Physiologic Role of Relaxin on Mammary Gland Growth in Rats[1] (42999)

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Abstract. **The role of relaxin in stimulating growth of the mammary gland was assessed in ovariectomized and intact male rats for a period of 20 days. In addition to relaxin alone, the ovarian mammogenic hormones estradiol and progesterone were used in combination with relaxin and with each other to evaluate responses of mammae. Indices for mammary growth included wet weight, dry fat-free tissue, DNA, RNA, total protein, and collagen. Quantitative estimates of DNA and collagen represented the best indicators of parenchymal and stromal growth, respectively. Because changes in body weights were significantly different among hormonally administered groups, these were included as well. In Ovariectomized young rats, relaxin alone and in combination with estradiol and progesterone increased all indices significantly ($P < 0.01$). The collagenous portion of total protein was high for the group receiving relaxin alone (62%) compared with the control group (46%). Relaxin administered along with estradiol and progesterone increased collagen accumulation to 73%, compared with 54% in the estradiol + progesterone group. Relaxin did not significantly increase growth indices when administered to male rats at 10 and 20 μg/day, while 30 μg stimulated a significant increase in total protein ($P < 0.05$), suggesting that 30 μg of relaxin/day may be considered the basal concentration needed to induce a physiologic response in males. Relaxin induced a growth effect on mammae by synergizing with progesterone and estradiol in order to stimulate parenchymal proliferation, as noted by a DNA increase, and to increase stromal distensibility of the mammary pad by invoking accumulation of collagen and total protein in substituting for mammary adipose tissue.** [P.S.E.B.M. 1989, Vol 192]

Mammary gland development involves changes in growth rate, morphology, and chemical composition of both parenchyma and stroma. Growth of the mammary apparatus during fetal life and before puberty is considered to occur at an isometric rate or parallel with the whole body (1). Under the influence of mammogenic hormones, mammary growth during puberty and pregnancy accelerates and is characterized as an allometric rate (2, 3). Hormones of the mammogenic complex include the ovarian steroids estrogen and progesterone and the adenohypophysial proteins prolactin and somatotropin. Accelerated secretion rates of these hormones contribute primarily to the pronounced growth of the mammary glands during pregnancy. Since ovariectomy abolishes the allometric growth of mammary glands at puberty (4), hormone replacement studies have identified estrogen as the primary hormone responsible for growth associated with estrous cycles (4, 5).

A hormone present in low levels during estrous cycles and in high levels just prior to parturition has been known to modify structures of the birth canal in preparation for parturition (6). Since this hormone, known as relaxin, is present at periods of rapid mammary growth, it has been implicated as an additional hormone in the mammogenic complex. Relaxin has been shown to stimulate mammary growth to a limited degree in mice (7, 8) and rats (9). Observations of mammary whole-mounts in rats showed that relaxin increased duct length (10, 11). It was postulated that relaxin acted by remodeling connective tissue during duct and lobule-alveolar development. To assess more accurately the role relaxin plays in stimulating mammary growth, this study was undertaken using intact and ovariectomized (OVX) rats. Criteria to measure growth included mammary wet weight, dry fat-free tissue (DFFT), DNA, RNA, protein, and collagen (2, 4).

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Materials and Methods

Animals. Sixty-four Sprague-Dawley OVX rats were obtained from Charles River Breeding Laboratories (Wilmington, MA). Twenty male rats (Fisher-144 strain) were used. Animals were housed individually in wire cages with feed (Purina Laboratory Chow 5008; Ralston-Purina Co., St. Louis, MO) and water provided *ad libitum*. Animal quarters were air conditioned (25 ± 1°C) with a light-dark cycle of 12 hr, lighting being from 7 AM to 7 PM.

Experimental Design. Eight groups of young OVX rats (n = 8) were treated as follows: Group 1, control (C); Group 2, progesterone; Group 3, estradiol; Group 4, progesterone plus estradiol; Group 5, relaxin plus progesterone; Group 6, relaxin plus estradiol; Group 7, relaxin plus estradiol plus progesterone; and Group 8, relaxin. In addition, four groups of male rats (n = 5) were assigned to three different doses of procine relaxin injection at 10, 20, and 30 μg, respectively, plus a control group.

Hormone Injections. Progesterone and estradiol benzoate were purchased from Sigma Chemical Co. (St. Louis, MO). Purified porcine relaxin was furnished by Dr. O. D. Sherwood (University of Illinois at Urbana). The estradiol and progesterone were dissolved in ethanol and sunflower oil was used as the carrier, while relaxin was in benzopurpurine vehicle. The control group received 0.2 ml of sunflower oil/day subcutaneously for 20 days and an additional 0.2 ml of benzopurpurine vehicle 2% (w/v) from Days 11 to 20. All hormones except relaxin were injected daily by subcutaneous route in a total volume of 0.2 ml of oil. Relaxin was injected alone or in conjunction with estradiol or progesterone from Days 11 to 20 in a 2% solution (w/v) of benzopurpurine in distilled water in female rats and for 20 consecutive days in male rats. Doses of hormones used on a daily basis were 5 mg of progesterone, 1 μg of estradiol, and 16.7 μg (50 guinea pig units [GPU]) of relaxin in OVX females and 10, 20, or 30 μg of relaxin in male rats. On the 21st day, the rats were sacrificed by cervical dislocation and abdominal-inguinal mammary glands were excised. The glands were stored at −20°C until extracted of fat and water as described previously (12). DNA and RNA were determined according to Webb and Levy (13) and Albaum and Umbreit (14), respectively. Collagen was determined by measuring hydroxyproline as described by Woessner (15), and protein concentration was quantified according to the Lowry method (16). All chemical analyses were determined colorimetrically using 25 mg of DFFT/sample. Rats were weighed daily throughout the experimental period, and the trend of body weight was recorded.

Statistical evaluation of the data included analysis of variance and Duncan's multiple range test. These were computerized using software by SAS (17). Daily body weight gains were regressed with injection time and the best fit curve was depicted.

Results

Changes in body weight along the treatment period of 20 days in OVX female rats are represented by the regression curves (Figs. 1 and 2). In OVX rats, a rectilinear growth in body weight was observed in control

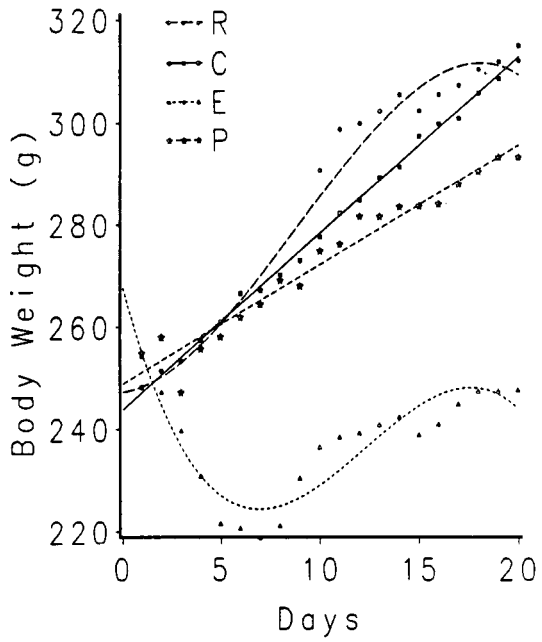

Figure 1. Linear and cubic regression curves represent body weight gain (g) for control (C) and progesterone (P) and estradiol (E) and relaxin (R), respectively.

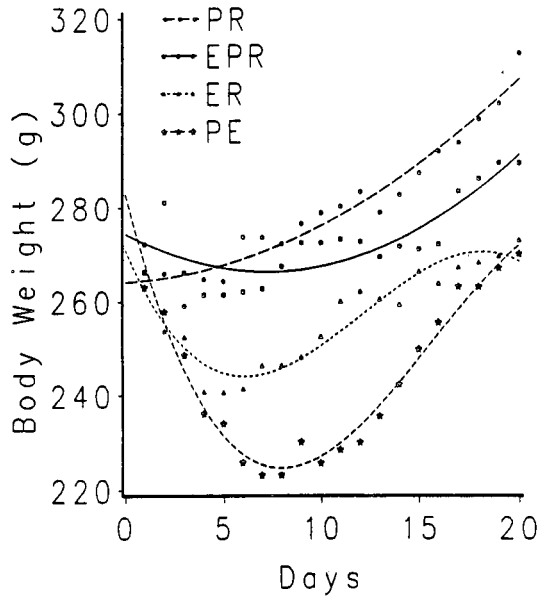

Figure 2. Quadratic and cubic regression curves represent body weight gain (g) for progesterone (P) + relaxin (R) and estradiol + progesterone + relaxin and estradiol + relaxin and progesterone + estradiol, respectively.

and progesterone groups. Groups receiving progesterone plus relaxin and progesterone plus estradiol plus relaxin showed a quadratic trend in body weight over the injection period. Cubic regression curves in body weight changes were demonstrated in the estradiol group and the relaxin group as well as the estradiol plus progesterone and the estradiol plus relaxin groups. Body weights of male rats averaged 375 g at the beginning and the end of the experiment with no treatment differences observed.

In OVX female rats, mammary gland growth indices tended to increase upon addition of the mammogenic hormones progesterone, estradiol, and relaxin (Tables I and II). Wet weights increased slightly in animals treated with estradiol or progesterone. There were further increases with combinations of two hormones. Relaxin alone produced the heaviest mammary glands. However, the imprecise nature of wet weight measurements resulted in no significant differences.

DFFT measurements were found to be more valuable than wet weight in evaluating mammary growth. Relaxin stimulated an increase in DFFT above controls and progesterone alone or estradiol alone. Progesterone alone had a level at 349.8 mg and relaxin in combination increased the DFFT to 428.0 mg. Similarly, estradiol alone resulted in DFFT at 364.0 mg, while adding relaxin enhanced an attainment to 458.1 mg. In addition, relaxin alone resulted in a percentage of DFFT to wet weight at 9.5% and the combination of estradiol + progesterone + relaxin at 9.8% (Table I).

Total DNA of the mammary gland increased in response to relaxin alone from 7.5 mg in controls to 16.7 mg. This was equal to the group given progesterone + estradiol at 16.5 mg and was surpassed only by progesterone + estradiol + relaxin at 18.3 mg. Although RNA was stimulated to 12.5 mg in the progesterone + estradiol group, compared with 2.9 mg in the control group, it reached only 6.4 mg with relaxin alone. However, the addition of relaxin to progesterone + estradiol raised RNA to a high of 23.5 mg.

Relaxin alone enhanced the accumulation of total protein from a control level of 138.6 to 493.1 mg. This was higher than any other hormonally treated group (Table II). Collagen increased significantly when the

Table I. Changes in Mammary Gland Growth Indices (Wet Weight, DFFT, DNA, and RNA) following Administration of Estradiol, Progesterone, or Relaxin and Their Combinations in Ovariectomized Rats

Treatment	Wet weight (g)	DFFT (mg)	DFFT ÷ wet weight (%)	Total DNA (mg)	Total RNA (mg)
Control	4.5 ± 0.2	262.5 ± 0.9a	5.8	7.5 ± 0.3a	2.9 ± 0.2a
Progesterone	4.8 ± 0.3	349.8 ± 0.9b, c	7.4	10.8 ± 0.6a, b	4.3 ± 0.2a, b
Estradiol	4.7 ± 0.5	364.0 ± 2.9b, c	8.0	12.4 ± 1.0a, b, c	4.9 ± 0.4a, b
Progesterone + estradiol	5.4 ± 0.6	487.9 ± 5.6c, d	9.3	16.5 ± 0.7b, c	12.5 ± 2.4c, d
Progesterone + relaxin	5.2 ± 0.5	428.0 ± 5.9c, d	8.5	14.9 ± 2.2b, c	6.9 ± 0.6b, c
Estradiol + relaxin	5.5 ± 0.5	458.1 ± 5.0c, d	8.6	13.4 ± 2.4b, c	8.7 ± 1.3c
Progesterone + estradiol + relaxin	5.7 ± 0.4	557.1 ± 5.3d	9.8	18.3 ± 2.3c	23.5 ± 0.3d
Relaxin	6.3 ± 0.5	563.7 ± 6.1d	9.5	16.7 ± 2.7b, c	6.4 ± 0.2b

Note. Mean ± SE. Means with the same letter are not different; $P < 0.001$ for DFFT, total RNA and $P < 0.01$ for total DNA, respectively, by analysis of variance and Duncan's multiple range test.

Table II. Effects of Estradiol, Progesterone, and Relaxin and Their Combinations on Protein and Collagen Accumulation in OVX Female Rats

Treatment	Total protein (mg)	Protein ÷ DFFT (%)	Total collagen (mg)	Noncollagen protein (mg)	Collagen ÷ protein (%)	Noncollagen ÷ protein (%)
Control	138.6 ± 1.2a	52.8	63.9 ± 4.0a	74.7 ± 2.3a	46	54
Progesterone	192.0 ± 3.3a	54.9	89.6 ± 3.8a	102.4 ± 3.5a	46	54
Estradiol	316.5 ± 4.1b	86.9	93.1 ± 2.3a	223.4 ± 2.9b	29	71
Progesterone + estradiol	386.0 ± 3.9b	79.2	210.0 ± 8.1b	176.0 ± 4.7b	54	46
Progesterone + relaxin	394.1 ± 4.6b	92.1	174.3 ± 7.6b	219.8 ± 5.2b	44	56
Estradiol + relaxin	317.2 ± 2.5b	69.2	176.4 ± 5.3b	140.8 ± 2.6a, b	57	43
Progesterone + estradiol + relaxin	451.0 ± 4.8c	80.9	331.1 ± 9.6c	119.9 ± 6.3a	73	27
Relaxin	493.1 ± 5.4c	87.5	310.1 ± 12.7c	183.0 ± 9.4b	62	38

Note. Mean ± SE. Means with the same letter are not different; $P < 0.001$ for total protein, total collagen and $P < 0.01$ for noncollagen protein, respectively, by analysis of variance and Duncan's multiple range test.

Table III. Mammary Growth Indices of Four Groups of Male Rats ($n = 5$) Subjected for 20 Days to Three Different Doses of Porcine Relaxin (0, 10, 20, and 30 μg daily)

Treatment[a]	Wet weight (g)	DFFT (mg)	Total DNA (mg)	Total RNA (mg)	Total protein (mg)	Protein ÷ DFFT (%)	Total collagen (mg)	Collagen ÷ protein (%)	Noncollagen protein (mg)
Control	12.1 ± 2.1	666.4 ± 12.4	14.6 ± 2.2	3.5 ± 0.9	398.3 ± 13.2[b]	59.8	205.1 ± 6.5	51	193.2 ± 5.6
10 μg	10.8 ± 1.5	648.3 ± 9.9	15.7 ± 1.9	3.2 ± 1.0	364.2 ± 9.8[b]	56.2	210.4 ± 5.9	58	149.2 ± 2.8
20 μg	11.4 ± 3.0	688.8 ± 11.1	16.8 ± 2.1	5.2 ± 1.6	463.7 ± 12.4[c]	67.3	234.0 ± 4.2	50	229.7 ± 8.2
30 μg	11.2 ± 2.2	677.1 ± 8.9	22.1 ± 4.2	8.6 ± 1.7	461.7 ± 14.3[c]	68.2	284.3 ± 3.3	62	177.4 ± 6.4

Note. Mean ± SE.
[a] 10 μg of relaxin = 30 GPU; 20 μg of relaxin = 60 GPU; 30 μg of relaxin = 90 GPU.
[b,c] Significantly different at $P < 0.05$.

combination of progesterone + estradiol was used, in contrast to progesterone or estradiol alone. The injection of relaxin alone raised the amount of collagen to a higher level. This was rivaled only by the combination of progesterone + estradiol + relaxin. The ratio of collagen to noncollagen protein was not changed from controls by progesterone or by progesterone + relaxin. However, it was reduced by estradiol alone and increased by relaxin alone (Table II).

Male rats treated with relaxin responded by showing gradual increases in mammary DNA, RNA, and collagen as the dose of relaxin was raised from 10 to 20 to 30 μg/day (Table III). The percentage of collagen to total protein was highest at 62% in the group receiving 30 μg of relaxin.

Discussion

Inconsistency in average daily body weight gain in OVX rats assigned to different treatments implicated the role of a single hormone administered alone or in combination with others on food intake. Dissimilarities among the treated groups and the control (Figs. 1 and 2) reveal the role of each hormone and its impact on hunger and satiety. Relaxin, the hormone of interest in this study, showed a growth enhancing effect. It stimulated higher body weight than control and progesterone groups. Estradiol had a detrimental effect on body weight gain, a characteristic noted in the literature (18, 19) that estradiol affects the appetite sensor. Two hormones that were able to reverse the effect of estradiol on body weight gain were progesterone and relaxin, with relaxin causing a higher magnitude of change than progesterone. Therefore, the important finding to emphasize was that relaxin had an anabolic action as it related to body weight. Relaxin stimulated the mammary apparatus in a positive way as well, as evidenced by the accumulation of DFFT, DNA, protein, and collagen.

Wada and Turner (20, 21) demonstrated the ability of relaxin to synergize with estrogen in stimulating lobule alveolar proliferation in mice. Relaxin was shown by Harness and Anderson (8) to synergize with prolactin to increase wet weight and with somatotropin (9) to increase DFFT in hypophysectomized rats. Wright and Anderson (11) morphologically illucidated the effect of relaxin on mammary duct proliferation in immature hypophysectomized female rats. They concluded that relaxin caused elongation of mammary milk ducts. In addition to those findings, this study showed that relaxin had a substantial effect on increasing wet weight along with DFFT and its percentage.

Measuring DNA as a growth index has its limitation because it does not allow quantification of various cell types within the mammary gland. Therefore, any change in the DNA index cannot be attributed to any compartment of the mammary apparatus. However, measurements of total protein and its collagenous portion showed that relaxin tended to alter collagen, the predominating compartment of the mammary stroma. This was not necessarily by increasing the total size of the mammary gland (as measured by wet weight index), but it took place through a remodeling mechanism. Degradation of the mammary fat pad and enhancement of collagen allowed further elongation of the ducts as noted by Wright and Anderson (11). The dual effect of relaxin was to stimulate connective tissue remodeling and to encourage duct epithelial cell proliferation.

Because relaxin was administered exogenously *in vivo* in this experiment, it was difficult to attribute a direct effect to relaxin as a sole source of hormonal regulation. Relaxin has been shown to stimulate prolactin secretion from the hypophysis in rats (22) and monkeys (23) and cortisol secretion in mice (24). Both could play an effective role in promoting mammary gland growth. Therefore, relaxin could be needed to produce a local effect on the mammary gland and at the same time to trigger the release of other mammogenic hormones.

In using light and electron microscopy as tools to study the effect of relaxin on mammary duct epithelial cells, Bani and Bigazzi (7) found that relaxin exerted a mitotic effect by stimulating branching of the ducts. This finding was consistent with the increase in DNA index in this study. This same group reported in 1986

(25) that relaxin alone was not able to alter the mammary fat pad, suggesting that its effect was only on epithelia and stroma other than adipose tissue.

Relaxin may serve a dual purpose in controlling the limits to which mammary parenchyma may proliferate. First, it regulates the remodeling processes of connective tissue as represented by collagen. Second, it exerts an action on epithelial cells to stimulate them to proliferate.

Relaxin is a potent growth promoter in the mammary gland and has actions on both parenchymal and stromal compartments. Relaxin also exerts positive and additive effects on progesterone and estradiol to accomplish the sudden acceleration of mammary gland growth prior to parturition.

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