The effects of the β-adrenergic agonist cimaterol (CL 263, 780) on mammary differentiation and milk protein gene expression

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This experiment was to elucidate the mechanisms responsible for the effects of the β-agonist cimaterol on mammary differentiation and the expression of milk protein genes. Ninety-six weanling female rats were randomly assigned to either a control or a test group (supplementation of cimaterol, 10 mg/kg). Mammary tissues from the test group showed an increase in the DNA, RNA, protein, RNA:DNA ratio, and protein:DNA ratio while exhibiting a decrease in the lipid content as compared with those of the control group. The amount of α-, β-, and γ- casein and whey acidic protein mRNAs was markedly increased from virgin to early lactating stages: there were 29-, 36-, 33-, and 60-fold increases, respectively. The levels of α-, β-, and γ-casein and WAP mRNAs of mammary tissues in the test group were about 1.3-, 1.2-, 1.3-, and 1.4-fold higher, respectively, than those of the control group during early lactating stages. The test group had increased milk protein secretion, retained protein, and amino acid uptake in mammary acinar culture (1.2, 1.1, and 1.3 times, respectively) compared with those of the control group in the early lactating stage. These results indicate that dietary supplementation of cimaterol may induce mammary differentiation and milk protein mRNA transcripts, and may contribute to an increase in the functional activity of the mammary gland.

Keywords: cimaterol; mammary differentiation; milk protein gene expression; milk protein mRNA transcripts; acinar cell culture

Introduction

Multiple factors, including hormones, growth factors, nutritional status, and interactions between epithelial and stromal cells, are involved in the regulation of mammary epithelial cell growth. Even though effects of catecholamines on endocrine secretions involved in animal growth and metabolism have been known for a long time, it has only been during the past few years that β-adrenergic agonists (β-agonists) such as cimaterol and clenbuterol have been shown to have significant effects on growth rate and body composition in rat[1,2,3] and livestock animals.[4,5,6,7] It is generally agreed that β-agonists significantly increase body protein and muscle mass and decrease body fat content. However, research is scarce on the effects of the β-agonists on mammary differentiation and milk protein gene expression.[8] Furthermore, information on if or how the β-agonist might regulate the expression of milk protein genes at the level of mRNA transcripts and protein synthesis and secretion are lacking. The objective of this research was to examine the effects of the β-agonist cimaterol on mammary differentiation and milk protein gene expression by dot hybridization technology for milk protein mRNA quantification and acinar cell culture for activities of milk protein synthesis and amino acid uptake.

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Methods and materials

Ninety-six weanling female rats, 21 days of age and averaging 52.4 g, were randomly assigned to two treatment groups: control and test [cimaterol-fed (10 mg/kg)]. The basal (control) diet was formulated to contain approximately 18% crude protein (N × 6.25) and 3,800 Kcal of digestible energy per kg of diet as shown in *Table 1*. Test diet was the basal diet supplemented with 10 mg/kg cimaterol. Animals were fed ad libitum for 16

Table 1 Formula and chemical composition of control diet

	Basal
Formulated level	
Crude protein (%)	18.0
Digestible energy (kcal/kg)	3800.0
Ingredient (%)	
Corn, yellow	56.8
Soybean meal	25.4
Isolated soy protein	2.1
Tallow	11.9
Tricalcium phosphate	0.8
Vitamin-mineral mixture[a]	3.0
Chemical composition (%)	
Moisture	12.80
Crude protein	18.02
Crude fat	13.06
Nitrogen free extract	50.99
Crude fiber	2.76
Crude ash	2.37
Calcium	0.92
Phosphorus	0.41

[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; pantothenate HCl, 5 mg; thiamine HCl, 5 mg; riboflavin, 4 mg; pyridoxine HCl, 8 mg; vitamin B_{12}, 0.06 mg; folic acid 1.2 mg; choline chloride, 1,200 mg; $CaHPO_4$, 22.2 g; NaCl, 1.53 g; K_2SO_4, 6.70 g; MgO, 0.68 g; $FeSO_4 \cdot 7H_2O$, 0.20 g; $CuSO_4 \cdot 5H_2O$, 0.24 g; $MgSO_4 \cdot H_2O$, 0.19 g; KI, 0.005 g; $MnSO_4 \cdot H_2O$, 1.21 g; $ZnCl_2$, 0.02g.

weeks. The rats were housed individually in a barrier facility. The temperature was maintained at about 22° C. A 12-hour light/dark cycle was used. The body weight of each rat was measured weekly. Five rats were sacrificed from each group at each different physiological stage [virgin (postweaning week 3); early and late pregnant (postweaning weeks 8 and 10, respectively); early and late lactating (postweaning weeks 11 and 13, respectively); and weaning (postweaning week 14)]. Mammary tissues were used for chemical analysis (DNA, RNA, protein, and lipid), milk protein mRNA quantification, and acinar culture works at each different physiological stage.

DNA, RNA, protein, and lipid analysis

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

Cytoplasmic mRNA

Rat α-, β-, and γ-casein, and whey acidic protein (WAP) complementary DNAs[14–16] were kind gifts of Dr. J. M. Rosen, Baylor College of Medicine, Houston, Texas, USA. Transformation was carried out as described by Norgard et al.[17] Small quantities of partially purified plasmid DNA were prepared by the mini-preparation boiling method.[18] Large scale preparation of plasmid DNA was performed by CsCl buoyant density centrifugation following amplification in *Escherichia coli* DH5 strain using the method of Norgard et al.[17]

Total cytoplasmic RNA[19,20] was extracted from fresh mammary tissue. After extraction and ethanol precipitation, RNA was quantified and dotted directly onto a nitrocellulose sheet as described before.[21,22] The sheet was baked in vacuo at 80° C for 2 hr, sealed in a plastic bag, and stored at 4° C until hybridization.[21]

Before hybridization, the integrity of the RNA was testified. Specificities of casein and WAP probes used in the experiment were confirmed by Northern analysis.[23] Nick translation and hybridization were essentially as described by Maniatis et al.[23] Hybridization was carried out with [^{32}P]-labeled nick-translated α-, β-, γ-casein or WAP complementary DNA probes that were cloned in PstI digested-pBR322. Dot blots[22,24] were done in 50% (vol/vol) formamide containing 0.75 M sodium chloride, 0.075 M sodium citrate (pH 7), 10% dextran sulfate, 0.02% BSA, 0.02% polyvinylpyrrolidone, and 0.02% ficoll. An autoradiogram was prepared by exposing the dried nitrocellulose sheet to XAR-5 X-ray film (Eastman Kodak Co.) at −70° C for 4 hr. The extent of hybridization was determined by densitometric scanning (Hoefer Scanning Densitometer GS 300, Hoefer Sci. Ins. San Francisco USA) of the autoradiograms.

Mammary acinar culture

Isolation and culturing of mammary acinar cells were done as described with some modifications.[8] The basic incubation medium was Eagle's minimal essential medium (MEM). Glucose and bovine serum were added to 1× MEM to a final concentration of 0.2% (wt/vol) and 5% (vol/vol), respectively. Lactogenic hormones, i.e., insulin (200 μU/mL), hydrocortisone (1.8×10^{-8} M), and prolactin (1 μg/mL) were added to the acinar culture media. The cells were plated on plastic culture dishes (about 10^6 cells/dish). Four dishes were set up for each observation with contents pooled after 18 hr incubation for subsequent analysis. Milk protein secretion in acinar culture was measured by a pulse-chase method as previously described.[8] The [^{3}H] lysine was added at 0.5 μCi/mL of medium. The amino acid uptake was measured according to the procedure of Park et al.[25] with unlabeled and [^{14}C] labeled-cycloleucine as the nonmetabolizable monitor.

Statistical analysis

All data were analyzed by Student's *t* test using the General Linear Model procedure developed by Statistical Analysis System (Cary, NC USA).[26]

Results

Chemical compositions on the basis of dry matter of rat mammary tissue trimmed of skin, supra-mammary lymph glands, and extraneous adipose tissue were measured to examine mammary proliferation and differentiation *(Table 2)*.

The levels of DNA, RNA, protein, the RNA:DNA ratio, and protein:DNA ratio of mammary tissue from the test (cimaterol-supplemented) group were higher than those of the control group. A low lipid content of mammary tissue in rats of test group was also found.

The total cytoplasmic mRNAs for α-, β-, γ-casein, and WAP from mammary tissue were evaluated by dot-hybridization technique *(Table 3)*. The total cytoplasmic RNA was extracted from mammary tissue of virgin (postweaning week 3); early and late pregnant (postweaning

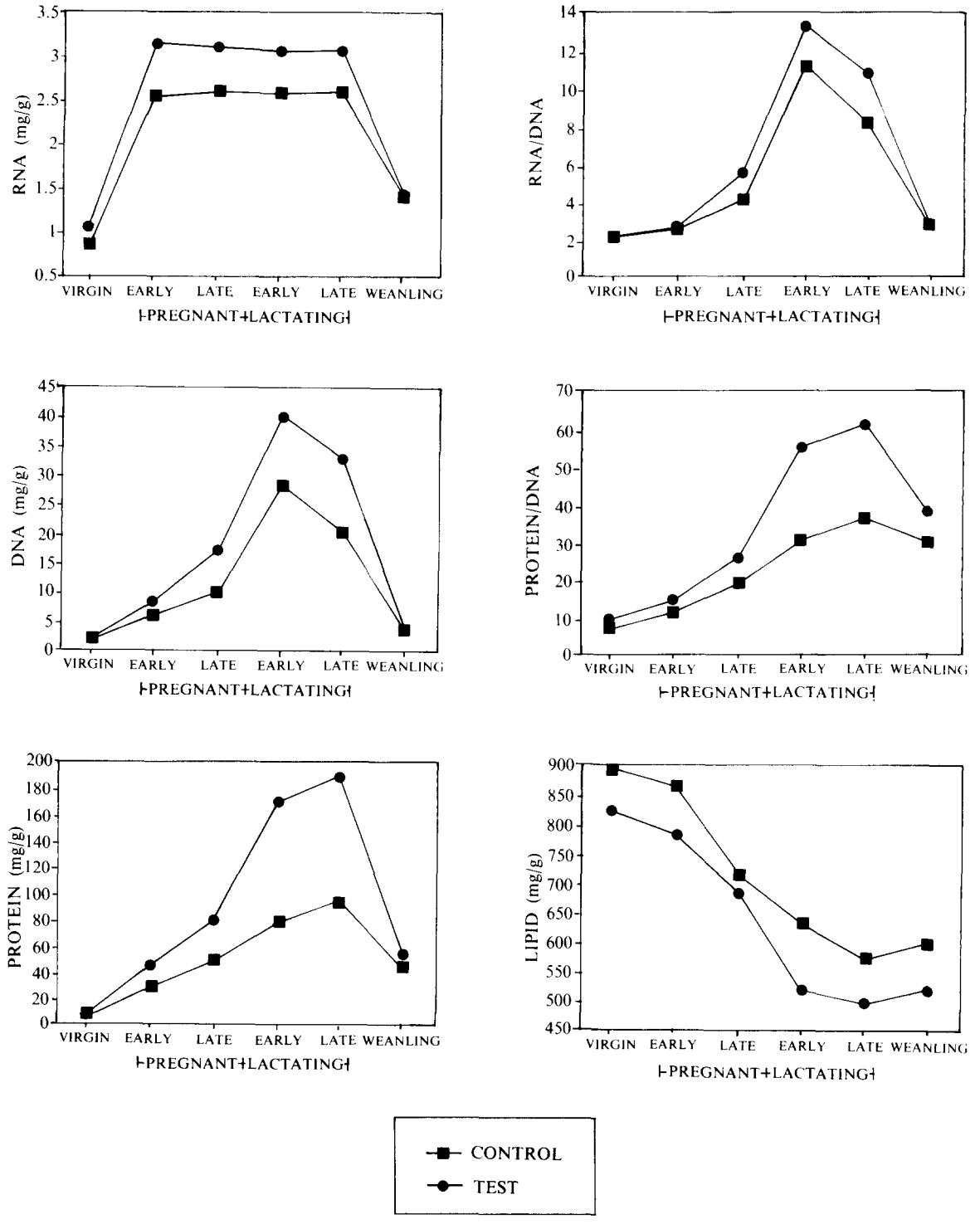

Figure 1 Chemical compositions of rat mammary gland at various physiological stages. Mammary tissues of rats were obtained from both control and test groups. Each value is the mean of five observations. mg/g denotes milligram per dry matter tissue g.

week 8 and week 10, respectively); early and late lactating (postweaning week 11 and week 13, respectively); weaning rats (postweaning week 14). Six μg of denatured RNA was dotted onto a nitrocellulose sheet and hybridized to specific [^{32}P]-labeled α-, β-, γ-casein, or WAP-complementary DNA, which was cloned with PstI digested-pBR322.

The total cytoplasmic milk protein mRNAs from mammary tissues in the test group were higher than those of the control counterpart during pregnant, lactating, and weaning stages. *Table 3* also shows that all three casein and WAP mRNAs of the control and test group were very low but detachable in virgin mammary gland.

The acinar culture studies with rat mammary tissues

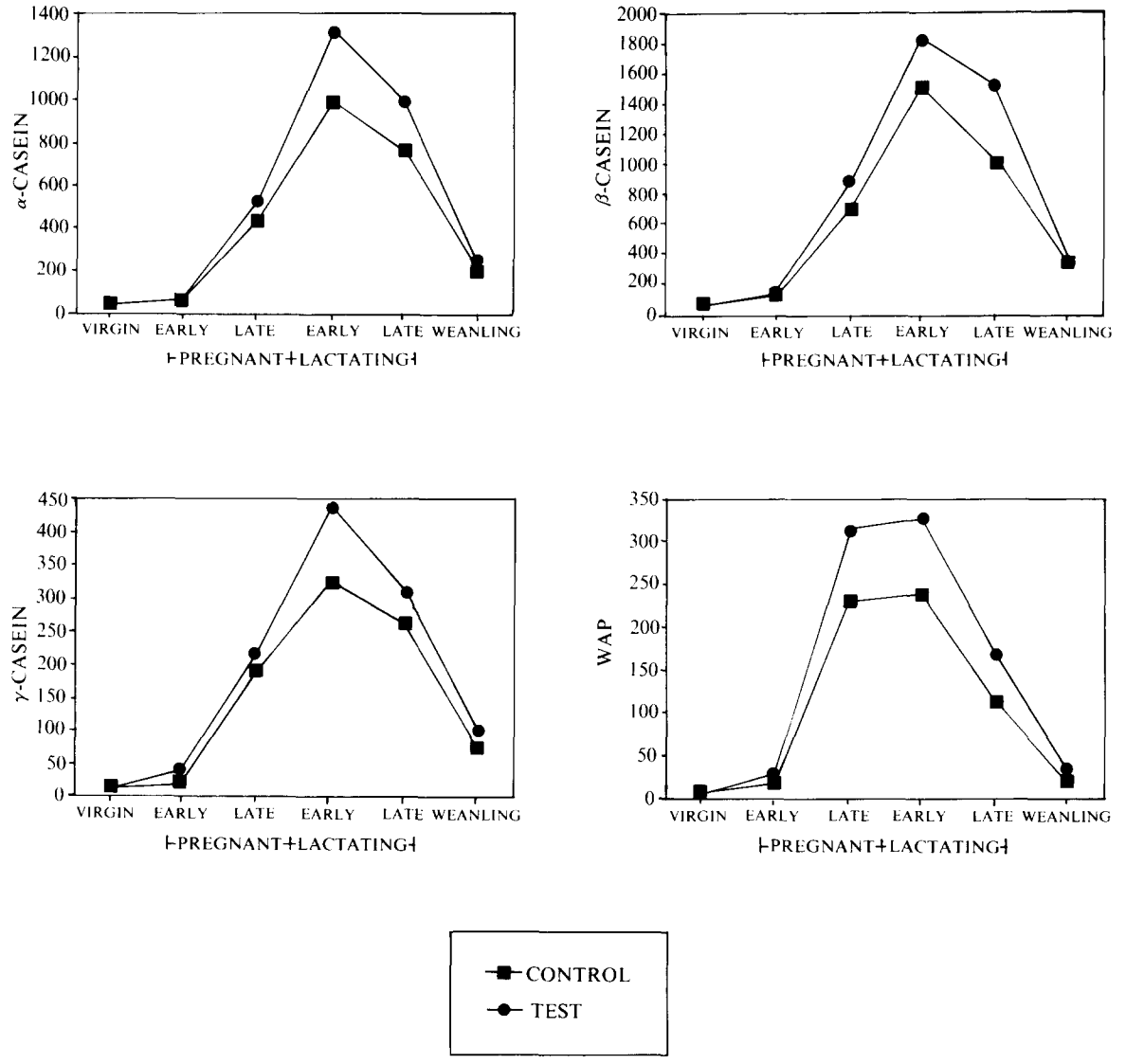

Figure 2 Milk protein cytoplasmic mRNA specific activity of rat mammary gland. Specific activity: Cytoplasmic total RNA was extracted from pooled mammary tissues for each group with guanidine/cesium chloride, dotted directly onto nitrocellulose, and hybridized with nick-translated α-, β-, γ-casein, and WAP cDNA probes. Relative levels of mRNAs were expressed as specific activity, where specific activity is relative intensity/μg RNA. Relative intensity of individual dots on autoradiograms was determined by scanning densitometry.

were conducted to determine if or how cimaterol alters milk protein synthesis and secretion, and amino acid uptake.

Milk protein synthesis and secretion in acini of the cimaterol group was about 12–18% over that of the control group in both late pregnant and early lactating stages *(Table 4)*. The increase in synthesis and secretion parallels the increases in the three casein and WAP mRNAs. Amino acid uptake of rats receiving cimaterol was increased in the present study by about 30% relative to that of the control group.

Discussion

The DNA in the test group mammary tissue was 19% higher than that in the control tissue from virgin to late lactating stages, as shown in *Table 2*. An increase in the DNA content indicates proliferation: it represents an increase in the number of epithelial cells induced to replicate DNA by cimaterol.[8] The increase in RNA in the test group tissue was even more pronounced, amounting to 46% of the control RNA value. The higher level of RNA in the mammary gland from test group ensures a greater primary biological function of the gland.[27] The increase in RNA by cimaterol reflects an increase in the rate of RNA synthesis and/or a decreased rate of RNA degradation in the alveolar cell. During the development from virgin to late lactating stages, RNA increased 12.7- and 16.1-fold, respectively, in both the control and test groups. Although the RNA:DNA ratio, protein:DNA ratio, and protein content increased from virgin to early lactating stages in both groups, the test group showed

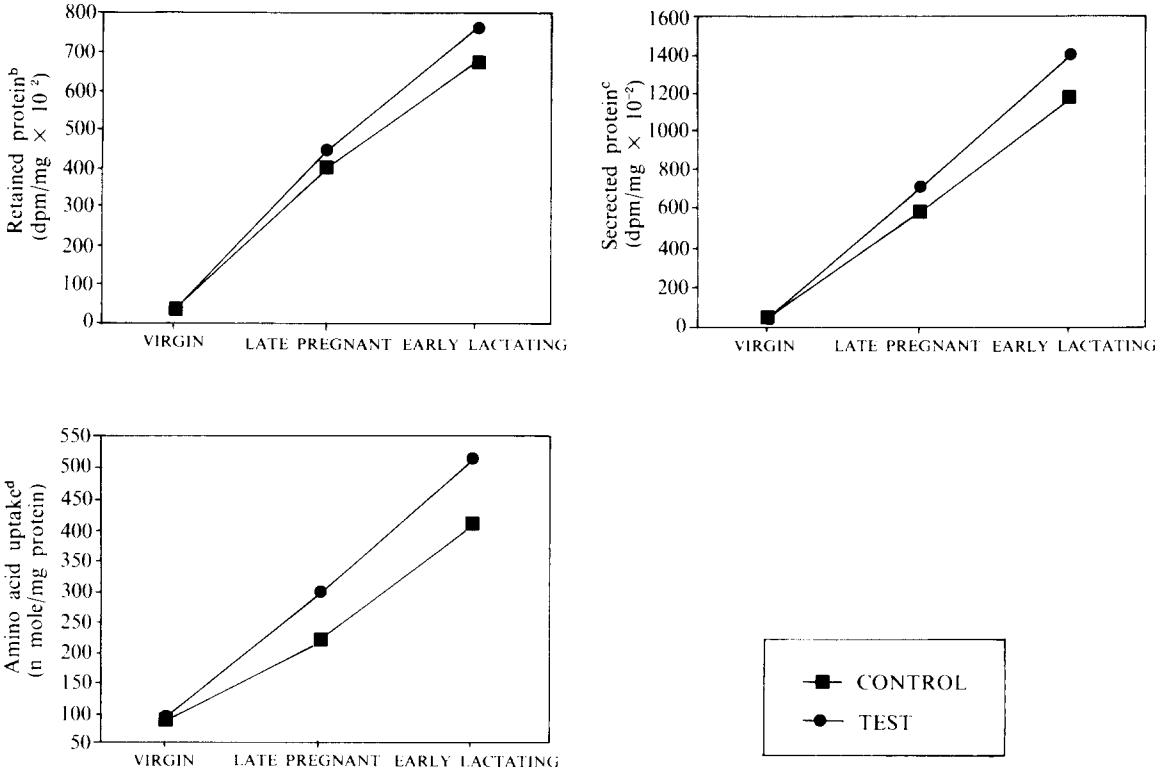

Figure 3 The effect of β-agonist supplementation on the amount of retained and secreted protein, and amino acid uptake in mammary acinar culture. Mammary tissues were collected in sterile BSS with antibiotics at the time of killing. Acinar cells were isolated by collagenase solutions. Acinar (equivalent to 10^6 cell/dish) suspensions were preincubated in basic 1X MEM for 24 hr or 90 min in a 5% CO_2 atmosphere at 37° C. Mammary acini were preincubated with basic 1X MEM for 24 hr at 37° C in a 5% CO_2 atmosphere. The basic MEM was then replaced with pulse medium containing [^{3}H] lysine (0.5 μCi/mL). After 15 min incubation for pulse label, acini were recovered and incubated at 37° C for 90 min in chase medium. The specific activity (dpm/mg protein × 10^2) of secreted protein and retained protein was determined as described previously. Each value is the mean of four observations with five replications. Mammary acini were preincubated in 1X MEM at 37° C for 90 min in a 5% CO_2 atmosphere. The MEM was then replaced with treatment media containing 0.5 mmol/L unlabeled cycloleucine and 0.5 μCi/mL [^{14}C] cycloleucine. After recovery of acini and washing with BSS, specific activities of acini were determined. Uptake of cycloleucine was calculated as: cycloleucine uptake (nmole/mg protein) = acini specific activity (dpm/mg protein)/medium specific activity (dpm/nmole). Each value is the mean of three observations with four replications.

Table 2 Chemical composition of rat mammary tissue at various physiological stages[a]

			Pregnant				Lactating							
	Virgin		Early		Late		Early		Late		Weaning			
Item[b]	Control	Test	Control	Test	Control	Test	Control	Test	Control	Test	Control	Test	SE	Prob.
DNA (mg/g)	0.82	0.92	2.54	3.16	2.60	3.11	2.58	3.07	2.60	3.08	1.43	1.47	0.23	0.023
RNA (mg/g)	1.63	2.03	6.03	8.28	10.32	17.32	28.41	40.36	20.63	32.76	3.48	3.78	1.01	0.012
Protein (mg/g)	5.41	8.30	28.46	44.81	49.36	80.31	78.32	169.81	94.81	189.32	43.26	56.12	4.51	0.037
RNA/DNA	1.99	2.21	2.37	2.62	3.97	5.57	11.01	13.15	7.93	10.64	2.43	2.57	1.13	0.017
Protein/DNA	6.60	9.02	11.20	14.18	18.98	25.82	30.36	55.31	36.47	61.47	30.25	38.18	2.39	0.003
Lipid (mg/g)	893	821	863	782	714	684	632	518	574	497	603	523	13.00	0.027

[a]Mammary tissues of rats were obtained from both control and test groups. Each value is the mean of five observations. Virgin-postweaning week 3; early pregnant-postweaning week 8; late weaning-postweaning week 10; early lactating-postweaning week 11; late lactating-postweaning week 13; weaning-postweaning week 14.
[b]mg/g denotes milligram per g dry matter tissue.

a greater increase in these parameters in early lactating mammary tissues (about 1.34, 1.69, and 2.0 times, respectively) as compared with those of the control group. Based on these results, it may be stated that the dietary supplementation of the β-agonist cimaterol may bring about hyperplasia, hypertrophy, and high functional activity in epithelial cells.

The synthesis of DNA, RNA, and protein is very characteristically regulated during the cell cycle.[28] DNA and histone components of the chromatin are synthesized only during the S phase. In contrast, cytoplasmic protein and RNA are synthesized continuously throughout interphase, i.e., G_1, S, and G_2 phases. However, during mitosis the synthesis of all DNA and RNA is

Table 3 Milk protein cytoplasmic mRNA specific activity of rat mammary tissue (specific activity[a])

Milk protein mRNA	Virgin		Pregnant				Lactating				Weaning		SEM	Prob.
			Early		Late		Early		Late					
	Control	Test	Control	Test	Control	Test	Control	Test	Control	Test	Control	Test		
α-casein	38.3	41.4	66.4	78.3	440.6	531.4	1003.8	1329.1	774.3	1000.1	213.2	241.4	12.3	0.037
β-casein	46.7	45.8	106.4	147.3	698.1	871.8	1508.4	1823.4	1023.4	1523.6	336.4	398.1	23.1	0.009
γ-casein	11.3	12.0	20.6	39.7	190.3	218.6	328.4	441.4	263.2	314.9	75.9	103.1	10.9	0.034
WAP	4.4	5.0	18.6	28.3	230.1	314.6	239.4	327.4	116.4	173.1	23.1	38.4	2.7	0.008

[a]Cytoplasmic total RNA was extracted from pooled mammary tissues from each group with guanidine/cesium chloride, dotted directly into nitrocellulose, and hybridized with nick-translated α-, β- and γ-caseins, and WAP complementary DNA probes. Relative levels of mRNAs were expressed as specific activity, where specific activity is relative intensity/μg RNA. Relative intensity of individual dots on autoradiograms was determined by scanning densitometry.

Table 4 The effect of cimaterol supplementation on the amount of retained and secreted protein and amino acid uptake in mammary acinar culture[a]

Item	Virgin		Late-pregnant		Early-lactating		SEM	Prob.
	Control	Test	Control	Test	Control	Test		
Retained protein[b] (dpm/mg × 10^{-2})	36.2	34.1	394.7	442.0	670.9	756.4	10.4	0.024
Secreted protein[c] (dpm/mg × 10^{-2})	35.8	44.4	597.4	710.4	1196.3	1411.6	19.8	0.001
Amino acid uptake[d] (n mole/mg protein)	84.7	91.3	216.8	298.7	410.3	518.4	18.4	0.002

[a]Mammary tissues were collected in sterile BSS with antibiotics at the time of killing. Acinar cells were isolated by collagenase digestion.
[b,c]Mammary acini (10^6 cells/dish) were preincubated in basic 1X MEM for 24 hr at 37° C in a 5% CO_2 atmosphere. The basic MEM was then replaced with pulse medium containing [^{3}H] lysine (0.5 μCi/mL). After 15 min incubation for pulse label, acini were recovered and incubated at 37° C for 90 min in chase medium. The specific activity (dpm/mg protein × 10^{-2}) of secreted protein and retained protein was determined as described previously. Each value is the mean of four observations with five replications.
[d]Mammary acini were preincubated in 1X MEM at 37° C for 90 min in a 5% CO_2 atmosphere.[33] The MEM was then replaced with treatment media containing 0.5 mmol/L unlabeled cycloleucine and 0.5 μCi/mL [^{14}C] cycloleucine. After recovery of acini and washing with BSS, specific activities of acini were determined. Uptake of cycloleucine was calculated as: cycloleucine uptake (nmole/mg protein) = acini specific activity (dpm/mg protein)/medium specific activity (dpm/nmole). Each value is the mean of four observations with five replications.

turned off; moreover, the rate of protein synthesis is greatly reduced. Cimaterol may influence the cell cycle, i.e., the modulation of endocrine and enzyme systems by cimaterol may change the rate of cell cycling or the rate at which cells pass from one phase to the next phase of the cycle. The effect may explain the differences in the chemical composition of the mammary gland. The present results demonstrate that the extent of hyperplasia and hypertrophy induced by cimaterol is clearly reflected by higher nucleic acid and protein content of the mammary gland *(Table 2)*.

Mammary tissue from animals fed cimaterol contained more parenchyma and less fat than their counterparts. This is in agreement with others.[8] This result explains that cimaterol has a direct role in control of lipolysis and lipogenesis. Bound to the adipocyte, β-receptors activate the adenylate cyclase cascade, which results in phosphorylation (activation) or hormone-sensitive lipase that catalyzes triglyceride hydrolysis. In addition, increased intracellular cAMP concentrations might inhibit fatty acid synthesis by attenuation of key regulatory enzymes.

Natural weaning results in involution of the mammary gland. During involution, a rapid loss of mammary epithelial cells occurs. As epithelial cells are lost, adipocytes regain lipids, as shown in *Table 2*. Thus, the stage changes in lipid concentrations were associated with the growth and differentiation of the mammary gland.

As shown in *Table 3*, the increase in the relative level of casein and WAP mRNA due to cimaterol was about 1.1–1.7 fold over the control value of rat mammary tissue in pregnant, lactating, and weanling stages. It can be postulated that the endogenous factor, cimaterol, may modulate the secretion rate of hormones [e.g., growth hormone[29]] or activity of enzymes [e.g., DNA polymerase[30]] regulating mammary differentiation. The altered hormones or enzymes may enhance the induction of casein and WAP mRNA by way of modulation of transcriptional rate, RNA processing and nucleocytoplasmic transport efficiency, and mRNA turnover rate. These results show that cimaterol can regulate the expression of milk protein genes.

The amounts of α-, β-, and γ-casein, and WAP mRNA were markedly increased from virgin to early lactating stages: 29-, 36-, 33-, and 60-fold increases, respectively. Because there was an increase in the RNA of the mammary gland averaging from 1.83–34.4 mg/g during this time *(Table 3)*, these values represent in-

creases of about 545-, 677-, 620-, and 1128-fold, respectively, in the concentrations of each mRNA when expressed per mass of mammary tissue.

A comparison of the level of each mRNA at different stages of development indicates that the proportions of each of the three casein mRNAs remained relatively constant throughout pregnancy and lactation, i.e., they have similar kinetics. This result implies that the concentrations of the three casein mRNAs appear to be coordinately regulated during functional differentiation.[31] These three caseins are members of a small, multigene family that is present as a gene cluster on a single chromosome.[32] A different kinetics of expression between casein and WAP genes was found to occur during virgin, pregnancy, and lactation. WAP mRNA activity was also differently lost during the late lactating period compared with the casein mRNA levels. This discoordinate regulation of expression of casein and WAP genes is consistent with the independent origin of the WAP gene and its location on different chromosomes from the members of the casein gene family.[33] It is worth noting that there may be coordinate regulation in expression of the three casein genes and discoordinate regulation between casein and WAP gene expression during functional differentiation of rat mammary glands.

As shown in *Table 4*, the dietary supplementation of cimaterol showed an increase in the amount of retained and secreted protein, and amino acid uptake by mammary acinar culture. An increased availability of intracellular amino acid precursors induced by the test group may enhance the synthesis of total milk protein.

We conclude that cimaterol contributed to the regulation of differentiated functions as well as milk protein gene expression in the mammary gland.

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