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Effects of Relaxin in Combination with Prolactin and Ovarian Steroids on Mammary Growth in Hypophysectomized Rats[1] (39935)

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Introduction. Hormonal regulation of mammary gland growth is thought to be predominantly under the control of prolactin, estrogens, and progesterone (1). Much work in delineating qualitative hormonal requirements for mammary growth in hypophysectomized rats or mice was accomplished using the technique of whole-mount observations (2). This method may be adapted to semiquantification at best using some arbitrary numerical scale to assign each gland a grade for the extent of lobule-alveolar development. However, a more acceptable method for quantification of mammary development is chemical DNA determination (3), in spite of the minor complication in measuring stromal as well as parenchymal tissue (4).

Relaxin has only recently been available in sufficient quantities to evaluate its role in mammary development, although earlier studies of a limited nature suggested that the hormone affected the mammary gland (5, 6). More recently, the role of relaxin on mammary development in ovariectomized rats was reported (7). The results suggested that relaxin synergized with estrogen and progesterone in the presence of anterior pituitary hormones to grow the mammary gland. The present study was designed to determine the role of relaxin on mammary growth in the absence of the pituitary and in the presence of purified prolactin.

Materials and methods. Albino female rats were hypophysectomized by and purchased from Charles River Laboratories, Wilmington, Massachusetts, when they were 160-g body weight (age = 60 days). They were maintained in an animal room at 25.6 ± 1° with artificial light from 6 AM to 8 PM. Water and Purina laboratory pellets were provided *ad libitum*. Animals were weighed daily to determine whether or not growth was occurring. Those animals which gained 10 g during 1 week were not included in the experiment. Those included were ovariectomized under ether anesthesia. Ten days later, a regime of daily sc injections of mammotropic hormones was initiated and continued for 19 days. The animals were sacrificed on the 20th day, and the six abdominal-inguinal mammary glands were excised. Immediately following excision, mammary wet weight was obtained and glands were frozen for subsequent determinations of dry fat-free tissue (DFFT) weight, DNA, and RNA. Animals were autopsied and the sella turcica was examined to determine completeness of hypophysectomy.

DFFT was obtained by extracting water and fat with a reflux apparatus, using ethanol for 48 hr and ethyl ether for 48 hr. Using 25-mg aliquots of DFFT, DNA was measured by the *p*-nitro-phenylhydrazine method of Webb and Levy (8), while RNA was determined by the orcinol method of Albaum and Umbreit (9).

The hormonal regime injected for 19 days in 10 rats per group was as follows: Group 1—control, 0.2 ml of sesame oil; Group 2—porcine relaxin (NIH-R-PL), 90 GPU/day in 0.2 ml of sesame oil; Group 3—prolactin (ovine, NIH-P-5-11), 25 IU (1 mg)/day in 0.2 ml of sesame oil; Group 4—prolactin plus relaxin; Group 5—prolactin plus estradiol benzoate (EB), 1 μg/day in 0.2 ml of sesame oil; Group 6—prolactin plus relaxin plus EB; Group 7—prolactin plus progesterone (P), 3 mg/day in 0.2 ml of sesame oil; Group 8—prolactin plus re-

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laxin plus P; Group 9—prolactin plus P plus EB; Group 10—prolactin plus relaxin plus P plus EB.

Statistical treatment of the data involved analysis of variance followed by Duncan's new multiple range test (10) at the 5% level of probability to determine treatment differences.

Results. In response to hormonal treatments, body wight changes of hypophysectomized rats ranged from a maximum increase of 15 g to a loss of as much as 9.8 g (Table I). This change represents the difference between body weights determined on Days 1 and 20 of treatment. In all cases, the rats which received prolactin, relaxin, or progesterone, alone or in combinations, gained body weight, but not significantly differently from controls. Rats receiving estradiol benzoate, regardless of the presence of other hormones, lost from 4.6 to 9.8 g during the injection period. These differences were not significantly different from each other, but were significantly less than all other groups not receiving estradiol.

Mammary wet weights ranged from a high of 5.12 g in the group receiving prolactin, progesterone, and relaxin to a low of 3.04 g in the group injected with prolactin, estradiol, and progesterone. Table I, column 2 shows the effect of treatment upon mammary wet weight expressed as a percentage of final body weight. This parameter ranged from 2.1% in four of the treatment groups to 3.0% in the group on prolactin, progesterone, and relaxin. Despite wide fluctuations in body weight changes, only the group receiving prolactin, progesterone, and relaxin had a significantly different wet weight percentage from controls.

After water and fat were removed from mammary tissue, DFFT weights ranged from 0.20 g in controls to 0.29 g in rats receiving prolactin, progesterone, and relaxin. Only the latter group had a significantly increased DFFT content when compared to all other groups. When DFFT weight was related to wet weight of mammary tissue (Table I), the results showed that %DFFT varied from a low of 5.1% in rats receiving relaxin alone to 6.9% in rats treated with prolactin, estradiol, and relaxin. All groups receiving prolactin, except the groups on prolactin plus progesterone and prolactin plus progesterone plus relaxin, had a significantly higher %DFFT than controls.

DNA was measured as grams of DNA per milligram of DFFT, as total DNA in the six mammary glands, or as DNA per unit of body weight (Table II). Concentration of DNA per milligram of DFFT ranged from a low of 23.3 μg in the group receiving prolactin, estradiol, and progesterone to a high of 36.5 μg in the group receiving prolactin plus relaxin. Only the former group was significantly different from the control group. Total DNA in the mammary

TABLE I. EFFECT OF RELAXIN, PROLACTIN, ESTROGEN, AND PROGESTERONE ON BODY WEIGHT AND MAMMARY GLAND WEIGHT IN HYPOPHYSECTOMIZED RATS (MEAN ± SE).[a]

Treatment	Body weight change (g)	Percentage mammary wet weight	Percentage mammary dried fat-free tissue
Control	$10.1 \pm 2.2^{l,m}$	2.2 ± 0.1^{k}	$5.3 \pm 0.3^{i,j}$
R[b]	15.0 ± 4.5^{l}	2.5 ± 0.2^{k}	5.1 ± 0.1^{j}
PRL[c]	$12.5 \pm 1.5^{l,m}$	2.1 ± 0.1^{k}	$6.1 \pm 0.3^{g,h}$
PRL + R	6.3 ± 2.3^{m}	2.1 ± 0.1^{k}	$6.3 \pm 0.2^{f,g,h}$
PRL + EB[d]	-7.4 ± 1.7^{n}	2.2 ± 0.2^{k}	$6.8 \pm 0.1^{f,g}$
PRL + EB +R	-5.6 ± 1.5^{n}	2.2 ± 0.1^{k}	6.9 ± 0.2^{f}
PRL + P[e]	7.9 ± 1.2^{m}	2.1 ± 0.1^{k}	$5.7 \pm 0.2^{h,i,j}$
PRL + P + R	$8.4 \pm 2.0^{l,m}$	3.0 ± 0.2	$5.9 \pm 0.2^{h,i}$
PRL + EB + P	-9.8 ± 1.3^{n}	2.1 ± 0.1^{k}	$6.7 \pm 0.3^{f,g}$
PRL + EB + P + R	-4.6 ± 2.1^{n}	2.3 ± 0.1^{k}	$6.5 \pm 0.3^{f,g,h}$

[a] Column values without common superscripts are different ($\alpha = 0.05$).
[b] R = relaxin, 90 GPU/day.
[c] PRL = prolactin, 1 mg/day.
[d] EB = estradiol benzoate, 1 μg/day.
[e] P = progesterone, 3 mg/day.

gland ranged from a low of 4.63 mg in those animals receiving prolactin plus estrogen plus progesterone to highs of 8.44 mg in the group of prolactin plus relaxin and 8.46 mg in the group receiving prolactin plus progesterone plus relaxin. These two groups and the group receiving prolactin, estradiol, and relaxin were significantly different from controls. When DNA was expressed on a per unit body weight basis, a fourth group was significantly higher than controls: the group receiving prolactin plus estradiol.

Mammary RNA varied in concentration from a low of 14.3 μg/mg of DFFT in the group on prolactin, estradiol, and progesterone to 17.9 μg in the control group (Table III). All treatment groups were statistically similar to controls, except the prolactin plus relaxin, prolactin plus progesterone, and prolactin plus estradiol plus progesterone groups. Total RNA in the mammary gland varied from a low of 2.87 mg in the group on prolactin, estradiol, and progesterone to a high of 5.17 mg in the group on prolactin, progesterone, and relaxin. Only rats receiving prolactin, progesterone, and relaxin were significantly different from controls. A similar result was found when RNA was expressed on a per unit body weight basis.

TABLE II. EFFECT OF RELAXIN, PROLACTIN, ESTROGEN, AND PROGESTERONE ON MAMMARY GLAND DNA CONTENT OF HYPOPHYSECTOMIZED RATS (MEAN ± SE).[a]

Treatment	DNA		
	μg/mg	Total mg	mg/100 g body wt
Control	$31.0 \pm 2.0^{n,o,p,q}$	$6.11 \pm 0.46^{l,m}$	$3.54 \pm 0.15^{h,i,j}$
R[b]	$33.2 \pm 1.8^{n,o,p}$	$7.31 \pm 0.65^{k,l}$	$4.15 \pm 0.31^{g,h,i}$
PRL[c]	$34.5 \pm 2.4^{n,o,p}$	$7.34 \pm 0.50^{k,l}$	$4.31 \pm 0.31^{f,g,h,i}$
PRL + R	36.5 ± 1.7^{n}	8.44 ± 0.53^{k}	$4.91 \pm 0.29^{f,g}$
PRL + EB[d]	$32.4 \pm 2.1^{n,o,p}$	$7.22 \pm 0.55^{k,l}$	$4.75 \pm 0.40^{f,g}$
PRL + EB + R	$35.3 \pm 1.4^{n,o}$	8.03 ± 0.45^{k}	5.22 ± 0.23^{f}
PRL + P[e]	$25.6 \pm 1.7^{q,r}$	5.30 ± 0.35^{m}	$3.37 \pm 0.35^{i,j}$
PRL + P + R	$28.7 \pm 1.1^{p,q,r}$	8.46 ± 0.78^{k}	$4.96 \pm 0.35^{f,g}$
PRL + EB + P	23.3 ± 1.2^{r}	4.63 ± 0.28^{m}	3.16 ± 0.18^{j}
PRL + EB + P + R	$30.0 \pm 2.1^{o,p,q,r}$	$6.89 \pm 0.48^{k,l}$	$4.37 \pm 0.31^{f,g,h}$

[a] Column values without common superscripts are different ($\alpha = 0.05$).
[b] R = relaxin, 90 GPU/day.
[c] PRL = prolactin, 1 mg/day.
[d] EB = estradiol benzoate, 1 μg/day.
[e] P = progesterone, 3 mg/day.

TABLE III. EFFECT OF RELAXIN, PROLACTIN, ESTROGEN, AND PROGESTERONE ON MAMMARY GLAND RNA CONTENT OF HYPOPHYSECTOMIZED RATS (MEAN ± SE).[a]

Treatment	RNA			
	μg/mg	Total mg	mg/100 g body wt	RNA/DNA
Control	17.9 ± 0.58^{m}	$3.56 \pm 0.25^{k,l}$	$2.07 \pm 0.15^{i,j}$	$0.60 \pm 0.03^{f,g}$
R[b]	$17.3 \pm 0.41^{m,n}$	3.85 ± 0.35^{k}	$2.18 \pm 0.16^{i,j}$	$0.54 \pm 0.03^{f,g,h}$
PRL[c]	$17.0 \pm 0.45^{m,n,o}$	$3.62 \pm 0.12^{k,l}$	$2.12 \pm 0.07^{i,j}$	$0.51 \pm 0.02^{g,h}$
PRL + R	$15.6 \pm 0.55^{n,o,p}$	$3.57 \pm 0.09^{k,l}$	$2.08 \pm 0.05^{i,j}$	0.44 ± 0.03^{h}
PRL + EB[d]	$16.6 \pm 0.81^{m,n,o}$	3.78 ± 0.37^{k}	2.47 ± 0.24^{i}	$0.53 \pm 0.04^{f,g,h}$
PRL + EB + R	$16.2 \pm 0.72^{m,n,o}$	$3.71 \pm 0.26^{k,l}$	2.41 ± 0.15^{i}	0.46 ± 0.02^{h}
PRL + P[e]	$15.3 \pm 0.20^{o,p}$	3.09 ± 0.21^{k}	1.85 ± 0.13^{j}	$0.57 \pm 0.05^{f,g}$
PRL + P + R	17.5 ± 0.71^{m}	5.17 ± 0.42	3.04 ± 0.03	$0.62 \pm 0.02^{f,g}$
PRL + EB + P	14.3 ± 0.47^{p}	2.87 ± 0.14^{l}	$1.96 \pm 0.09^{i,j}$	0.63 ± 0.03^{f}
PRL + EB + P + R	$16.7 \pm 0.69^{m,n,o}$	3.90 ± 0.33^{k}	2.51 ± 0.24^{i}	$0.58 \pm 0.04^{f,g}$

[a] Column values without common superscripts are different ($\alpha = 0.05$).
[b] R = relaxin, 90 GPU/day.
[c] PRL = prolactin, 1 mg/day.
[d] EB = estradiol benzoate, 1 μg/day.
[e] P = progesterone, 3 mg/day.

The ratio of RNA to DNA, a measure of cellular synthetic activity, varied from a low of 0.44 in the group receiving prolactin plus relaxin to a high of 0.63 in the group on prolactin plus estradiol plus progesterone. Groups on prolactin plus relaxin and prolactin plus estradiol plus relaxin were significantly lower than controls, while all other groups were similar to controls.

Discussion. Relaxin has been shown in this and previous studies to have a stimulatory action on mammary gland development. In a previous study, relaxin was shown to stimulate mammary gland DNA in ovariectomized rats (7). In the present study, using hypophysectomized-ovariectomized rats, relaxin treatment alone resulted in a nonsignificant increase in mammary DNA per 100 g body weight. In combination with prolactin, however, relaxin treatment resulted in increased DNA which was statistically higher than control values. Relaxin alone increased DNA per 100 g body weight 17% above control values. Prolactin alone increased this parameter 22% above controls, while the combination of the two hormones increased DNA 39%. These results indicate that relaxin affects mammary gland DNA in conjunction with prolactin.

Prolactin plus estrogen treatment resulted in a significant 34% increase in DNA levels expressed as milligrams per 100 g body weight. When given in combination with prolactin and estrogen, relaxin increased DNA an additional 13% above control levels. This hormone combination resulted in the highest observed DNA value of all treatment groups. Statistically, the prolactin plus estrogen group was not higher than the relaxin only group, but the prolactin plus estrogen plus relaxin group was higher. Here, again, relaxin appeared to be increasing mammary growth in a synergistic manner.

In combination with prolactin and progesterone or prolactin, estrogen, and progesterone, relaxin treatment resulted in 45 and 44% elevations, respectively, in mammary DNA levels above the corresponding nonrelaxin treatment. Although only the prolactin plus progesterone plus relaxin group was significantly higher than controls, both groups receiving relaxin were significantly higher than their nonrelaxin counterpart. Relaxin, in some manner, enabled these treatment animals to respond to steroid stimulation. However, the present study does not allow speculation as to the specific mechanism.

Mammary gland RNA levels were not altered as extensively as DNA levels. For example, only the group receiving prolactin, progesterone, and relaxin had significantly increased total mammary RNA compared with that of controls. The lack of responsiveness as well as the low absolute levels of RNA may be attributed to the fact that only one of the numberous pituitary hormones was replaced. Hypophysectomy, therefore, serves as a tool in this study to emphasize the complexity of pituitary hormone interactions to affect optimal mammogenesis. The observation that prolactin plus progesterone plus relaxin significantly increases mammary RNA per 100 g body weight above control levels is not consistent with results from castrated females (7) and may well be a manifestation of the incompleteness of replacement therapy.

In terms of the RNA to DNA ratio, the results demonstrate that the only two groups significantly different from control values are the prolactin plus relaxin and prolactin plus estrogen plus relaxin groups. These values are lower than those of controls and therefore reemphasize the observation that, in hypophysectomized-ovariectomized rats, relaxin acts synergistically to stimulate mammary gland DNA to a greater extent than RNA.

Summary. Hypophysectomized-ovariectomized albino rats weighing 160 g were injected daily for 19 days with 90 GPU of relaxin and/or other mammotropic hormones, including prolactin (25 IU), estradiol benzoate (1 μg), and progesterone (3 mg). Prolactin alone or in combination did not increase body weight. Mammary wet weight was increased significantly by prolactin, progesterone, and relaxin, but not by other combinations. Relaxin was without effect on mammary wet weight, but synergized with prolactin and progesterone to increase mammary dry fat-free tissue. It also synergized with prolactin or prolactin and progesterone to stimulate DNA and

RNA of the mammary tissue, but did not increase the ratio of RNA to DNA. It is concluded that relaxin has limited mammotropic action by itself, but synergizes effectively with anterior pituitary and ovarian steroid mammotropins to stimulate mammary development.

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