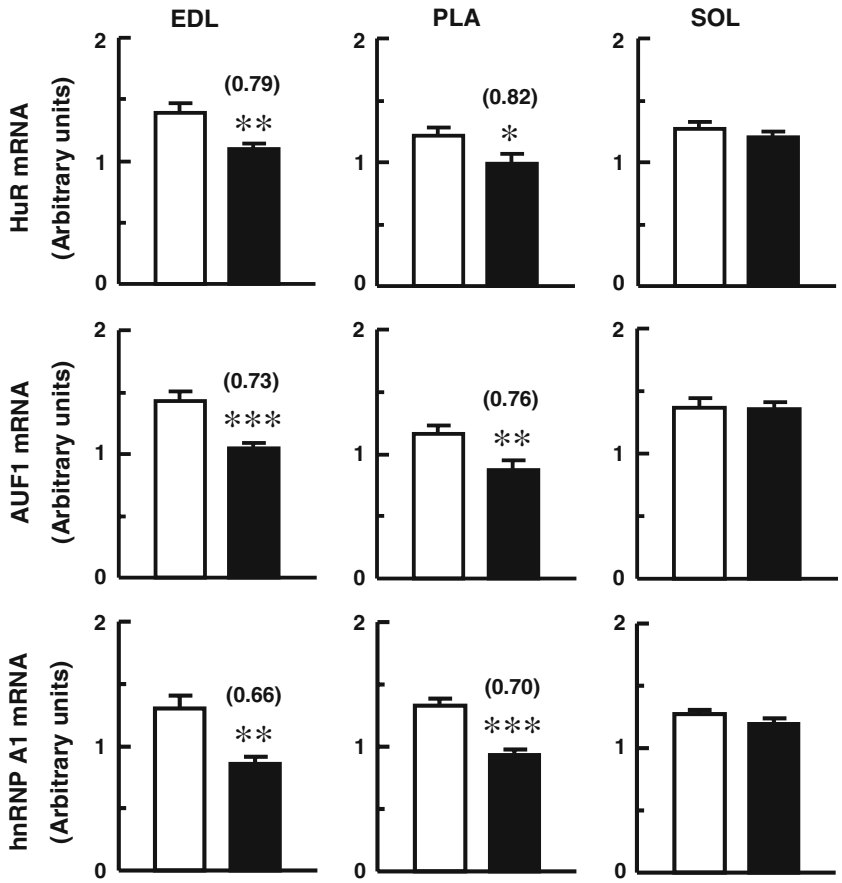

Fig. 5 Effects of clenbuterol on expression of HuR, AUF1, and hnRNP A1 mRNAs in EDL, PLA, and SOL muscles. The values are shown as the mean ± SEM ($n = 10$/group). The amount of each mRNA was calculated and normalized by the value of 18S rRNA gene. *Open bars*, control group; *closed bars*, clenbuterol-administered group. Values in parentheses are values for the clenbuterol-administered group relative to the control group. Statistics: $*p < 0.05$, $**p < 0.01$ and $***p < 0.001$ (vs. the control group)

Table 2 Summary of results

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

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. NS, not significant between both groups. Values in parentheses are values for the clenbuterol-administered group relative to the control group

[a] Our previous data [1]

transcriptional regulatory factor mRNAs are specific to fast-twitch fiber-rich muscles such as EDL and PLA muscles.

Discussion

The purpose of this study was to elucidate the effects of the β_2-agonist, clenbuterol (dose = 1.0 mg/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.

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 between 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 the 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 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 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 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.

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References

1. Sato S, Nomura S, Kawano F, Tanihata J, Tachiyashiki K, Imaizumi K (2008) Effects of the β_2-agonist clenbuterol on β_1- and β_2-adrenoceptor mRNA expressions of rat skeletal and left ventricle muscles. J Pharmacol Sci 107:393–400
2. Stevens L, Firinga C, Gohlsch B, Bastide B, Mounier Y, Pette D (2000) Effects of unweighting and clenbuterol on myosin light and heavy chains in fast and slow muscles of rat. Am J Physiol Cell Physiol 279:C1558–C1563
3. Kitaura T, Tsunekawa N, Hatta H (2001) Decreased monocarboxylate transporter 1 in rat soleus and EDL muscles exposed to clenbuterol. J Appl Physiol 91:85–90
4. Pellegrino MA, D G, Bortolotto S, Boschi F, Pastoris O, Bottinelli R, Polla B, Reggiani C (2004) Clenbuterol antagonizes glucocorticoid-induced atrophy and fiber type transformation in mice. Exp Physiol 89:89–100
5. Yimlamai T, Dodd SL, Borst SE, Park S (2005) Clenbuterol induces muscle-specific attenuation of atrophy through effects on the ubiquitin-proteasome pathway. J Appl Physiol 99:71–80
6. Akutsu S, Shimada A, Yamane A (2006) Transforming growth factor βs are upregulated in the rat masseter muscle hypertrophied by clenbuterol, a β_2 adrenergic agonist. Br J Pharmacol 147:412–421
7. Burniston JG, Clark WA, Tan LB, Goldspink DF (2006) Dose-dependent separation of the hypertrophic and myotoxic effects of the β_2-adrenergic receptor agonist clenbuterol in rat striated muscles. Muscle Nerve 33:655–663
8. Shi H, Zeng A, Ricome A, Hannon KM, Grant AL, Gerrard DE (2006) Extracellular signal-regulated kinase pathway is differentially involved in β-agonist-induced hypertrophy in slow and fast muscles. Am J Physiol Cell Physiol 292:C1681–C1689
9. Choo JJ, Horan MA, Little RA, Rothwell NJ (1992) Anabolic effects of clenbuterol on skeletal muscle are mediated by β_2-adrenoceptor activation. Am J Physiol Endocrinol Metab 263:E50–E56
10. Hesketh JE, Campbell GP, Lobley GE, Maltin CA, Acamovic F, Palmer RM (1992) Stimulation of actin and myosin synthesis in rat gastrocnemius muscle by clenbuterol; evidence for translational control. Comp Biochem Physiol C 102:23–27
11. Hinkle RT, Hodge KM, Cody DB, Sheldon RJ, Kobilka BK, Isfort RJ (2002) Skeletal muscle hypertrophy and anti-atrophy effects of clenbuterol are mediated by the β_2-adrenergic receptor. Muscle Nerve 25:729–734
12. Navegantes LC, Resano NM, Baviera AM, Migliorini RH, Kettelhut IC (2004) Effect of sympathetic denervation on the rate of protein synthesis in rat skeletal muscle. Am J Physiol Endocrinol Metab 286:E642–E647
13. Mak JC, Nishikawa M, Shirasaki H, Miyayasu K, Barnes PJ (1995) Protective effects of a glucocorticoid on downregulation of pulmonary β_2-adrenergic receptors in vivo. J Clin Invest 96:99–106
14. Collins S, Bouvier M, Bolanowski MA, Caron MG, Lefkowitz RJ (1989) cAMP stimulates transcription of the β_2-adrenergic receptor gene in response to short-term agonist exposure. Proc Natl Acad Sci USA 86:4853–4857
15. Collins S, Altschmied J, Herbsman O, Caron MG, Mellon PL, Lefkowitz RJ (1990) A cAMP response element in the β_2-adrenergic receptor gene confers transcriptional autoregulation by cAMP. J Biol Chem 265:19330–19335
16. Adcock IM, Stevens DA, Barnes PJ (1996) Interactions of glucocorticoids and β_2-agonist. Eur Respir J 9:160–168
17. Cornett LE, Hiller FC, Jacobi SE, Cao W, McGraw DW (1998) Identification of a glucocorticoid response element in the rat β_2-adrenergic receptor gene. Mol Pharmacol 54:1016–1023
18. Hadcock JR, Wang HY, Malbon CC (1989) Agonist-induced destabilization of β-adrenergic receptor mRNA. J Biol Chem 264:19928–19933
19. Port JD, Huang LY, Malbon CC (1992) β-Adrenergic agonists that down-regulate receptor mRNA up-regulate a Mr 35,000 protein(s) that selectively binds to β-adrenergic receptor mRNAs. J Biol Chem 267:24103–24108
20. Tholanikunnel BG, Granneman JG, Malbon CC (1995) The Mr 35,000 β-adrenergic receptor mRNA-binding protein binds transcripts of G-protein-linked receptors which undergo agonist-induced destabilization. J Biol Chem 270:12787–12793
21. Pende A, Tremmel KD, DeMaria CT, Blaxall BC, Minobe WA, Sherman JA, Bisognano JD, Bristow MR, Brewer G, Port JD (1996) Regulation of the mRNA-binding protein AUF1 by activation of the β-adrenergic receptor signal transduction pathway. J Biol Chem 271:8493–8501
22. Blaxall BC, Pellett AC, Wu SC, Pende A, Port JD (1992) Purification and characterization of β-adrenergic receptor mRNA-binding protein. J Biol Chem 274:4290–4297
23. Piñol-Roma S, Dreyfuss G (1992) Shuttling of pre-mRNA binding proteins between nucleus and cytoplasm. Nature 355:730–732
24. Fan XC, Steitz JA (1998) Overexpression of HuR, a nuclear-cytoplasmic shuttling protein, increases the in vivo stability of ARE-containing mRNAs. EMBO J 17:3448–3460
25. Peng SS, Chen CY, Xu N, Shyu AB (1998) RNA stabilization by the AU-rich element binding protein, HuR, an ELAV protein. EMBO J 17:3461–3470

26. Loflin PT, Chen CY, Xu N, Shyu AB (1999) Transcriptional pulsing approaches for analysis of mRNA turnover in mammalian cells. Methods 17:11–20
27. Wang W, Furneaux H, Cheng H, Caldwell MC, Hutter D, Liu Y, Holbrook N, Gorospe M (2000) HuR regulates p21 mRNA stabilization by UV light. Mol Cell Biol 20:760–769
28. Wang W, Caldwell MC, Lin S, Furneaux H, Gorospe M (2000) HuR regulates cyclin A and B1 mRNA stability during cell proliferation. EMBO J 19:1–12
29. Brennan CM, Steitz JA (2001) HuR and mRNA stability. Cell Mol Life Sci 58:266–277
30. Bolognani F, Perrone-Bizzozero NI (2008) RNA-protein interactions and control of mRNA stability in neurons. J Neurosci Res 86:481–489
31. Shirato K, Tanihata J, Motohashi N, Tachiyashiki K, Tomoda A, Imaizumi K (2007) β_2-Agonist clenbuterol induced changes in the distribution of white blood cells in rats. J Pharmacol Sci 104:146–152
32. Kawano F, Tanihata J, Sato S, Nomura S, Shiraishi A, Tachiyashiki K, Imaizumi K (2009) Effects of dexamethasone on the expression of β_1-, β_2- and β_3-adrenoceptor mRNAs in skeletal and left ventricle muscles in rats. J Physiol Sci 59:383–390
33. Shirato K, Motohashi N, Tanihata J, Tachiyashiki K, Tomoda A, Imaizumi K (2006) Effects of two types of inactivity on the number of white blood cells in rats. Eur J Appl Physiol 98:590–600
34. Nomura S, Ichinose T, Jinde M, Kawashima Y, Tachiyashiki K, Imaizumi K (2008) Tea catechins enhance the mRNA expression of uncoupling protein 1 in rat brown adipose tissue. J Nutr Biochem 19:840–847
35. The Physiological Society of Japan (2004) Guiding principles for the care and use of animals in the field of physiological sciences. J Physiol Soc Jpn 54:98
36. Lefkowitz RJ, Caron MG (1988) Adrenergic receptor. Models for the study of receptors coupled to guanine nucleotide regulatory proteins. J Biol Chem 263:4993–4996
37. Riabowol KT, Fink JS, Gilman MZ, Walsh DA, Goodman RH, Feramisco JR (1988) The catalytic subunit of cAMP-dependent protein kinase induces expression of genes containing cAMP-responsive enhancer elements. Nature 336:83–86
38. Mellon PL, Clegg CH, Correll LA, McKnight GS (1989) Regulation of transcription by cyclic AMP-dependent protein kinase. Proc Natl Acad Sci USA 86:4887–4891
39. Montminy MR, Savarino KA, Wagner JA, Mandel G, Goodman RH (1986) Identification of a cyclic-AMP-responsive element within the rat somatostatin gene. Proc Natl Acad Sci USA 83:6682–6686
40. Silver BJ, Bokar JA, Virgin JB, Vallen EA, Milsted A, Nilson JH (1987) Cyclic AMP regulation of the human glycoprotein hormone alpha-subunit gene is mediated by an 18-base-pair element. Proc Natl Acad Sci USA 84:2198–2202
41. Caamaño CA, Morano MI, Dalman FC, Pratt WB, Akil H (1998) A conserved praline in the hsp90 binding region of the glucocorticoid receptor is required for hsp90 heterocomplex stabilization and receptor signaling. J Biol Chem 273:20473–20480
42. Ricketson D, Hostick U, Fang L, Yamamoto KR, Darimont BD (2007) A conformational switch in the ligand-binding domain regulates the dependence of the glucocorticoid receptor on Hsp90. J Mol Biol 368:729–741
43. Good PJ (1995) A conserved family of elav-like genes in vertebrates. Proc Natl Acad Sci USA 92:4557–4561
44. Ma WJ, Cheng S, Campbell C, Wright A, Furneaux H (1996) Cloning and characterization of HuR, a ubiquitously expressed Elav-like protein. J Biol Chem 271:8144–8151
45. Dreyfuss G, Matunis MJ, Piñol-Roma S, Burd CG (1993) hnRNP proteins and the biogenesis of mRNA. Annu Rev Biochem 62:289–321