Effects of the cimaterol (CL 263,780) on growth and cellular metabolisms in the mammary gland of rats

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This study was conducted to examine the effects of cimaterol feeding on growth performances and mammary growth and differentiation. Attempts were also made to investigate the cellular metabolisms of protein and fat in the mammary gland at the various physiological stages (virgin, pregnancy, early lactation, and late lactation). Ninety-six female Sprague Dawley rats (approximately 52.3 g) were randomly assigned to three treatments: CON (feeding the diet without cimaterol), CIM I (feeding the diet with cimaterol [10 mg/kg] from weanling to parturition), CIM II (feeding the diet with cimaterol from breeding to parturition). Cimaterol improved ($P < 0.01$) body weight gain and feed intake and reduced ($P < 0.01$) feed efficiency. In the mammary gland, DNA, RNA, and protein content were increased, and lipid content was decreased, by feeding cimaterol. Also, functional activity (RNA/DNA) and size (protein/DNA) of mammary gland cells were increased by cimaterol. It was clear that the lipogenic activity in the mammary gland was increased with lactogenesis, although cimaterol had no effect on lipogenic activity. But cimaterol was effective in increasing lipolytic activity. As a result of in vitro acinar mammary cell culture, cimaterol showed a direct effect on increasing protein synthetic activity ($P < 0.01$). It was observed through this study that cimaterol functions not only on energy repartitioning but also on growth and mammary development and differentiation in rats.

Keywords: cimaterol; mammary gland; lipogenesis; lipolysis; protein synthetic activity; acinar cell culture

Introduction

β-Adrenergic agonists are chemical analogs of the naturally occurring catecholamines. Many β-adrenergic agonists are extremely potent and chemically stable. In addition, some β-adrenergic agonists (cimaterol, clenbuterol, etc.) are orally active, so in-feed formulations are possible.

Recent studies have consistently demonstrated that dietary supplements of β-adrenergic agonists improve growth performance, feed efficiency, and carcass composition in meat animals.[1-6] Cimaterol altered the carcass composition by increasing muscle protein accretion while reducing fat deposition, and this change was accomplished without additional feed consumption.[6]

There are several cellular mechanisms by which β-adrenergic agonists could increase the rate of muscle growth and depress adipose tissue growth; these were summarized in detail by Ricks et al.[3] Briefly, the growth of any tissue is the result of the balance between synthesis and degradation. In muscle, net growth implies that myofibrillar protein synthesis occurs at a rate greater than that of myofibrillar protein degradation. Adipose tissue growth primarily involves the net accretion of lipid, ie, the balance between triacylglycerol biosynthesis and lipolysis.

But, at the present, research information about effects of β-adrenergic agonist on mammary growth, development, and differentiation during pregnancy and lactation are lacking. The objectives of this study were to characterize the effects of cimaterol on the changes in efficiency of animal growth and the contents of DNA, RNA, protein, and lipid in mammary gland; to determine whether cimaterol feeding modulates mammary development and differentiation; and to compare effects of cimaterol on milk protein synthetic activity in mammary alveolar cell culture, and on in vitro lipogenic and lipolytic activity in mammary gland.

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Materials and methods

Materials

Cimaterol (CL 263,780) was generously provided by University of California, Davis. [^{3}H] lysine, [^{14}C] glucose, and Insta gel (cocktail solution) were purchased from Armersham International plc. Fetal bovine serum was purchased from Gibco Laboratories Life Technologies, Inc. Collagenase (type I), Hyaluronidase (type I), and bovine serum albumine (fatty acid free) were purchased from Sigma Chemical Company.

Animals and diets

Ninety-six female Sprague Dawley (SD) rats aged 3 weeks (approximately 52.3 g) were supplied by Animal Breeding Laboratory of Seoul National University. Animals were housed individually in stainless-steel cages in a light-cycle room with 8:00 AM to 8:00 PM light. Temperature and relative humidity were held at 22° C ± 2° and 60% ± 10%, respectively. The estrous cycles of rats were determined by microscopic examinations of vaginal smears. Pregnancy was confirmed by the presence of sperm in a vaginal smear and vaginal plugs. After parturition, litters were fixed with six pups and raised through day 21.

The formula and chemical composition of experimental diets are shown in *Table 1*. To certify the effect of cimaterol, the dietary fat of experimental diet was formulated 3 times higher than that recommended by the National Research Council.[7] Diets were stored at 4° C.

Experimental design

Depending on different levels of cimaterol (0, 10 mg/kg) and feeding periods, there were three treatments: control, cimaterol I, and II. Control group (*Table 1*) was fed diet without cimaterol meeting the National Research Council[7] requirements during entire experimental period. In cimaterol groups, cimaterol was mixed in the control diet at 10 mg/kg. Cimaterol group I (CIM I) was fed diet containing cimaterol from weanling to parturition for 10 weeks. Cimaterol group II (CIM II) was fed diet containing cimaterol from pregnancy to parturition for 3 weeks.

Table 1 Formula and chemical composition of experimental diet

	Control
Formulated level	
Crude protein (%)	18.0
Digestible energy (kcal/kg)	3800.0
Ingredient (%)	
Corn, yellow	55.5
Soybean meal	27.3
Fish meal	2.0
Tallow	12.2
Vitamin-mineral mixture[a]	3.0
Chemical composition (%)	
Moisture	9.57
Crude protein	18.85
Crude fat	14.79
Nitrogen free extract	51.82
Crude fiber	2.43
Crude ash	2.54
Calcium	0.86
Phosphorus	0.39

[a] Vitamin-mineral mixture (per kg): vitamin A, 5,000 IU; vitamin D, 1,000 IU; vitamin E, 36 mg; vitamin K, 0.06 mg; panthothenate HCl, 5 mg; thiamin HCl, 5 mg; riboflavin, 4 mg; pyridoxine HCl, 8 mg; vitamin B_{12}, 0.06 mg; folacin, 1.2 mg; cholinechloride, 1200 mg; $CaHPO_4$, 22.2 g; NaCl, 1.53 g; K_2SO_4, 6.70 g; MgO, 0.68 g; $FeSO_4$ $7H_2O$, 0.20 g; $CuSO_4$ $5H_2O$, 0.024 g; $MgSO_4$ H_2O, 0.19 g; KI, 0.005 g; $MnSO_4$ H_2O, 1.21 g; $ZnCl_2$, 0.20 g.

Chemical analysis

Diets were sampled biweekly and composited monthly. These samples were analyzed for proximate analyses by AOAC[8] procedures. Rats were decapitated, and mammary gland was collected according to physiological stages (virgin, pregnancy, early lactation, and late lactation).

Sampled mammary gland was weighed, frozen, and freeze dried. Dry matter (DM) was recorded. A portion of mammary tissue collected in sterile balanced salt solution (BSS) with calf serum and antibiotics was used for acinar cell culture works. A portion of mammary gland collected in sterile Krebs-Ringer bicarbonate (KRB) buffer was used for determination of lipogenic and lipolytic activity.

Mammary gland samples for protein, DNA, and RNA assay were extracted by potassium acetate and 3:1 (vol/vol) ethyl ether:ethanol according to the modified method of Merchant et al.[9] Protein was measured according to the method of Lowry et al.[10] with bovine serum albumin (BSA) as standard. The DNA was determined according to Labarca and Paigen[11] with calf thymus DNA as standard. The RNA[12] was measured with yeast RNA as standard. Total lipids were determined gravimetrically after extraction with a hexane-isopropanol (2:1) using the basic procedure described by Folch et al.[13]

Mammary acinar culture

Preparation of alveoli. Immediately after mammary tissue was obtained, the tissue was bathed in sterile BSS containing antibiotics and calf serum. The cell dispersion was accomplished by the method of Choi.[14] Mammary tissue was trimmed free of large pieces of connective, lymph, adipose tissues, and blood vessels and minced with sterile scissors to less than 5 mm^3 in a solution of 400 U/mL of collagenase (Type I), 400 U/mL hyaluronidase (Type I), 5% (vol/vol) fetal bovine serum and 0.15% (vol/vol) trypsin in 1X Minimal Essential Media (MEM). This tissue was dissociated with the collagenase-hyaluronidase solution for 3 hours at 37° C by continuous stirring. The cells were centrifuged at 1,000*g* at 4° C for 5 minutes, washed twice in BSS and resuspended in 1X MEM. The cell suspension was filtered through four layers of cheese cloth. The cells were plated on plastic tissue culture dishes (approximately 10^6 cells/dish). In all studies, four dishes were set up for each observation with contents pooled after incubation for subsequent analysis.

Culture medium. The basic medium used was Eagle's MEM[15] as modified by Smith et al.[16] Glucose and bovine serum were added to 1X MEM to final concentrations of 0.2% (wt/vol) and 5% (vol/vol), respectively. Antibiotics (penicillin 10,000 IU, amphotericin-B 25 mcg, streptomycin 10,000 IU per 100 mL media) were added to all media. The pH of the media was adjusted to 7.4 by addition of 7.5% sodium bicarbonate. The isotope used most frequently for labeling the cell culture was [^{3}H] lysine. Routinely, 0.5 uCi of the tracer was added to 1 mL media for the purpose of determining in vitro synthetic activity of milk protein.

Milk protein synthetic activity. At the termination of the 18-hour incubation, cells were collected, pooled (four dishes for each observation of each treatment), and centrifuged at 1,000*g* at 4° C for 10 minutes. The amount of milk protein synthesis was measured by the method of Choi et al.[17] Specific activity

incorporated into protein was counted in Insta gel by a liquid scintillation counter (LS 100C).

Measurement of lipogenic activity. Lipogenic activity was measured by the method of Mersmann et al.[18] Mammary gland was sliced with scissors. The amount of tissue slices per vial was 10–20 mg. Tissue slices were incubated for 120 minutes at 37° C in 3 mL of medium under a gaseous atmosphere of 5% CO_2 in O_2. The incubation medium (KRB buffer) contained 25 mmol/L HEPES, 5.0 mmol/L glucose, 3% BSA and 0.5 uCi [^{14}C] glucose. Incubations were terminated by placing vials on ice. After taking out tissue slices from the medium, total lipids in the tissue slices were extracted by the method of Dole and Meinertz.[19] The extracts were dried. Radioactivity incorporated into total lipids was determined in a liquid scintillation counter (LS 100C).

Measurement of lipolytic activity. Lipolytic activity was measured in Krebs-Ringer bicarbonate buffer with one-half of the indicated Ca^{++}, containing 4% fatty-acid-poor fraction V bovine serum albumin and 5.56 mmol/L glucose. Tissue slices (approximately 100 mg) were incubated for 120 minutes at 37° C in 3 mL of medium under a gaseous atmosphere of 5% CO_2 in O_2. Incubations were terminated by placing vials on ice. The medium was filtered through cheesecloth to remove the tissue and stored at −20° C until analysis. Non-esterified fatty acids (NEFA) in the medium were extracted and titrated according to the method of Kelly.[20]

Statistical analysis

All data for the present study were subjected to two way analysis of variances by Duncan test,[21] by using General Linear Models (GLM) procedure developed by Statistical Analysis System.[22]

Results

The effects of cimaterol on the growth performance of rats for 0–11 weeks are summarized in *Table 2*. Responses of rats to feeding cimaterol were significant ($P < 0.01$) on all growth performances including body weight gain, feed intake, and feed efficiency. The ranking for body weight gain and feed intake with significant difference was CIM I > CIM II > CON. Feed efficiency was the highest in CON and lowest in CIM II.

To evaluate mammary development and differentiation of the rat mammary glands, the contents of DNA, RNA, protein, and lipid in the gland were measured. Chemical compositions on the basis of DM of rat mammary glands are shown in *Table 3*. The mammary glands of the virgin stage were above 80% lipid and correspondingly low in DNA, RNA, protein, RNA:DNA ratio, and protein:DNA ratio. There was a trend toward an increase in DNA, RNA, and protein with a decrease in lipid throughout the experiment. There were also significant effects of cimaterol on increasing DNA, RNA, and protein content with a decrease in lipid content ($P < 0.01$). But there were no consistent effects on RNA:DNA ratio and protein: DNA ratio by cimaterol treatment and physiological stage, respectively. Interaction between cimaterol treatment and physiological stage was influenced on all criteria with significant difference ($P < 0.01$). Both CIM I and CIM II were higher in DNA, RNA, and protein than control group, while lower in lipid content. CIM II was the highest in DNA, RNA, and protein, with the lowest lipid content.

Changes of lipogenic and lipolytic activity in mammary gland are shown in *Table 4*. At the lactational stage, lipogenic activity in mammary gland was acutely increased with significant difference ($P < 0.01$). This result was caused by lactogenesis in mammary gland.

At the time of pregnancy and lactational stage, changes of protein synthetic activity are shown *Table 5*. Protein synthetic activity was measured by the incorporation of radioactive amino acids (^{3}H-lysine) into trichloroacetic acid-insoluble material by in vitro acinar cell cultures. Protein synthetic activity depended on supplementation of cimaterol and physiologic status. At the time of pregnancy, CIM I and CIM II group increased 1.6-fold and 1.5-fold, respectively, in protein synthesis over control group by cimaterol feeding. There was an increase of more than 1.4-fold over control in protein secretion. Amount of secreted and retained protein were increased by more than 10-fold in lactation than in pregnancy.

Discussion

Data from measurement of growth performance are in accordance with a number of previous reports about stimulation of growth in rats treated with beta-ago-

Table 2 Production measurements averaged by treatment (0–11 weeks)

Item	Control	CIM I[a]	CIM II[b]	Pooled SE[c]	Probability[d]
Average body weight (g)					
Initial	52.3	52.5	52.3	0.5	0.9378
Final	195.2	223.8	210.9	2.3	0.0001
Weight gain	142.0	167.9	159.8	4.2	0.0001
Feed intake (g)	731.1	890.1	885.9	21.5	0.0020
Feed efficiency	5.1	5.3	5.5	0.1	0.0001

[a] Cimaterol group I (CIM I) fed a cimaterol diet (10 mg/kg) from weanling to parturition (0–10 weeks).
[b] Cimaterol group II (CIM II) fed a cimaterol diet (10 mg/kg) from pregnancy to parturition (7–10 weeks).
[c] SEM, where $n = 20$.
[d] Significance level of F-test for cimaterol treatment.

Table 3 Chemical composition of rat mammary tissue at various physiological stages

	Virgin			Pregnancy			Early lactation			Late lactation				
Item[a]	CON	CIM I[b]	CIM II[c]	CON	CIM I	CIM II	CON	CIM I	CIM II	CON	CIM I	CIM II	SE[d]	Probability[e]
DNA (mg/g)	0.2	1.6	0.2	3.9	4.1	3.6	4.1	5.1	3.1	6.7	8.4	8.3	0.2	0.0001
RNA (mg/g)	0.6	1.0	0.6	4.3	6.0	13.5	15.1	17.3	16.2	15.8	18.2	14.5	0.1	0.0001
Protein (mg/g)	33.6	66.8	33.6	109.5	239.3	228.7	223.4	305.5	220.9	215.8	425.1	378.7	5.3	0.0001
RNA/DNA	3.0	0.6	3.0	1.1	1.5	3.8	3.7	3.4	5.2	2.4	2.2	1.8	0.3	0.0001
Protein/DNA	168.0	41.8	168.0	28.1	58.4	63.5	54.5	59.9	71.3	32.2	50.6	45.6	12.4	0.0001
Lipid (mg/g)	879.4	769.3	879.4	671.7	621.9	394.3	541.3	449.4	319.7	350.5	334.0	316.6	35.2	0.0001

Analysis of variance mean square:

Source of variables	df	DNA	RNA	Protein	RNA/DNA	Protein/DNA	Lipid
C, cimaterol treatment	2	4.2*	16.3*	39407.1*	6.3*	2886.2*	53247.6*
S, physiological stage	3	76.1*	499.4*	138189.8*	9.4*	10314.8*	435903.0*
C × S, interaction	6	1.1*	23.3*	6831.3*	2.4*	3708.8*	20847.6*
Error	24	0.070	0.057	85.602	0.238	458.494	3707.516

[a] mg/g denotes mg per dry matter tissue g.
[b] Cimaterol group I (CIM I) fed a cimaterol diet (10 mg/kg) from weanling to parturition (0–10 weeks).
[c] Cimaterol group II (CIM II) fed a cimaterol diet (10 mg/kg) from pregnancy to parturition (7–10 weeks).
[d] n, 4; SE, standard error of the mean; *: $P < 0.01$.
[e] Significance level of F-test for cimaterol treatment × physiological stage.

Table 4 Lipogenic and lipolytic activity in mammary tissue at various physiological stages[a]

	Virgin			Pregnancy			Early lactation				
Item	CON	CIM I[b]	CIM II[c]	CON	CIM I	CIM II	CON	CIM I	CIM II	SE[d]	Probability[e]
Lipogenic activity[f]	3.3	1.9	3.3	3.1	0.5	1.0	72.8	69.9	66.2	4.88	0.0001
Lipolytic activity[g]	3.5	4.9	3.5	5.4	5.6	5.8	11.4	14.9	17.2	0.35	0.0001

Analysis of variance mean square:

Source of variables	df	Lipogenic activity	Lipolytic activity
C, cimaterol treatment	2	28.2	14.4*
S, physiological stage	2	18197.8*	384.4*
C × S, interaction	4	13.0	11.0*
Error	27	95.327	0.501

[a] Each value is the mean of 4 replication.
[b] Cimaterol group I (CIM I) fed a cimaterol diet (10 mg/kg) from weanling to parturition (0–10 weeks).
[c] Cimaterol group II (CIM II) fed a cimaterol diet (10 mg/kg) from pregnancy to parturition (7–10 weeks).
[d] SE, standard error of the mean; *: $P < 0.01$.
[e] Significance level of F-test for cimaterol treatment × physiological stage.
[f] nmol glucose incorporated into total lipids/mg.
[g] ueq non-esterified fatty acid (NEFA) released/mg.

nists.[23-26] It is likely that the improved feed intake and body weight gain were achieved by dietary supplementation of cimaterol. Consequently, stimulating feed intake by dietary cimaterol might lead to improved body-weight gain. Effects on body weight gain reflect the relative effects on the rates of protein and fat deposition. In general, cimaterol is a β-agonist that alters carcass composition by partitioning energy away from fat deposition and towards protein accretion. Of course, responses to β-agonists differ depending on species, drug dose, and investigators. In chemical composition of whole body, dietary cimaterol significantly decreased fat content of rat whole body.[27] Several studies have reported that β-agonists had no significant effects on feed consumption or decreased feed consumption in livestock[3,4,28] and rats.[25]

The growth and development of mammary secretory cell is one of the major factors affecting milk production.[29,30] Of the total mammary development in rats, approximately 11% occurs before pregnancy, 41% occurs during pregnancy, and the remainder occurs during lactation, provided an intense milking stimulus is applied to the animal. In this study, mammary gland development and differentiation were evaluated by chemical method. A constant DNA concentration in mammary cells[31] makes DNA an excellent indicator of cell number. Determination of DNA as a measure of total cell numbers has facilitated the understanding of factors controlling mammary growth and

Table 5 Protein synthetic activity in acinar cell culture at various physiological stages

Item[a]	Pregnancy			Early lactation				
	CON	CIM I[b]	CIM II[c]	CON	CIM I	CIM II	SE[d]	Probability[e]
Secreted protein (dpm/mg × 10^{-2})	4.8	7.0	6.8	57.8	63.6	65.3	0.8	0.0001
Retained protein (dpm/mg × 10^{-2})	12.8	20.3	18.6	133.5	150.9	164.2	1.7	0.0001

Analysis of variance mean square:

Source of variables	df	Secreted protein	Retained protein
C, cimaterol treatment	2	52.2*	695.6*
S, physiological stage	1	18838.4*	105019.7*
C × S, interaction	2	15.6*	314.3*
Error	18	2.31	11.84

[a] Acinar cells isolated from rat mammary tissue were plated and incubated for 18 hr in the culture medium containing lactogenic hormones [insulin (200 μU/mL) + hydrocortisone (1.8 × 10^{-8} M) + prolactin (1 μg/mL)]; Each value is the mean of 4 observations with 4 culture dishes/observation.
[b] Cimaterol group I (CIM I) fed a cimaterol diet (10 mg/kg) from weanling to parturition (0–10 weeks).
[c] Cimaterol group II (CIM II) fed a cimaterol diet (10 mg/kg) from pregnancy to parturition (7–10 weeks).
[d] SE, standard error of the mean; *: $P < 0.01$.
[e] Significance level of F-test for cimaterol treatment × physiological stage.

development.[32] RNA concentration in several tissues has been associated with the intensity of protein synthesis[31] and the protein:DNA ratio provides a measure of cell size.[33] Hackett and Tucker[34] suggested that RNA may be used as an index of functional activity of the mammary gland. In this study, RNA:DNA ratio has a tendency to increase with mammary development and differentiation. This concurs with the fact that RNA:DNA ratios in mammary gland increase gradually during pregnancy and more sharply after parturition in preparation for the synthesis and secretion of milk. These increases in RNA concentration are due in part to increased rates of RNA synthesis, but also could be due to decreased rates of RNA degradation as shown by large increases in the cytoplasmic ribonuclease inhibitor.[35]

Considering the fact that the DNA content of control group at virgin stage might be underestimated, the protein:DNA ratio that provides a measure of cell size increases gradually throughout the experimental periods. Lipid content in mammary glands decreased with development. This may indicate the differentiation of mammary epithelial cells at the expense of the fat pad.[36] Cimaterol acts as stimulator of lipolysis, rather than decreasing lipid content in mammary gland.

At the lactational stage, lipogenic activity in mammary gland was acutely increased with significant difference ($P < 0.01$). It was clear that the lipogenic activity in mammary gland was increased with lactogenesis, although cimaterol had no effect on lipogenic activity. But cimaterol was effective in increasing lipolytic activity. Of course, an experimental diet high in dietary fat was partially responsible for increasing lipolytic activity.

In in vitro acinar cell culture, cimaterol had a direct effect on increase of protein synthetic activity ($P < 0.01$). Comparisons between the pregnancy and lactational stages in protein synthetic activity indicated that protein synthetic activity of mammary gland is acutely increased by lactogenesis.

In conclusion, these data support the hypothesis that action of cimaterol modulates mammary development and differentiation. We concluded that available energy for biosynthesis might be transported more efficiently into tissues (mammary gland) other than adipose tissue.

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