Actions of a beta-adrenergic agonist on muscle protein metabolism in intact, adrenalectomized, and dexamethasone-supplemented adrenalectomized rats

Cheng-Chung Liu, Jan-ying Yeh, Bor-rung Ou, and Neil E. Forsberg

Department of Animal Sciences, Oregon State University, Corvallis, OR USA

The objectives were to investigate the possibility that glucocorticoids potentiate actions of beta-adrenergic agonists in skeletal muscle and to elucidate mechanisms by which glucocorticoids and beta-adrenergic agonists effect control of muscle growth. Forty-eight male rats were assigned to one of six treatments, which consisted of a sham-adrenalectomized control, sham-adrenalectomized supplemented with cimaterol, adrenalectomized, adrenalectomized supplemented with cimaterol, adrenalectomized supplemented with dexamethasone, and adrenalectomized supplemented with cimaterol and dexamethasone. After 8 days, muscle samples were collected for assay of nucleic acid contents and proteinase (cathepsins and calpains) and calpastatin activities. Both glucocorticoid status and cimaterol influenced muscle growth and metabolism. Adrenalectomy reduced muscle RNA content and dexamethasone restored RNA. Cimaterol increased RNA levels, increased total DNA content, reduced calpain activities, and increased calpastatin activity. This implies that cimaterol has potential to regulate both protein synthesis and degradation. We did not find evidence for glucocorticoids potentiating actions of cimaterol. Instead, we determined that cimaterol antagonized certain growth-inhibiting properties of the glucocorticoids. Effects of cimaterol on muscle growth and on metabolic parameters were highly dependent on glucocorticoid status suggesting that the variations in muscle responses to beta-adrenergic agonists, which have been detected in other studies, may be due to variations in glucocorticoid status of experimental animals.
(J. Nutr. Biochem. 5:43–49, 1994.)

Keywords: muscle; cimaterol; beta-adrenergic agonist; glucocorticoid; proteinase; calpain; cathepsin

Introduction

The mechanisms by which beta-adrenergic agonists cause muscle hypertrophy are not fully understood. Because beta-adrenergic agonists often do not affect protein synthesis or degradation in cultured muscle cells, some investigators have proposed that their actions are mediated indirectly by other hormones. Although actions of beta-agonists are independent of thyroid hormone,[1] insulin,[2] testosterone,[3] and the growth hormone axis,[4] Sharpe et al.[5] reported that the growth-promoting actions of clenbuterol in the rat were dependent on the presence of an intact adrenal-cortical hypophysis and proposed that glucocorticoids played permissive roles in the growth-promoting actions of beta-adrenergic agonists. Others,[3] however, have proposed that actions of beta-adrenergic agonists are glucocorticoid independent. Objectives of the proposed research were to evaluate the glucocorticoid dependence for anabolic actions of cimaterol and to evaluate mechanisms by which beta-adrenergic agonists and glucocorticoids modulate muscle growth. Accordingly, effects of glucocorticoid and cimaterol status on muscle growth and on proteinase and proteinase inhibitor activities were investigated.

Methods and materials

Forty-eight male Sprague-Dawley rats, weighing approximately 250 g, were obtained from Charles River Breeding

Published as technical report number 9668 of the Oregon Agricultural Experiment Station.
Supported in part by United States Department of Agriculture grant 88-37265-3896.
Address reprint requests to Dr. Neil E. Forsberg at the Department of Animal Sciences, Withycombe Hall, Oregon State University, Corvallis, OR 97331-6702 USA.
Received March 30, 1993; accepted July 30, 1993.

Laboratories (Wilmington, MA USA). Of these, 32 had received adrenalectomies (Adx) and 16 had received sham-adrenalectomies (sham-Adx). Upon arrival, rats were placed in individual stainless steel metabolic cages in a temperature-controlled room with a 13 hr:11 hr light:dark cycle and were allowed ad libitum access to a powdered control diet (*Table 1*). Water containing sodium chloride (0.85% wt/vol) was made available to all rats throughout the study. Two days following their arrival, rats were assigned to one of six treatments. Eight sham-Adx rats were assigned to the control diet and a diet of the same composition supplemented with 25 ppm cimaterol (American Cyanamid Co., Princeton, NJ USA; cimaterol diet). Eight Adx rats were assigned to the following treatments: control diet alone,[6] cimaterol diet alone, control diet plus daily injections of Dex and cimaterol diet plus daily injections of Dex. Diets were made available ad libitum. Dexamethasone was administered at a level of 1.2 μg/100 g BW/day and was based on replacement dose used in the study of Sharpe et al.[5] Dexamethasone was dissolved in corn oil and injected twice daily subcutaneously at 12 hr intervals. Animals not receiving Dex received twice daily injections of vehicle. The study was conducted in two complete blocks with four animals per treatment used within each block. Animals were maintained on their treatments for 8 days during which time feed intake was recorded. On day 8 of the study, rats were weighed then euthanized by intraperitoneal injection of T-61 Euthanasia solution (Hoechst-Roussel Agri-Vet Co., Somerville NJ, USA). Musculature associated with the left upper hind-limb (biceps femoris, semitendinosus, vastus lateralis, and caudofemoralis) and lower hind-limb (gastrocnemius, plantaris, soleus, posterior tibialis, and extensor digitorum longus) was removed and weighed. Right hind-limb muscles were also taken. Tissues were frozen between blocks of dry ice then wrapped in aluminum foil and plastic and stored at −90° C until further analysis. Weights of liver, heart, and kidney were also determined.

Assessment of tissue DNA, RNA, and protein concentrations

DNA contents of muscle samples were determined as outlined by Labarca and Paigen.[7] Total RNA was determined as outlined by Munro and Fleck.[8] Protein concentration in tissue samples was determined as outlined by Bradford.[9]

Table 1 Composition of rat diets

Ingredient	Percent
Sucrose	47.4
Casein	20.0
Cornstarch	15.0
Corn oil	5.0
AIN Mineral mix	3.5
AIN Vitamin mix	1.0
Fiber	2.4
Corn cob meal	5.2
DL-methionine	0.3
Choline bitartrate	0.2

Diet is based on the AIN-76 diet.[6]
Cimaterol was provided by American Cyanamid Co. (Princeton, NJ USA) in a corn-cob meal carrier. Corn-cob meal lacking cimaterol was added to the control diet. The cimaterol-containing diet contained sufficient corn-cob meal to allow a final cimaterol concentration of 25 ppm.

Proteinase and proteinase inhibitor assays

Cathepsin B (EC 3.4.21.1) activity was measured according to the method of Barrett and Kirschke.[10] Samples of muscle were homogenized in 6 vol of ice-cold buffer (155 mM KH_2PO_4; 4.5 mM citric acid; 4 mM EDTA; pH 6.0) using a Polytron (30 sec, 0.7 × maximum speed, Brinkman Instruments Co., Westbury, NY USA). The sample was kept on ice at all times. Tissue homogenate (40 μL) was mixed with 170 μL distilled deionized (DDI) water. To this 500 μL buffer and 250 μL dithiothreitol (2 mM) were added and equilibrated for 10 min at 37° C. Forty μL of CBZ-alanyl-arginyl-arginyl-methoxy-β-naphthylamine (Enzyme Systems Products Ltd., Livermore, CA USA; 5 mM in dimethylsulfoxide) were added. Muscle samples were incubated at 37° C for 100 min. Reactions were stopped by addition of 2 mL HCl (1N) and centrifuged at 1000*g* for 12 min. The fluorescence of the released β-naphthylamine (excitation 292 nm, emission 410 nm) in the supernatant was determined with a Perkin-Elmer Model 650-10S fluorimeter (Perkin-Elmer, Norwalk, CT USA).

Cathepsin D (EC 3.4.23.5) activity was determined according to the method of Takakashi and Tang[11] using bovine hemoglobin as a substrate. A portion of the homogenate (100 μL) from the preceding assay was mixed with 1.9 mL of sodium formate buffer (0.25 M; pH 3.2) and 0.5 mL of hemoglobin substrate (5% hemoglobin in DDI water; wt/vol). Reaction mixtures were incubated for 20 min at 37° C, after which 2 mL of 10% (wt/vol) trichloric acid (TCA) were added to stop the reaction. Supernatant was filtered through Whatman No. 50 paper and $absorbance_{280}$ was measured using a spectrophotometer.

Cathepsin L (EC 3.4.22.15) activity was determined according to the method of Barrett and Kirschke[10] using azocasein (Sigma Chemical Co., St. Louis, MO USA) as a substrate. Tissue homogenates (0.25 mL) were obtained as outlined above and mixed with 0.25 mL of Buffer α (0.1 M sodium acetate buffer; pH 5.0; 1 mM EDTA; 40 mM cysteine; 0.1% (wt/vol) pepstatin). After 5 min at room temperature, 0.5 mL of azocasein:urea substrate solution (2% azocasein wt/vol); 6 M urea dissolved in Buffer α lacking cysteine) was added and the reaction incubated at 40° C for 30 min. Five mL of TCA (3% wt/vol) were added to stop the reaction and the reaction mixture was filtered through Whatman No. 1 filter paper. The $absorbance_{366}$ of the filtrate was determined using a spectrophotometer.

Each of the preceding enzyme assays was repeated in duplicate or triplicate using lower hind-limb muscle for each sample and appropriate blank and/or zero time control samples were processed. Preliminary studies were conducted to examine linearity of assays with time. Enzyme activities were expressed as a proportion of muscle protein.[9]

Calpain (EC 3.4.22.17) and calpastatin assays

Muscle μ- and m-calpain activities in upper hind-limb muscle were determined following their chromatographic separation using phenyl-Sepharose column chromatography.[12] Muscle samples (3 g) were homogenized using a Polytron (Brinkman Instrument Co., Westbury, NY USA; 0.4 × maximum speed, 30 to 40 sec) in 5 vol of ice-cold buffer (50 mM Tris-HCl (pH 7.5), 1 mM EDTA, 10 mM β-mercaptoethanol (βME) and 150 nM pepstatin A) then centrifuged at 10,000*g* for 30 min at 4° C. Twenty μL of 1 mM leupeptin, 0.6 mL of 5 M NaCl, 1 mL of phenyl-Sepharose CL-4B (prewashed with Buffer α; 20 mM Tris-HCl (pH 7.5), 0.1 mM $CaCl_2$, 10 mM βME, 20 μM leupeptin) plus 0.25 M NaCl were added to the supernatant. This mixture was agitated for 5

min, after which 0.4 mL of 0.1 M $CaCl_2$ was added followed by an additional 10 min of agitation. This suspension was poured onto a 0.8 × 4 cm plastic column and washed successively with 2 mL each of Buffer α containing 0.25 M NaCl, Buffer α alone then Buffer α without leupeptin. m-Calpain was eluted with 4 mL of Buffer B (20 mM Tris-HCl (pH 7.5), 1 mM EGTA, 10 mM βME) supplemented with 0.1 M NaCl. The column was washed with 2 mL of Buffer B. μ-Calpain was eluted with 4 mL of Buffer B. All of the above procedures were carried out at 4° C. Buffer volumes required for adequate separation of calpains from calpastatin and of μ-calpain from m-calpain were determined in preliminary studies.

Calpain activities were measured using Hammarsten casein (U.S. Biochemical Corp., Cleveland, OH USA) as a substrate. The reaction mixture contained 2 mM $CaCl_2$ (final concentration), 3 mL of column eluant, and 1 mL of casein solution (8 mg/mL casein in 20 mM Tris-HCl, pH 7.5; 10 mM βME). Control samples contained 2 mM EDTA, which replaced $CaCl_2$. After incubating at 25° C for 30 min, 1 mL of 36% TCA (wt/vol) was added, TCA-soluble products were measured as outlined by Bradford.[9] One unit of calpain activity was defined as the amount of enzyme that caused a calcium-dependent change of 0.1 unit of absorbance of 595 nm in 30 min at 25° C. Linearity of the progress of the assay was established in preliminary studies.

Calpastatin assays were determined as outlined by Nakamura et al.[13] Skeletal muscle (upper hind-limb muscles; 0.25 g) was homogenized with a Polytron (0.4 × maximum speed; 30 to 40 sec) in 5 vol of ice-cold Tris HCl (20 mM; pH 7.5) containing 5 mM EDTA. Homogenates were centrifuged at 10,000*g* for 20 min at 4° C. Supernatants were heated at 100° C for 10 min to inactivate endogenous calpains and other proteinases. After heat treatment, the supernatant was centrifuged at 10,000*g* for 10 min at 4° C. Aliquots of the supernatant were added to partially purified stock rat muscle m-calpain (three units) containing 2 mM $CaCl_2$ and incubated at 25° C for 5 min. Following this, hydrolysis of casein was assessed as outlined for calpain assays. One unit of calpastatin was defined as the amount that inhibited one unit of rat muscle m-calpain.

Statistical analysis

Data were analyzed by analysis of variance using a completely randomized block design.[14] Where differences among treatments were detected, a Student-Newman-Keul multiple range test was used to evaluate differences between individual treatment means. A level of significance of 5% was adopted for all comparisons.

Results

Effects of glucocorticoid status and of cimaterol on food intake, body weight gain, and the food to gain ratio are shown in *Table 2*. At both the beginning and completion of the study, Adx rats were smaller than rats that received sham-adrenalectomies. This was unavoidable due to requirements for surgical recovery, shipping, and quarantine. Food intake was reduced 11% by adrenalectomy and was restored to normal levels by Dex administration. Cimaterol reduced food intake by 18% in Adx rats and by 15% in Adx rats that received Dex, but did not affect intake in sham-Adx rats. Rate of gain was reduced by 21% by adrenalectomy. Dexamethasone further reduced body weight gain by 19% in Adx rats. Cimaterol increased weight gain by 46% in Adx rats that received Dex therapy but did not affect gain in sham-Adx or Adx rats. It should be noted, however, that cimaterol tended to increase the rate of gain in both of these groups. Adrenalectomy did not affect the food to gain ratio. However, Dex treatment markedly increased the food to gain ratio (i.e., reduced efficiency of food conversion to body mass) compared with both sham-Adx and Adx groups. Cimaterol reduced the food to gain ratio (i.e., improved efficiency) by 24% in Adx rats and by 44% in Adx rats that received Dex but did not affect this ratio in sham-Adx rats.

Effects of glucocorticoid status and of cimaterol on muscle weights and on muscle constituents are shown in *Table 3*. Although Adx rats had smaller body weights than sham-Adx rats, their muscle weights did not differ. Dexamethasone reduced muscle weight associated with the left upper hind-limb by 10% but did not affect muscle weight associated with the left lower hind-limb. Cimaterol increased muscle weights in upper and lower hind-limbs of sham-Adx rats by 15% to 16%, was without effect on muscle weights in Adx rats, and increased muscle weights in Adx rats that received Dex therapy by 15% to 17%. Cimaterol did not affect weights of liver, heart, or kidney in any treatment group (data not shown).

Effects of the treatments on DNA concentration, although significant ($P < 0.05$), were small. DNA concentration was increased by 4% by adrenalectomy and restored by Dex. Cimaterol reduced DNA concentration in sham-Adx rats by 4% and in Adx rats by 8%, but

Table 2 Effect of glucocorticoid status and cimaterol on rat growth

	Experimental treatment					
	Sham	Sham + cim	Adx	Adx + cim	AdxD	AdxD ± Cim
Initial weight (g)	294.8 ± 12.2^{a}	296.9 ± 8.7^{a}	265.8 ± 21.9^{b}	268.5 ± 10.2^{b}	268.4 ± 10.6^{b}	264.1 ± 12.4^{b}
Final weight (g)	352.8 ± 20.6^{a}	362.5 ± 14.2^{a}	312.9 ± 23.7^{b}	315.0 ± 28.1^{b}	307.4 ± 11.2^{b}	318.6 ± 14.8^{b}
Average daily intake (g/day)	24.4 ± 2.0^{a}	22.6 ± 1.0^{ab}	21.6 ± 1.2^{b}	17.6 ± 3.3^{c}	24.9 ± 1.9^{a}	21.2 ± 1.3^{b}
Average daily gain (g/day)	8.5 ± 0.5^{ab}	9.4 ± 1.3^{a}	6.7 ± 0.9^{c}	7.1 ± 1.8^{bc}	5.4 ± 1.1^{d}	7.9 ± 1.2^{bc}
Food/gain ratio	2.9 ± 0.1^{bc}	2.4 ± 0.3^{c}	3.3 ± 0.3^{b}	2.5 ± 0.2^{c}	4.8 ± 1.0^{a}	2.7 ± 0.4^{bc}

Values are means ± SE. Values in the same row that do not share a common superscript differ significantly ($P < 0.05$).
Cim, Cimaterol; Adx, Adrenalectomized; AdxD, Adrenalectomized and receiving Dex therapy and control diet; AdxD + Cim, Adrenalectomized and receiving Dex therapy and the cimaterol diet.

Table 3 Effect of glucocorticoid status and cimaterol on muscle weights and DNA and RNA contents in rats

	Experimental treatment					
	Sham	Sham + cim	Adx	Adx + cim	AdxD	AdxD + Cim
Muscle sample:						
–left upper (g)	2.01 ± 0.16^{b}	2.33 ± 0.14^{a}	1.94 ± 0.12^{b}	2.00 ± 0.19^{b}	1.74 ± 0.08^{c}	2.04 ± 0.09^{b}
–left lower (g)	2.73 ± 0.18^{b}	3.13 ± 0.17^{a}	2.46 ± 0.25^{bc}	2.67 ± 0.29^{b}	2.36 ± 0.21^{c}	2.72 ± 0.25^{b}
DNA (mg/g tissue)	1.38 ± 0.06^{b}	1.32 ± 0.04^{c}	1.44 ± 0.04^{a}	1.33 ± 0.02^{c}	1.33 ± 0.04^{c}	1.33 ± 0.03^{c}
DNA (total mg)	3.77 ± 0.30^{b}	4.14 ± 0.30^{a}	3.54 ± 0.29^{b}	3.54 ± 0.40^{b}	3.14 ± 0.31^{c}	3.60 ± 0.30^{b}
RNA (mg/g tissue)	1.06 ± 0.06^{d}	1.36 ± 0.09^{b}	0.72 ± 0.06^{e}	1.16 ± 0.08^{c}	1.09 ± 0.07^{d}	1.43 ± 0.12^{a}
RNA (total mg)	2.89 ± 0.36^{bc}	4.27 ± 0.34^{a}	1.79 ± 0.27^{d}	3.11 ± 0.47^{b}	2.57 ± 0.25^{c}	3.90 ± 0.51^{a}

Values are means ± SE of musculature associated with upper and lower hind limb. Values in the same row that do not share a common superscript differ significantly ($P < 0.05$). Abbreviations for treatments are given in the legend to *Table 2*.

Table 4 Effects of glucocorticoid status and cimaterol on activities of several proteolytic enzymes in muscle of rats

	Experimental treatment					
	Sham	Sham + cim	Adx	Adx + cim	AdxD	AdxD + Cim
Cathepsin B*	1.32 ± 0.07^{c}	1.48 ± 0.06^{b}	1.31 ± 0.05^{c}	1.80 ± 0.06^{a}	1.24 ± 0.09^{c}	1.45 ± 0.07^{b}
Cathepsin D†	8.11 ± 1.05^{a}	8.03 ± 0.53^{a}	5.44 ± 0.94^{c}	9.01 ± 0.93^{a}	8.02 ± 0.79^{a}	7.72 ± 1.03^{b}
Cathepsin L‡	6.84 ± 0.45^{b}	7.70 ± 0.36^{a}	7.59 ± 0.33^{a}	7.49 ± 0.65^{a}	6.37 ± 0.39^{c}	6.91 ± 0.63^{b}
μ-Calpain§	17.57 ± 2.39^{a}	12.43 ± 2.34^{b}	11.77 ± 1.95^{b}	18.60 ± 2.79^{a}	10.28 ± 1.83^{b}	12.52 ± 1.35^{b}
m-Calpain§	37.18 ± 3.82^{b}	31.86 ± 3.05^{c}	34.21 ± 1.54^{c}	43.54 ± 3.50^{a}	32.84 ± 1.99^{c}	37.99 ± 3.68^{b}
Calpastatin¶	122.97 ± 15.5^{d}	168.20 ± 19.7^{c}	146.40 ± 24.7^{d}	221.11 ± 23.9^{a}	125.40 ± 18.4^{d}	190.31 ± 21.1^{b}

Values are means ± SE. Values in the same row that do not share a common superscript differ significantly ($P < 0.05$).
Abbreviations for treatments are given in the legend to *Table 2*. All values are given as units of activity per g protein.
*1 unit of cathepsin B activity is defined as nmoles of MNA released/g protein/100 min.
†1 unit of cathepsin D activity is defined as the change in absorbance units at 280 nm per g protein per 20 min.
‡1 unit of cathepsin L activity is defined as the change in absorbance units at 366 nm per g protein per 30 min.
§1 unit of calpain activity is defined as the change in absorbance units at 595 nm per g protein per 30 min.
¶1 unit of calpastatin activity inhibited 1 unit of m-calpain activity.

not in Adx rats receiving Dex. Total DNA, calculated as DNA concentration multiplied by muscle weight, was reduced 11% to 17% by Dex treatment compared with either the sham-Adx or the Adx groups. Cimaterol increased DNA content by 10% to 15% in sham-Adx and Adx rats receiving Dex, but did not affect DNA content in Adx rats. RNA concentration was reduced by 32% by adrenalectomy and was restored by Dex therapy. Cimaterol increased RNA concentration by 28% to 61% irrespective of glucocorticoid status (*Table 3*). The largest effect of cimaterol on RNA concentration was detected in Adx rats. Total RNA content was reduced 38% by adrenalectomy. Administration of Dex to Adx rats increased RNA content to near the levels detected in sham-Adx rats. Cimaterol increased muscle total RNA content by 48% to 74% irrespective of glucocorticoid status.

Effects of glucocorticoid and cimaterol status on muscle proteinase and proteinase inhibitor activities are shown in *Table 4*. Cathepsin B activity was unaffected by glucocorticoid status. Cimaterol increased cathepsin B activity by 12% to 37% in muscle irrespective of glucocorticoid status. Cathepsin D activity was reduced 33% by adrenalectomy and restored to normal levels by Dex therapy. Cimaterol did not affect cathepsin D activity in sham-Adx rats, increased cathepsin D activity 66% in Adx rats, and reduced cathepsin D activity by 4% in Adx rats treated with Dex. Cathepsin L activity was increased 11% by adrenalectomy and restored by Dex therapy. Cimaterol increased cathepsin L activity by 13% in sham-Adx rats and in Adx rats supplemented with Dex by 8%, but did not affect cathepsin L activity in Adx rats. m-Calpain activity exceeded μ-calpain activity by two to three fold (*Table 4*) but μ-calpain activity was more sensitive to glucocorticoid status and cimaterol treatment. μ- and m-calpain activities responded similarly to glucocorticoid and cimaterol treatments. μ- and m-calpain activities were reduced 32% and 8%, respectively, by adrenalectomy. Dexamethasone therapy did not restore their activities. Cimaterol reduced μ- and m-calpain activities in sham-Adx rats by 29% and 14%, respectively, and increased their activities by 58% and 27%, respectively, in Adx rats. In Adx rats that received Dex therapy, cimaterol increased m-calpain activity by 16% but did not significantly affect μ-calpain activity. Although cimaterol increased μ- and m-calpain activities in Adx rats supplemented with Dex, calpain activities in these animals were less than activities detected in cimaterol-supplemented adrenalectomized animals. Calpastatin activity exceeded combined activities of the calpains by two to three fold. Calpastatin activity was unaffected by glucocorticoid status. How-

ever, irrespective of glucocorticoid status, cimaterol increased calpastatin activity by 37% to 52%.

Discussion

Glucocorticoid status and cimaterol exerted characteristic effects on growth parameters. Adrenalectomy reduced food intake, rate of gain, and efficiency of converting food to body mass. Although Dex increased intake, it further reduced rate of gain by reducing efficiency of converting food to body mass. Hence, this dose of Dex must represent a dose that promoted tissue catabolism rather than a replacement dose. Although cimaterol reduced food intake in Adx rats, rate of gain was unaffected because cimaterol also improved efficiency of food conversion. Similarly, in Dex-supplemented Adx rats, cimaterol depressed intake but increased rate of gain by improving efficiency of food conversion. Cimaterol's ability to improve efficiency was independent of glucocorticoids because the effect was noted in Adx rats.

Mechanisms by which glucocorticoids and cimaterol alter muscle growth

Glucocorticoids are growth-inhibiting steroids and their effects on muscle growth have been related to effects both on protein synthesis and degradation.[5] Glucocorticoids maintained food intake but decreased efficiency of food utilization. Glucocorticoids also regulated muscle ribosome levels. Specifically, adrenalectomy reduced total RNA content and dexamethasone restored RNA. If dexamethasone reduces muscle growth via an effect on protein synthesis, it must therefore reduce efficiency of synthesis. In addition to this, Dex treatment reduced DNA concentration and content. Dexamethasone could therefore reduce muscle growth via an effect on muscle cell number. Limitations to this possible explanation are that muscle cell hyperplasia occurs predominately prenatally, and other cell types in the sample could have accounted for changes in DNA.

We did not find evidence that glucocorticoids modulate muscle growth through changes in activities of proteolytic enzymes. These observations are consistent with another study in this laboratory in which it was reported that injections of dexamethasone (1 mg/kg BW/day) into rabbits did not affect muscle calpain or calpastatin activities.[15] However, adrenalectomy increased calpastatin activity and dexamethasone reduced calpastatin activity in adrenalectomized, cimaterol-supplemented animals. These observations indicate that a change in calpain-dependent proteolysis by dexamethasone could be mediated via changes in calpastatin levels. However, we believe that the excess of calpastatin relative to calpains, which was present even in dexamethasone-supplemented animals, prevents the regulation of calpastatin level from being an effective means of regulating calpain activity. It is of interest that adrenalectomy reduced μ- and m-calpain activities. This demonstrates that endogenous glucocorticoids, at normal concentrations and below, regulate calpain activities, but that synthetic glucocorticoid (dexamethasone) is unable to regulate muscle calpain activities. It is also of interest that cathepsin D activity closely followed glucocorticoid status. Adrenalectomy reduced its activity and Dex treatment increased its activity. These changes indicate that cathepsin activities are regulated independently of one another, and that cathepsin D could play a role in glucocorticoid-dependent changes in muscle proteolysis.

Mechanisms by which cimaterol influences muscle growth were revealed in this study. First, cimaterol increased efficiency of food conversion to body mass despite instances in which it reduced food intake. As a basis for this, cimaterol had large effects on RNA levels in muscle tissue, suggesting that it may increase capacity for muscle protein synthesis. In the presence of glucocorticoids (either endogenous or injected), cimaterol also increased total DNA content of muscle, suggesting that it increased muscle cell number. The limitation of this observation, however, is that effects of cimaterol on muscle total DNA content were small and there were many other cell types in the muscle samples besides muscle cells. In addition to this, we determined that cimaterol could stimulate growth by control of calcium-dependent proteolysis. Specifically, cimaterol reduced μ- and m-calpain activities and increased calpastatin activity. These observations suggest that cimaterol reduced the potential for calcium-dependent digestion of muscle proteins, and that protein degradation was reduced.

Others have noted that the response of the calpain system to beta-adrenergic agonists is species-specific.[16] Our previous studies have shown that calpains and calpastatin are co-regulated. Maturation in rabbits[17] and sheep[18] and cimaterol treatment[19] and fasting[20] of rabbits effect coordinated changes in muscle μ- and m-calpain and calpastatin activities or their messenger RNA levels. Co-regulation of calpains suggests that they participate in the same metabolic process, and co-regulation of calpastatin with calpains suggests that a mechanism which prevents uncontrolled proteolysis may also be incorporated into control of the calpain system. μ- and m-calpains were co-regulated by cimaterol and glucocorticoid status, suggesting that they may participate in related metabolic processes. However, in this study calpastatin was regulated independently of the calpains. These observations may be compared to those of Higgins et al.[21] and Kretchmar et al.[22] who reported that beta-agonists reduced sheep muscle μ-calpain but increased m-calpain and calpastatin activities. Reasons for the differences between our studies could be related to species differences, agonist type, or duration of exposure. Whereas our rats were exposed to cimaterol for 8 days, sheep in their studies were exposed to either clenbuterol or L-644,969 for 6 weeks prior to muscle sampling.

Effects of cimaterol on the cathepsins were inconsistent and, therefore, shed no light on a mechanism of action. The different responses of the cathepsins to cimaterol again demonstrate that expression of these proteases is independently regulated.

Do glucocorticoids potentiate actions of beta-adrenergic agonists?

Several authors have questioned whether beta-adrenergic agonists mediate their actions indirectly via other hormones. However, growth-promoting properties of beta-agonists have been detected in thyroidectomized,[1] insulin-supplemented diabetic,[2] and castrated[3] rats. Clenbuterol's actions in cattle are not mediated by the growth hormone axis.[4] Moreover, anabolic actions were detected in denervated muscle.[23–25] Hence, actions of β-agonists are independent of thyroid hormone, insulin, testosterone, and growth hormone and of input from the central nervous system. An exception was reported by Sharpe et al.,[5] who proposed that clenbuterol's growth-promoting actions in rats were potentiated by glucocorticoids. More recently, however, Rothwell and Stock[3] reported that actions of clenbuterol were glucocorticoid independent. Based on our study, we conclude that cimaterol antagonizes growth-inhibiting actions of glucocorticoids, but also exerts control of muscle protein metabolism through mechanisms that are independent of glucocorticoids. The rationale for this is presented below.

Cimaterol increased muscle weights in sham-Adx rats and in Dex-supplemented Adx rats but did not increase muscle weights in Adx rats. These observations, which are similar to those reported by Sharpe et al.,[5] suggest that glucocorticoids potentiate actions of clenbuterol on muscle growth. However, an alternative explanation is that cimaterol antagonized growth-inhibiting actions of glucocorticoids. Our data permit us to distinguish between these two possibilities. Specifically, if glucocorticoids potentiate actions of cimaterol, Dex treatment of cimaterol-fed Adx rats should have increased muscle weights. However, muscle weights of cimaterol-fed Adx rats and Dex-supplemented Adx rats were equal. Hence, we conclude that cimaterol enhanced muscle weights, in part, by antagonizing growth-inhibiting actions of glucocorticoids.

A mechanism by which cimaterol antagonized growth-inhibiting actions of glucocorticoids may be partially related to total DNA content. Cimaterol did not affect DNA content of Adx rat muscle but reversed the Dex-dependent reduction in DNA content in Adx rats. Cimaterol also increased DNA content of sham-Adx rat muscle. Although we cannot be certain that the changes in DNA occurred in muscle cells, the observation suggests that Dex reduced muscle growth, in part, by reducing DNA content, and that cimaterol antagonized this effect.

Cimaterol reduced μ- and m-calpain activities in sham-Adx rats but increased their activities in Adx rats and in Adx rats that received Dex. This is a complex interaction, and we have insufficient data to explain it. It appears that, in the presence of endogenous glucocorticoids, beta-adrenergic agonists reduce calpain activity but, in their absence, the opposite is true. Exogenous glucocorticoid administration did not restore the ability of cimaterol to reduce calpain activities, suggesting that the replacement therapy did not fully mimic actions of endogenous glucocorticoids. These data also indicate that variations in glucocorticoid status of experimental animals influence the response of the calpains to a beta-adrenergic agonist challenge. This may explain why some studies have determined that agonists increase calpain activities while others, including this study, have reported the opposite.

Glucocorticoid-independent actions of cimaterol

Although actions of cimaterol on muscle weights were detected only in the presence of glucocorticoids, other actions of cimaterol were detected in the presence and absence of glucocorticoids, demonstrating that some actions of beta-adrenergic agonists are independent of glucocorticoids. Specifically, cimaterol increased RNA content and concentration and cathepsin B and calpastatin activities in sham-Adx rats, Adx rats, and Dex-supplemented Adx rats. It reduced feed intake and the food to gain ratio in both Adx rats and Dex-supplemented Adx rats.

In summary, this study has shown that glucocorticoids and cimaterol may interact in control of some aspects of muscle growth. Specifically, we propose that cimaterol antagonizes growth-inhibiting actions of glucocorticoids. Glucocorticoids can interact with beta-adrenergic agonists in control of muscle DNA content and in control of μ- and m-calpain activities. Glucocorticoids may reduce muscle growth by reducing efficiency of protein synthesis, but they do not alter growth through changes in activities of proteolytic enzymes. Cimaterol may stimulate growth by increasing muscle capacity for protein synthesis and by reducing the potential for calcium-dependent proteolysis. In response to cimaterol and glucocorticoids, μ- and m-calpain are co-regulated, but calpains were not co-regulated with the lysosomal enzymes or with calpastatin.

References

1 Forsberg, N.E. and Wehr, N.B. (1990). Effects of cimaterol on muscle protein metabolism and its actions in hypothyroid and hyperthyroid rats. *Nutr. Res.* **7,** 149–164

2 McElligott, M.A., Mulder, J.E., Chaung, L.Y., and Barreto, Jr., A. (1987). Clenbuterol-induced muscle growth: investigation of possible mediation by insulin. *Am. J. Physiol.* **253,** E370–E375

3 Rothwell, N.J. and Stock, M.J. (1988). Increased body weight gain and body protein in castrated and adrenalectomized rats treated with clenbuterol. *Br. J. Nutr.* **60,** 355–360

4 Maltin, M.A., Delday, M.I., Hay, S.M., Innes, G.M., and Williams, P.E.V. (1990). Effects of bovine pituitary growth hormone alone or in combination with the beta-agonist clenbuterol on muscle growth and composition in veal calves. *Br. J. Nutr.* **63,** 535–545

5 Sharpe, P.M., Hayes, N.B., and Buttery, P.J. (1986). Glucocorticoid status and growth. In *Control and Manipulation of Animal Growth,* (P.J. Buttery, D.B. Lindsay, and N.B. Haynes, eds.), p. 207–222, Butterworths, London, UK

6 American Institute of Nutrition. (1977). Report of the American Institute of Nutrition. Ad hoc committee on standards for nutritional studies. *J. Nutr.* **107,** 1340–1347

7 Labarca, C. and Paigen, K. (1980). A simple, rapid and sensitive DNA assay procedure. *Anal. Biochem.* **102,** 344–352

8 Munro, H.N. and Fleck, A. (1969). Analysis of nitrogenous constituents. Measurements of nucleic acids in tissues. In *Mam-*

malian Protein Metabolism. Vol. 3, p. 477, Academic Press, New York, NY USA

9 Bradford, M.M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein using the principle of protein dye-binding. *Anal. Biochem.* **72,** 248–254

10 Barrett, A.J. and Kirschke, H. (1981). Cathepsin B, cathepsin D and cathepsin L. *Meth. Enzymol.* **80,** 535–565

11 Takahashi, T. and Tang, J. (1981). Cathepsin D from porcine and bovine spleen. *Meth. Enzymol.* **80,** 565–569

12 Gopalakrishna, R. and Barsky, S.H. (1985). Quantitation of tissue calpain activity after isolation by hydrophobic chromatography. *Anal. Biochem.* **148,** 413–423

13 Nakamura, M., Imahori, K., and Kawashima, S. (1988). Tissue distribution of an endogenous inhibitor of calcium-activated neutral protease and age-related changes in its activity in rats. *Comp. Biochem. Physiol.* **89B,** 381–384

14 Steel, R.G.D. and Torrie, J.H. (1980). *Principles and Procedures of Statistics,* McGraw-Hill Book Co., Inc., New York, NY USA

15 Yeh, J.Y., Ou, B.R., and Forsberg, N.E. (1994). Effects of dexamethasone on muscle protein degradation and calpain and calpastatin activities and gene expression in rabbits. *J. Endocrinology* (in press)

16 Bardsley, R.G., Allcock, S.M.J., Dawson, J.M., Dumelow, N.M., Higgins, J.A., Lasslett, Y.V., Lockley, A.K., and Buttery, P.J. (1992). Effect of β-agonists on expression of calpain and calpastatin activity in skeletal muscle. *Biochimie* **74,** 267–273

17 Ou, B.R. and Forsberg, N.E. (1991). Determination of skeletal muscle calpain and calpastatin activities during maturation. *Am. J. Physiol.* **261,** E677–E683

18 Ou, B.R., Meyer, H.H., and Forsberg, N.E. (1991). Effects of age and castration on activities of calpains and calpastatin in sheep skeletal muscle. *J. Anim. Sci.* **69,** 1919–1924

19 Forsberg, N.E., Ilian, M.A., Ali-Bar, A., Cheeke, P.R., and Wehr, N.B. (1989). Effects of cimaterol on rabbit growth and myofibrillar protein degradation and on calcium-dependent proteinase and calpastatin activities in skeletal muscle. *J. Anim. Sci.* **67,** 3313–3321

20 Ilian, M.A. and Forsberg, N.E. (1992). Gene expression of calpains and their specific inhibitor, calpastatin, in skeletal muscle of fed and fasted rabbits. *Biochem. J.* **287,** 163–171

21 Higgins, J.A., Lasslett, Y.V., Bardsley, R.G., and Buttery, P.J. (1988). The relation between dietary restriction or clenbuterol (a selective β2 agonist) treatment on muscle growth and calpain proteinase (EC 3.4.22.17) and calpastatin activities in lambs. *Br. J. Nutr.* **60,** 645–652

22 Kretchmar, D.H., Hathaway, M.R., Epley, R.J., and Dayton, W.R. (1989). In vivo effect of a β-adrenergic agonist on activity of calcium-dependent proteinases, their specific inhibitor, and cathepsins B and H in skeletal muscle. *Arch. Biochem. Biophys.* **275,** 229–235

23 Maltin, C.A., Reeds, P.J., Delday, M.I., Hay, S.M., Smith, F.G., and Lobley, G.E. (1986). Inhibition and reversal of denervation-induced atrophy by the beta-agonist growth promoter, clenbuterol. *Biosci. Rep.* **6,** 811–818

24 Maltin, M.A., Hay, S.M., Delday, M.I., Reeds, P.J., and Palmer, R.M. (1989). Evidence that the hypertrophic action of clenbuterol on denervated rat muscle is not propranolol-sensitive. *Br. J. Pharmacol.* **96,** 817–822

25 Zeman, R.J., Ludemann, R., and Etlinger, J.D. (1987). Clenbuterol, a beta-2 agonist, retards atrophy in denervated muscles. *Am. J. Physiol.* **252,** E152–E155