Temporal Pattern of cAMP Concentrations and α-Actin mRNA Expression in Skeletal Muscle of Cimaterol-Fed Rats

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ABSTRACT : Twenty four female Sprague-Dawley rats weighing about 190 g were used to examine changes in muscle cAMP concentrations and steady-state levels of skeletal muscle α-actin mRNA during chronic administration of cimaterol, a β-adrenergic agonist. Cimaterol was mixed in a powdered rat diet at 10 mg/kg diet. At 3 and 21 days after the start of treatment, skeletal muscle and heart samples were collected for the measurement of cAMP concentrations and skeletal muscle α-actin mRNA levels. Cimaterol increased ($p < 0.01$) body weight gain gradually during the first seven days of the trial period, but not thereafter. Most skeletal muscle weights and the ratio of muscle weight to body weight were increased ($p < 0.05$) by cimaterol treatment both at 3 and 21 days. Heart weight was also increased ($p < 0.05$) by cimaterol treatment at 3 and 21 days, but the ratio of heart weight to body weight was increased ($p < 0.05$) only at 3 day.

Cimaterol decreased ($p < 0.05$) cAMP concentration of gastrocnemius muscle at both 3 and 21 days after treatment. However, cimaterol tended ($p = 0.07$) to increase cAMP concentration at 3 days in the heart. Cimaterol tended ($p = 0.08$) to increase the steady-state level of α-actin mRNA by 60% in gastrocnemius muscle at 3 days but had no effect at 21 days. The results indicate that the pattern of hypertrophic response to chronic dietary administration of cimaterol is different between cardiac and skeletal muscle. In skeletal muscles it appears that the hypertrophy induced by cimaterol is partly due to stimulated myofibrillar protein synthesis at a pre-translational level.

(**Key Words**: Beta-Adrenergic Agonist; Cimaterol, cAMP, Actin mRNA, Skeletal Muscle; Heart)

INTRODUCTION

Chronic administration of β-adrenergic agonists (BAA) improves carcass composition and enhances skeletal muscle growth in many animal species (review by Moloney et al., 1991). Recent results suggest that the hypertrophic effect of BAA is mediated by stimulation of the β_2-adrenoceptor because the hypertrophic effect of BAA could be completely prevented by the non-selective β-antagonist, propranolol, or the β_2-selective antagonist, ICI 118,551 (MacLennan and Edwards, 1989; Choo et al., 1992). Furthermore, chronic administration of the β_2-selective antagonist, butoxamine or ICI 118,551, reduced muscle growth in normally growing rats (Zeman et al., 1988; Sillence et al., 1991), suggesting a physiological role of the β_2-adrenoceptor in the normal growth of skeletal muscles.

Although the mechanism responsible for BAA-induced muscle hypertrophy has been studied in terms of changes in protein metabolism (review by Yang and McElligott, 1989; Kim and Sainz, 1992), currently we have no clear understanding of the mechanism(s) leading from the activation of β-adrenoceptors to changes in protein metabolism. Because the action of β-adrenoceptors is mediated by the second messenger cyclic AMP (cAMP) in eliciting numerous cellular functions, it is tempting to speculate that the skeletal muscle hypertrophic effect of BAA is also mediated by cAMP as a second messenger. While some studies measure changes in cAMP concentration in skeletal muscles after short-term or long term administration of BAAs (Merican et al., 1983; Chasiotis, 1985; Alderson et al., 1987; MacLennan and Edwards, 1989; Moore et al., 1994; Kim et al., 1995), no confirmation is available about the temporal pattern of cAMP changes in skeletal muscles during chronic administration of BAA. The objective of this study was to measure the cAMP changes in skeletal muscles of rats during chronic administration of the BAA, cimaterol. In

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addition, steady-state levels of skeletal muscle α-actin mRNA were monitored during cimaterol treatment.

MATERIALS AND METHODS

Animals

Twenty four female Sprague-Dawley rats weighing approximately 190 g were purchased and maintained at the Laboratory Animal Services, University of Hawaii. Animals were randomly assigned to two blocks, 3 days and 21 days. Each block has two treatment groups, control (CON) and cimaterol-fed (CIM). Six rats were used per treatment group. Cimaterol was used as a model BAA compound and mixed in a powdered rat chow at 10 mg/kg diet. Control animals were given powdered rat chow without cimaterol. Rats were caged individually with free access to feed and water, and body weights were monitored three times a week to check the growth performance. At 3 and 21 days after the start of treatment, rats were sacrificed by CO_2 inhalation, then gastrocnemius, plantaris, soleus and heart were disseceted immediately, weighed and frozen in liquid nitrogen for subsequent analysis. The two sampling points were chosen because previous studies had shown that rats are responsive to BAA at around 3 days after administration, but the growth-promoting and skeletal muscle hypertrophic effects of BAA completely disappear at around 14 days (Kim, 1988). This project was approved by the University of Hawaii Animal Care and Use Committee.

cAMP assay

Cyclic AMP (cAMP) concentration was measured from gastrocnemius and heart muscle samples. To extract cAMP, approximately 0.5 g of muscle tissue was homogenized with 10 volumes of acidic ethanol (1 ml of 1N HCl/100 ml ethanol). The homogenate was centrifuged at 6,000 g for 20 minutes. Supernatant fluid was collected and divided into 2 tubes of 1 ml each. The extracts were dried under a stream of air in a hot water bath at 70-80℃. The dried extracts were reconstituted in 50 mM Tris buffer containing 5 mM EDTA at pH 7.5. The reconstituted sample was assayed for cAMP concentration using a commercially available cAMP kit (Amersham International, Arlington Heights, IL).

Preparation of ^{32}P-labeled oligonuceotide probes

Skeletal muscle α-actin oilgonuceotide probe of 20 bases was synthesized from the Biotechnology Instrumentation Facility, University of Hawaii. The oligonuceotide sequence (5' to 3') was GCAACCATAGCACGATGGTC (Gustafson et al., 1986), and the synthetic nucleotides were constructed complimentary to 3' nontranslated sequence of rat cDNA clones reported by Shani et al. (1981). The probe was labeled at the 3' end using terminal transferase (Sigma Chemical Co., St. Louis, MO) to a specific radioactivity of 6.7×10^9 dpm/μg of oligonucleotide with [α-^{32}P]dCTP (6000 Ci/mmol; New England Nuclear, Boston, MA).

RNA isolation and dot-blot analysis

Total RNA was isolated from the plantaris muscles according to the guanidinium isothiocyanate/phenol method (Chomczynski and Sacchi, 1987). Reagents were from the REX™ Total RNA Extraction Kit (United States Biochemical Corporation, Cleveland, Ohio), and maufacturer-recommended procedures were followed. RNA quantity and quality were determined by absorbance analysis at 260/280 nm, and aliquots of total RNA samples at appropriate concentrations were stored at −70℃.

For dot blot analysis, 15 μg of total RNA was denatured by incubating at 65℃ for 5 minutes in 100 μl of denaturing solution (66 μl of formamide, 21 μl of 37% formaldehyde, and 13 μl of 10 × MOPS buffer), then after being chilled on ice, an equal volume of cold 20 × SSC solution was added to the denatured RNA solution. The RNA solution was applied to a nylon membrane (Hybond-N$^+$, Amersham International, Arlington Heights, IL) in a dot-blot apparatus (Bio-Rad, Hercules, CA). RNA was immobilized by placing the membrane RNA side up on a fiter paper soaked in 0.05 N NaOH solution for 5 minutes, after which the membrane was rinsed by immersion in 5 × SSC solution for 1 minute with gentle agitation. The membrane was wrapped in Saran Wrap® and stored at 4℃.

For quantification of skeletal muscle α-actin mRNA, the membrane was prehybridized at 59℃ for 1 hr in a hybridization solution (5 × SSPE, 5 × Denhardt's solution, and 0.5% SDS) containing 50 μg/ml denatured herring sperm DNA (Sigma Chemical Co., St. Louis, MO). Following prehybridization, hybridization was performed for 24 hr by adding ^{32}P-labeled actin oligonucleotide probe directly to the prehybridization solution at a concentration of approximately 2.5 ng/ml in a volume of abut 4 ml per bag. After hybridization, membrane was washed twice by incubating the membrane for 10 min in 2 × SSPE, 0.1% SDS solution at room temperature. A final 15 minutes washing was carried out at 59℃ in 1 × SSPE, 0.1% SDS solution, then the excess fluid was dripped from the membrane. Membrane was wrapped in Saran Wrap, and autoradiography was carried out with an intensifying screen at −70℃ for 4 days. After develop-

ment of the film, the levels of mRNA were estimated by scanning densitometry using the Image QuaNT program (Molecular Dynamics, Sunnyvale, CA).

Data analysis

Data were analysed by the one way ANOVA procedure at the separate 3- and 21- d sampling time points using the MINTAB (1989) program.

RESULTS AND DISCUSSION

A significant increase in body weight gain was observed in the cimaterol-fed rats during the first seven days of the feeding period as compared to the control animals, but no difference was observed between the two groups thereafter (figure 1 and table 1). This attenuation of the growth promoting effect of cimaterol during the chronic administration period is in accord with pervious studies (review by Kim and Sainz, 1992). A previous study by Kim et al. (1992) indicated that the attenuation is due to downregulation of β-adrenoceptors, because the reduction in receptor density preceded the attenuation of weight gain when they measured receptor densities at various time points after initiating BAA treatment. Table 2 summarizes the effect of cimaterol on skeletal muscle and heart weights. The weights of gastrocnemius and plantaris muscles were increased ($p < 0.01$) by cimaterol at both 3 and 21 days. The increase was greater at 21 days than at 3 days in both muscles (8 vs 25% in gastrocnemius and 10 vs 37% in plantaris). The weight of the soleus was significantly increased by cimaterol only at 21 days. In most cases, the ratio of muscle weight to body weight was also increased ($p < 0.05$) by cimaterol treatment (table 2). The increase in the ratio was more pronounced at 21 days than at 3 days in all skeletal muscles. Heart weight at 3 and 21 days was increased by cimaterol treatment. The increase was greater at 3 days than 21 days (14 vs 8%). However, the ratio of heart weight to body weight was increased only at 3 days.

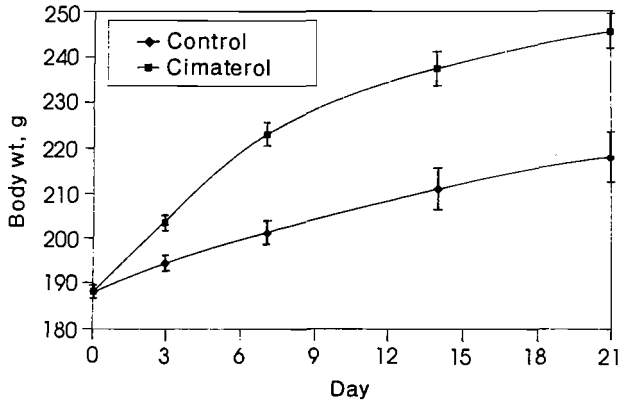

Figure 1. Effect of cimaterol administration on growth of juvenile female rats. Error bars are standard error of the mean.

Table 1. Effect of cimaterol on body weight gain[1]

Period	n	Control	Cimaterol
0 - 3 day	12	6.7 ± 1.67	15.0** ± 1.08
3 - 7 day	12	6.5 ± 1.66	19.6** ± 1.68
7 - 14 day	6	11.5 ± 1.75	13.5 ± 2.06
14 - 21 day	6	7.0 ± 2.06	8.2 ± 1.23

[1] 10 ppm of cimaterol was mixed in rat chow. Mean values ± SEM, values are different at. **$p < 0.01$.

Table 2. Effects of cimaterol on skeletal muscle and heart weights, and skeletal muscle and heart weights expressed as a percentage to body weight[1]

	3 Day		21 Day	
	CON	CIM	CON	CIM
Weight, g				
Gastrocnemius	2.21 ± 0.031	2.39** ± 0.038	2.55 ± 0.047	3.16** ± 0.062
Plantaris	0.443 ± 0.0094	0.487**± 0.0079	0.489 ± 0.0163	0.668**± 0.0123
Soleus	0.156 ± 0.0049	0.167 ± 0.0039	0.169 ± 0.0088	0.209**± 0.0036
Heart	0.681 ± 0.0014	0.777**± 0.0135	0.747 ± 0.0129	0.808* ± 0.0173
Weight as a percentage to body weight				
Gastrocnemius	1.14 ± 0.019	1.18^{+} ± 0.013	1.16 ± 0.0141	1.286** ± 0.0084
Plantaris	0.228 ± 0.0049	0.240* ± 0.0029	0.224 ± 0.0035	0.272** ± 0.0038
Soleus	0.080 ± 0.0025	0.082 ± 0.0020	0.077 ± 0.0025	0.085* ± 0.0015
Heart	0.350 ± 0.0061	0.383**± 0.0038	0.344 ± 0.0150	0.329 ± 0.0079

[1] 10 ppm of cimaterol was mixed in rat chow, Mean values ± SEM (n = 6).
Values within day are different at $^{+}p < 0.1$; *$p < 0.05$; **$pp < 0.01$.

While the hypertrophic effects of BAA on skeletal muscle are consistent among studies, previous reports show that the effects on cardiac muscle are not consistent: some studies report an increase of heart mass by BAA treatment (Reeds et al., 1986; Sillence et al., 1991), but others report that heart mass is not affected by BAA treatment (Emery et al., 1984; Kim et al., 1987; MacLennan and Edwards, 1989). Some factors such as species difference, use of different BAA compounds, and dose may be associated with the inconsistent results. However, considering our current result which demonstrated a significant increase in the percentage of heart weight to body weight at 3 days but not 21 days of cimaterol treatment, the differences among observations may be related to the temporal nature of the response to BAA. It appears, therefore, that the pattern of the hypertrophic response to BAA is different between skeletal and cardiac muscles.

Cyclic AMP acts as a second messenger in mediating many cellular functions regulated by BAA, and short-term or single administration of BAA increased cAMP concentration in target organs including skeletal muscles (Merican et al., 1983; Chasiotis, 1985; MacLennan and Edwards, 1989; Moore et al., 1994). However, long-term chronic administration of BAA decreased tissue cAMP concentrations, probably due to downregulation of β-adrenoceptors (Bramuglia et al., 1993; Kim et al., 1995). Since the changes in muscle cAMP concentrations have not been studied during chronic administration of BAA in the same experiment, we designed this experiment to measure the cAMP concentration at 3 days, when an active hypertrophic response is occurring, and after 21 days, when the hypertrophic response has ceased. The hypothesis was that if cAMP acts as a second messenger for the BAA-induced hypertrophy of skeletal or cardiac muscles, we would see elevated tissue cAMP concentrations during a period of tissue hypertrophy, but not during a period when no hypertrophy is occurring. Table 3 summarizes the cAMP concentrations in gastrocnemius and heart muscles. Cimaterol decreased cAMP concentration in gastrocnemius muscle at 3 and 21 days after treatment.

The decrease was greater at 21 days than at 3 days (38% vs. 18%). Since cAMP is a generic second messenger for a number of hormones in tissue, the cAMP level in skeletal muscle may not be the net result of β-adrenoceptor activation. Nevertheless, if cAMP acts as a second messenger for the BAA-induced hypertrophy of skeletal, elevated tissue cAMP concentrations will be observed during a period of tissue hypertrophy. However, decreased concentration of cAMP was observed at 3 days of treatment in the gastrconemius muscle. Thus, the result suggests that either cAMP may not act as a second messenger in the mechanism for muscle hypertrophy induced by BAA, or sustained elevation of cAMP up to 3 days may not be necessary to trigger biochemical changes to induce a hypertrophic response in skeletal muscles upon chronic administration of BAA.

Table 3. Effects of cimaterol on cAMP concentrations in gastrocnemius muscle and heart (pmol/g muscle)

	3 Day		21 Day	
	CON	CIM	CON	CIM
Gastrocnemius	350± 7.3	287*± 7.3	301± 7.4	187*± 8.1
Heart	239± 35.1	319⁺± 21.1	–	–

Mean values ± SEM (n = 6), values within day are different at $^{+}p < 0.1$; $^{*}p < 0.05$.

In contrast to the skeletal muscle, cAMP concentration in heart muscle tended ($p = 0.07$) to increase at 3 days after the start of treatment. Currently, we cannot provide any explanation for the difference in cAMP response between skeletal and heart muscles, but this may relate to a different mechanism by which BAA induces hypertrophy in skeletal and cardiac muscles, respectively. When Maltin et al. (1992) measured changes in protein metabolism in skeletal and cardiac muscles of rats during chronic administration of clenbuterol, improved translational efficiency (the amount of protein synthesized per unit of RNA) was observed in skeletal muscles during the early hypertrophy (2-3 days), but in cardiac muscles no change in translational efficiency was observed. They, therefore, suggested the possibility of different underlying mechanisms for BAA-induced hypertrophy of skeletal and cardiac muscles. This different mechanism of action may explain the observed differences in the pattern of hypertrophy between cardiac and skeletal muscles during chronic administration of BAA in this study.

Even though not all studies agree (Hesketh et al., 1992), it has been indicated that BAA-induced hypertrophy of skeletal muscle is partly due to pre-translational enhancement of myofibrillar protein synthetic capacity because increased steady-state levels of myofibrillar protein mRNA were observed after BAA administration (Smith et al., 1989; Helferich et al., 1990; Koohmaraie et al., 1991; Grant et al., 1993). In this study, cimaterol tended ($p = 0.08$) to increase the steady-state level of α-actin mRNA by 60% at 3 days but not at 21 days (figure 2), thus confirming and extending the previous results

showing that the increased level of mRNA is specific for the highly-responsive period (day 3) but not for the non-responsive period (day 21). Similarly, Grant et al. (1993) reported in pigs that ractopamine increased the relative abundance of skeletal muscle α-actin mRNA during the early period (2 and 4 wk) of treatment but not in the later period (6 wk) of treatment. This temporal nature of steady-state level of skeletal muscle mRNA is consistent with the temporal pattern of protein synthetic rate, RNA concentration, and skeletal muscle hypertrophic response observed during chronic administration of BAA (review by Kim and Sainz, 1992). Cumulative evidence supports the hypothesis that the skeletal muscle hypertrophy induced by BAA is partly due to stimulated myofibrillar protein synthesis at a pre-translational level, especially during the early administration period.

In summary, results of this study indicate that the pattern of hypertrophic response to chronic administration of cimaterol is different between cardiac and skeletal muscles. Even though the implication of the difference in cAMP repsonses to BAA administration between skeletal and cardiac muscles is not clear, this difference may relate to different mechanisms of action for BAA-induced hypertrophy in skeletal and cardiac muscles.

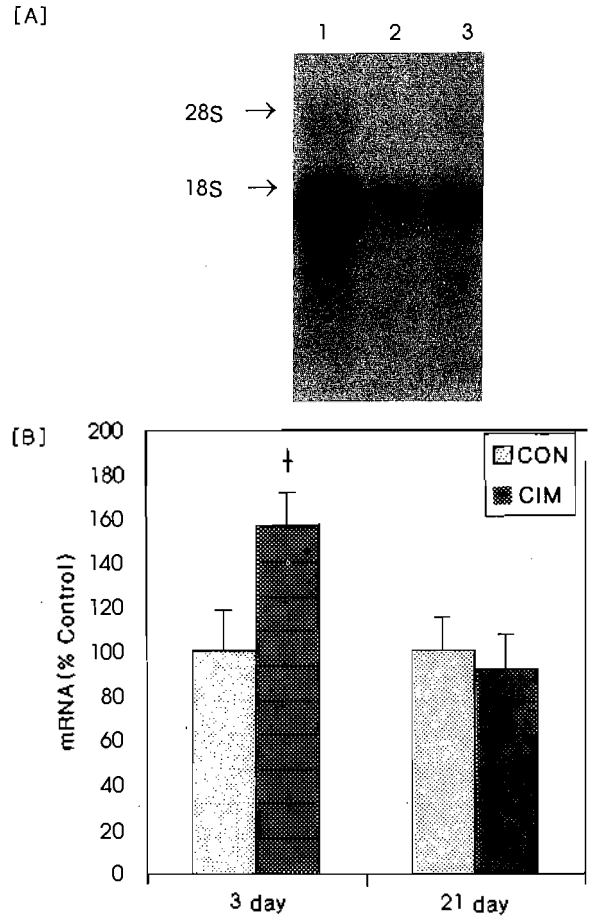

Figure 2. Effect of cimaterol administration on steady-state levels of skeletal muscle α-actin mRNA. [A] Oligonucleotide probe specific for rat skeletal muscle α-actin mRNA was tested by Northern blot analysis of total rat skeletal muscle (lane 1), liver (lane 2), and heart (lane 3) RNA. Blots were prepared by separating the total RNA in a formaldehyde, agarose gel. The hybridization and washing conditions were the same as for the dot blot procedure described in the Materials and Methods section. [B] This panel represents the result of quantitation of mRNAs by scanning densitometry of dot blot analysis, expressed as percentages of control. Error bars are standard error of the mean. +, $p < 0.1$.

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