

Collagen Studies in Late Pregnant Relaxin Null Mice¹

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ABSTRACT

The relaxin knockout (rlx $-/-$) mouse was used to assess the effect, during pregnancy, of relaxin with regard to water, collagen content, growth, and morphology of the nipple (N), vagina (V), uterus, cervix (C), pubic symphysis (PS), and mammary gland (MG). The results presented here indicate that during pregnancy, relaxin increases the growth of the N, C, V, and PS. Large increases in water content in the PS (20%) occurred in pregnant (Day 18.5) wild-type (rlx $+/+$) mice but not in rlx $-/-$ animals. This indicates that in the PS, relaxin might increase the concentration of a water-retaining extracellular matrix component (hyaluronate). In the pregnant rlx $+/+$ mouse, collagen content decreased significantly in the N and V but not in other tissues. There were no significant changes in the rlx $-/-$ mouse. This contrasts with findings in the rat, in which relaxin has been found to cause decreases in collagen concentrations in the V, C, and PS. Histological analysis showed that the collagen stain was more condensed in the tissues (V, C, PS, N, and MG) of rlx $-/-$ mice than in those of rlx $+/+$ mice. This phenomenon indicates that the failure of collagen degradation and lack of growth in the N underlie the inability of the rlx $-/-$ mice to feed their young, as reported previously. Vaginal and cervical luminal epithelia, which proliferated markedly in the rlx $+/+$ pregnant mice, remained relatively atrophic in the rlx $-/-$ mice. As proliferation and differentiation of uterine and vaginal epithelia are thought to be induced by a paracrine stromal factor that acts upon estrogen stimulation, our results indicate that relaxin may be this paracrine factor.

cervix, female reproductive tract, mammary glands, pregnancy, relaxin, uterus, vagina

INTRODUCTION

Relaxin (rlx) is a two-chain peptide classified as a member of the insulin family of peptide hormones. It is predominantly expressed in the corpora lutea of the ovaries of rats, mice, and pigs during pregnancy [1–4]. In the rat and the pig, rlx has been shown to facilitate the safe delivery of the young by remodeling the connective tissue of the cervix, vagina, and pubic symphysis (PS) at term [5, 6]. In the mouse, rlx is produced and secreted by the corpora lutea through the second half of the 19 days of pregnancy [7]. In our laboratory, previous studies using rlx null mutant

(rlx $-/-$) mice demonstrated that relaxin is required for mammary gland and nipple development during pregnancy [8]. Relaxin null mutant mice were fertile, and their gestation length and litter sizes appeared to be normal. The majority of rlx $-/-$ females delivered normally, although their PSs did not soften and elongate. The rlx $-/-$ mothers failed to suckle their young because they had extremely small and hard nipples to which the pups could not attach and suckle following parturition. Two days after delivery, histological examination of the nipples revealed that the connective tissue of the rlx $-/-$ mice was tighter and that the collagen stain was more condensed compared with that of the rlx $+/+$ animals. These observations indicated that there is an rlx-dependent collagen breakdown in the target tissues during normal pregnancy in the mouse.

Relaxin has been reported to induce the growth and softening of the cervix and vagina during the second half of pregnancy in rats [9–12]. When pregnant rats were treated with a monoclonal antibody to rlx (5 mg i.v. daily) from Day 12 through Day 22 of pregnancy, the vagina wet and dry weights, length, diameter, inner surface area, and DNA content decreased significantly compared with PBS-treated control rats [12]. Furthermore, the cervix and vagina showed morphological changes [6, 13], with increased collagen fiber density and fewer layers of epithelial cells.

In vitro studies on cultured human fibroblasts indicate that rlx decreases collagen synthesis and increases collagen degradation [14]. In the rat, even though the PS does not elongate greatly during pregnancy, when rlx levels are at their highest, the total collagen of the PS does decrease without changing the proportion of collagen types [15]. These data indicate that in the rat, rlx is involved in collagen degradation in its target tissues. Direct injection of rlx into the rat nipple was reported to increase nipple growth [16]. The lack of mammary nipple growth and the failure of the PS relaxation observed in rlx $-/-$ mice [8] indicate that rlx in mice may have a role similar to that observed the rat [15, 17].

In both ovariectomized rats and pigs, rlx has been shown to increase the growth of the uterus [18, 19]. During pregnancy, rlx was reported to increase the growth of the uterus and vagina in the pig [20]. Our previous studies [8] showed that rlx was involved in collagen reorganization within the nipple, and a study on rats showed that rlx affected collagen content in the vagina [6]. Therefore, we hypothesize that at term, rlx may affect collagen degradation in other rlx-targeting tissues in the mice. In order to investigate the role of rlx with regard to growth and connective tissue remodeling in the mouse during pregnancy, we measured (at term) the wet weight, dry weight, and total collagen content in the mammary gland, nipple, vagina, cervix, uterus, and PS. We also examined the histology of these tissues.

MATERIALS AND METHODS

All animal experiments were approved by the Howard Florey Institute's Animal Experimental Ethics Committee,

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which adheres to the Australian Code of Practice and the Rules of the National Health and Medical Research Council for the care and use of animals for scientific purposes.

Animals

All 45 female mice used in these studies had the same breeding background (129sv \times C57BLK6J) and were age matched. They were maintained in a disease-free, temperature-controlled environment with a 12L:12D lighting schedule and free access to laboratory chow (Barastock, Pakenham, Victoria, Australia) and water. The genotype of each generated animal was identified by polymerase chain reaction, as described previously [8]. All experiments were carried out in four different groups. Twelve virgin wild-type mice were used in the control group (CTL). Eleven *rlx* $+/+$, 10 *rlx* \pm , and 12 *rlx* $-/-$ mice were used (respectively) in each pregnant (Day 18.5) group. The female mice of the pregnant groups were mated, and pregnancy was timed from midnight of the night preceding the demonstration of a vaginal plug. At Day 18.5 of pregnancy, animals were killed.

Tissue Collection and Histological Analysis

All the animals were killed with an overdose of penthrane (methoxyflurane, Medical Developments, Springvale, Australia). The right abdominal mammary nipples and mammary glands were removed, and the PS was dissected out cleanly. The uterus, cervix, and vagina of each animal were subsequently removed. For the collagen assay, all tissues were briefly rinsed in isotonic saline, blotted, and weighed (wet weight) and were then placed in liquid nitrogen and stored at -80°C until use. For morphology, all tissues from two to three animals in each group were fixed overnight in 4% paraformaldehyde (Pharmacia, Uppsala, Sweden). The tissues were then processed, embedded in paraffin, and sectioned at $5\ \mu\text{m}$. Staining was accomplished with hematoxylin and eosin (H&E) or with the Masson trichrome procedure, as previously described [8].

Determination of Total Collagen Content

The wet weights of each PS, cervix, uterus, vagina, mammary gland, and nipple sampled from control, pregnant *rlx* $+/+$, *rlx* \pm , and *rlx* $-/-$ groups were measured. The samples were lyophilized to enable measurement of their dry weight and water content. The tissues were rehydrated in 50 mM Tris-HCl and 0.15 M NaCl buffer containing proteinase inhibitors (10 mM *N*-ethylmaleimide, 0.1 mM phenylmethylsulphonyl fluoride, 1 mM benzamidine hydrochloride, and 10 mM EDTA) for 24 h at 4°C . Afterwards, samples were defatted in a chloroform:methanol (2:1) solution for 24 h before being rehydrated again for 60 h at 4°C . The samples were frozen in liquid nitrogen and diced in a mortar with a pestle. The diced tissues were hydrolyzed in 6 M HCl at 110°C for 24 h for total collagen content analysis, as described previously [15]. A scaled-down version of the procedure detailed by Bergman and Loxley [21] was used to measure the hydroxyproline content of each sample before the hydroxyproline values were converted to collagen content by multiplying by a factor of 6.94 [22, 23].

Determination of Soluble and Insoluble Collagen

Collagen was extracted from diced tissues using the method described by Bateman et al. [24]; all procedures

were carried out at 4°C . The samples were first extracted with 50 mM Tris-HCl buffer (pH 7.5) containing 0.15 M NaCl and proteinase inhibitors (as described above) for 24 h to extract the newly synthesized collagen. The samples were centrifuged (at $13\,000 \times g$ for 45 min), and the supernatants (neutral salt-soluble fraction) were frozen at -80°C ; the pellets were extracted with 0.5 M acetic acid for 24 h to extract the newly cross-linked collagen. Samples were spun as described above, and their supernatants were frozen at -80°C (acetic acid-soluble fraction). The pellets were then freeze-dried, weighed, and subjected to limited pepsin digestion (enzyme:substrate ratio: 1:10) twice for 24 h to extract the maturely cross-linked collagen (pepsin-soluble fraction). Aliquots of the neutral salt and acetic acid extracts were pepsin-digested to yield the mature collagen. The amount of soluble collagen present in the serially extracted fractions was measured by hydroxyproline assay, as described above. The types of soluble collagen were determined by SDS-PAGE.

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis

Soluble and pepsin-digested collagen chains were analyzed on 5% (w/v) acrylamide gels containing 3.5% (w/v) acrylamide stacking gels. The collagen chains were dissolved in a loading buffer containing 0.05 M Tris-HCl (pH 6.8), 2 M urea, 20% (w/v) sucrose, 0.1% (w/v) SDS, and 0.1% (w/v) bromophenol blue. Interrupted electrophoresis with delayed reduction of the disulfide bonds of type III collagen was used to separate the $\alpha 1(\text{I})$ chains from the $\alpha 1(\text{III})$ collagen chains using a type I and III collagen standard from human dermis [25].

The gels were stained overnight with 0.1% (w/v) Coomassie brilliant blue R-250 and were destained with 30% (v/v) methanol containing 7% acetic acid; samples were then photographed and dried.

Statistical Analysis

Results were analyzed using the Student's *t*-test; a *P* value of less than 0.05 was considered significant. All data are presented as mean \pm SEM.

RESULTS

Wet Weight, Dry Weight, and Water Content of the Tissues

We found that the wet and dry weights (Table 1) of the nipple, vagina, cervix, and PS in wild-type pregnant mice were much higher ($P < 0.01$) than their respective counterparts derived from *rlx* $-/-$ and control (nonpregnant) groups. The wet and dry weights of the nipples of *rlx* $-/-$ pregnant mice had lower values than those associated with the nonpregnant controls, and this discrepancy needs to be further investigated.

The wet and dry weights (Table 1) of the uterus were higher ($P < 0.01$) in all pregnant animal groups when compared with those of the control mice, but there was no difference in uterine weight between different genotype pregnant animals.

In the case of the mammary gland (Table 1), both wet and dry weights did not increase significantly in all pregnant mice compared with similar measures in the control group, and there was no difference between *rlx* $+/+$ and *rlx* $-/-$ pregnant mice.

Table 1 shows that in the PS, the water content of preg-

TABLE 1. Changes in weight and water content of tissues derived from control mice and mice that were rlx +/+, rlx +/-, and rlx -/- and that were 18.5 days pregnant.^a

Tissue	Control	rlx +/+	rlx +/-	rlx -/-
Wet weight (mg)				
Pubic symphysis	0.83 ± 0.05 (12)	3.11 ± 0.3 ^{c,e} (11)	2.34 ± 0.23 ^{c,e} (9)	0.91 ± 0.12 (12)
Cervix	22.13 ± 3.16 (12)	39.45 ± 1.94 ^{c,e} (11)	36.26 ± 2.26 ^{c,e} (10)	27.43 ± 1.91 (12)
Uterus	131.8 ± 15.8 (12)	645.7 ± 55.4 ^c (11)	736.0 ± 56.8 ^c (10)	689.5 ± 56.0 ^c (12)
Vagina	38.62 ± 3.55 (11)	71.19 ± 5.73 ^{c,e} (11)	48.32 ± 3.56 ^e (10)	30.28 ± 2.23 (12)
Mammary gland	704.0 ± 104.8 (12)	809.8 ± 57.6 (11)	631.1 ± 30.3 (10)	660.7 ± 53.4 (12)
Nipple	0.33 ± 0.03 (6)	1.32 ± 0.14 ^{c,e} (11)	1.0 ± 0.09 ^{c,e} (10)	0.29 ± 0.02 ^c (12)
Dry weight (mg)				
Pubic symphysis	0.31 ± 0.02 (12)	0.45 ± 0.04 ^{c,d} (10)	0.32 ± 0.03 (9)	0.28 ± 0.05 (9)
Cervix	4.46 ± 0.64 (12)	7.42 ± 0.39 ^{c,e} (10)	6.91 ± 0.44 ^{c,d} (10)	5.52 ± 0.31 (10)
Uterus	21.57 ± 1.81 (12)	103.7 ± 9.4 ^c (10)	125.1 ± 9.1 ^c (10)	104.9 ± 8.1 ^c (10)
Vagina	7.28 ± 0.77 (11)	14.22 ± 1.26 ^{c,e} (10)	9.65 ± 0.55 ^{b,e} (10)	5.92 ± 0.45 (10)
Mammary gland	165.2 ± 26.8 (5)	183.4 ± 23.1 (5)	175.6 ± 14.3 (5)	196.5 ± 22.3 (5)
Nipple	0.1 ± 0.01 (5)	0.32 ± 0.04 ^{c,e} (10)	0.23 ± .03 ^{b,e} (10)	0.08 ± 0.01 ^c (9)
Water content (%)				
Pubic symphysis	62.1 ± 2.6 (12)	84.8 ± 1.0 ^{c,e} (10)	85.6 ± 1.3 ^{c,e} (9)	66.1 ± 2.5 (9)
Cervix	79.8 ± 0.4 (12)	80.9 ± 0.2 ^b (10)	80.9 ± 0.3 ^b (10)	80.1 ± 0.5 (10)
Uterus	82.8 ± 0.8 (12)	83.5 ± 0.4 (10)	82.9 ± 0.4 (10)	83.8 ± 0.4 (10)
Vagina	81.3 ± 0.4 (12)	80.1 ± 0.3 ^b (10)	79.8 ± 0.5 ^b (10)	80.0 ± 0.4 ^b (10)
Mammary gland	79.8 ± 4.0 (5)	77.6 ± 2.3 (5)	70.4 ± 2.5 (5)	71.3 ± 2.9 (5)
Nipple	65.8 ± 6.9 (5)	76.0 ± 0.6 ^c (10)	76.1 ± 0.8 ^c (10)	75.2 ± 3.4 (10)

^a All values are means ± SEM. Animal numbers are shown in parentheses.

^b $P < 0.05$.

^c $P < 0.01$ compared with control group values.

^d $P < 0.05$.

^e $P < 0.01$ compared with rlx -/- group values.

nant rlx +/+ and rlx ± animals was much greater ($P < 0.01$) than that associated with the rlx -/- and the virgin control mice. The water content of the nipple in the rlx +/+ and rlx ± mice was also higher than that of the control mice, but the water content of the nipple was similar to that observed in rlx -/- animals. In the cervix, uterus, and vagina, the water content values for each group were very similar. In the mammary gland, there was no statistically significant difference between groups.

Collagen Content

The collagen content was standardized using total collagen content divided by the dry weight of the tissue to obtain the concentration of collagen in the tissue (%). We then compared the percentage of collagen in different groups. In the nipple and vagina, the collagen content (%) values in the rlx +/+ ($n = 5$) and rlx ± ($n = 5$) groups were similar and lower ($P < 0.05$), respectively, than the collagen content values of the control ($n = 4$) and rlx -/- ($n = 5$) groups (Fig. 1, A and B). In the PS (Fig. 1C), the collagen content values were 37%, 41%, and 49% in the pregnant rlx +/+, rlx ±, and rlx -/- mice, respectively, and 39% in the virgin control mice. However, there was no statistical significance between groups. In the cervix (Fig. 1D), all the groups had similar values (~20%). The total collagen content values of the uterus (Fig. 1E) were 6%, 4%, and 6% in rlx +/+, ±, and -/- pregnant mice (respectively), compared to 8% in the control mice. The collagen content was about 2% in mammary glands (Fig. 1F) of all experimental groups.

The Collagen Types in Different Tissues

Because of the small size of the collected tissues from these mice, the amount of soluble (neutral salt and acetic acid fractions) collagen determined from each sample (by hydroxyproline analysis) was very low. Hence, we could not achieve consistent meaningful data to compare the sol-

uble collagen content in different tissues between groups. However, the pepsin-digested collagen types of these tissues were clearly demonstrated by the PAGE. Figure 2 shows the presence of type I and type V collagen in all samples of different tissues from different animal groups. Type I collagen was represented by the $\alpha 1$ (I) and $\alpha 2$ (I) chains, whereas type V collagen was represented by the $\alpha 1$ (V) and $\alpha 2$ (V) chains. Little or no type III collagen [$\alpha 1$ (III)] was detected. The covalently cross-linked α -chain dimers are $\beta 11$ and $\beta 12$.

Histological Analysis

Examination of vaginal (Fig. 3, A–D) and cervical (Fig. 3, E and F) sections from 18.5-day pregnant animals stained using the Masson trichrome technique showed a much denser stain of collagen fibers in rlx -/- tissues than in rlx +/+ tissues. The luminal lining of stratified squamous epithelium in both cervix and vagina was much thicker in the rlx +/+ mice than in their rlx -/- counterparts. Sections through the PS of 18.5-day pregnant mice also showed denser collagen staining in the rlx -/- animals (Fig. 3G) compared with the rlx +/+ mice (Fig. 3H). The arrangement of the collagen fibers in the wild-type mice had a much looser pattern compared to that observed in the knock-out mice. Sections through the body of the uterus of rlx +/+ pregnant animals were not consistently different from those with an rlx -/- genotype (data not shown); the result is difficult to interpret and needs to be further studied. In the mammary gland, there was a stronger collagen stain along the mammary duct and in between the alveoli in the knock-out mice than in their wild-type counterparts (data not shown).

DISCUSSION

Growth and Collagen Content

The results presented in this study indicate that the pregnancy-induced size increase in nipple and PS that was ob-

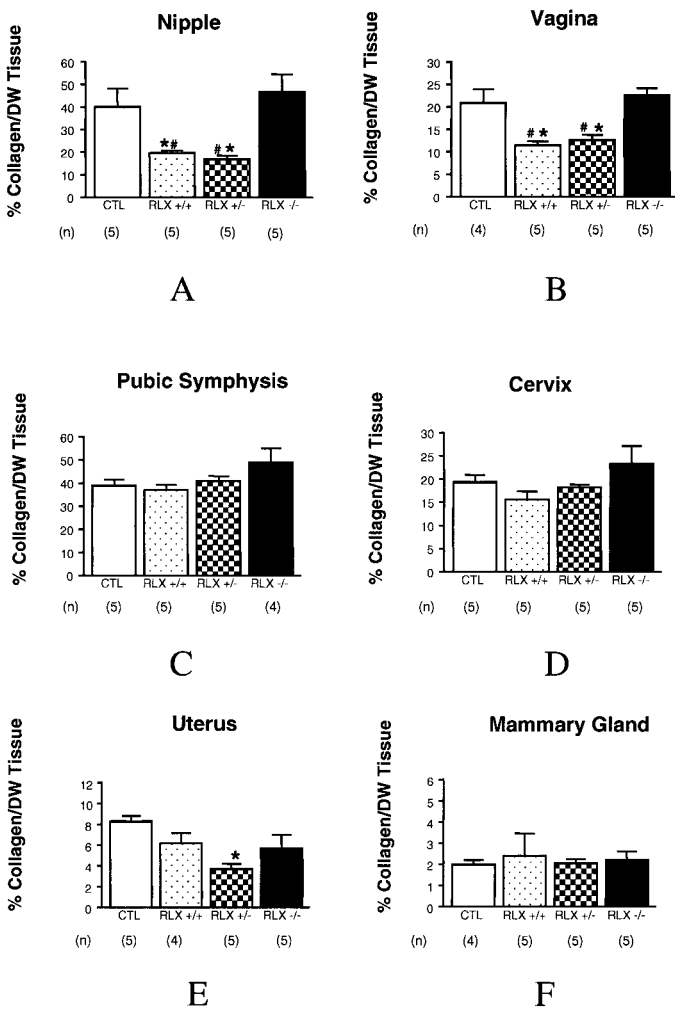


FIG. 1. Comparison of total collagen content/dry weight (DW) tissue (%) between control (CTL), 18.5-day pregnant wild-type (rlx +/+), heterozygous (rlx ±), and knock-out (rlx -/-) mice. n = numbers of animals used in each group. Error bars represent SEM. *A difference of $P < 0.05$, when compared with CTL values. # A difference of $P < 0.05$, when compared with rlx -/- mice. The collagen content (%) of different tissues are shown in A) nipple; B) vagina; C) pubic symphysis; D) cervix; E) uterus; and F) mammary gland.

served in the wild-type mouse [8] is due to an rlx-stimulated increase in the wet and dry weights in these tissues. The growth (weight increase) of the vagina and cervix, observed in rlx +/+ but not in rlx -/- mice, which is similar to that observed in the monoclonal antibody-treated pregnant rats [9, 10, 12], is rlx dependent.

It is well established that administration of porcine rlx to estrogen-primed nonpregnant pigs and rats promotes uterine growth [5]. In contrast, when monoclonal antibody was used to neutralize the endogenous rlx in pregnant rats, uterine growth was not affected [9]. The results presented here indicate that during pregnancy, the increase in the uterine weight (both wet and dry weights) in the mouse is not due to the effect of rlx, as there is no weight difference between the wild-type and rlx-deficient mice at the end of pregnancy.

The growth of the mammary gland (in terms of the wet and dry weight changes) did not differ significantly between pregnant and nonpregnant mice, and there was no difference between rlx +/+ and rlx -/- animals. The results presented previously showed that the mammary gland

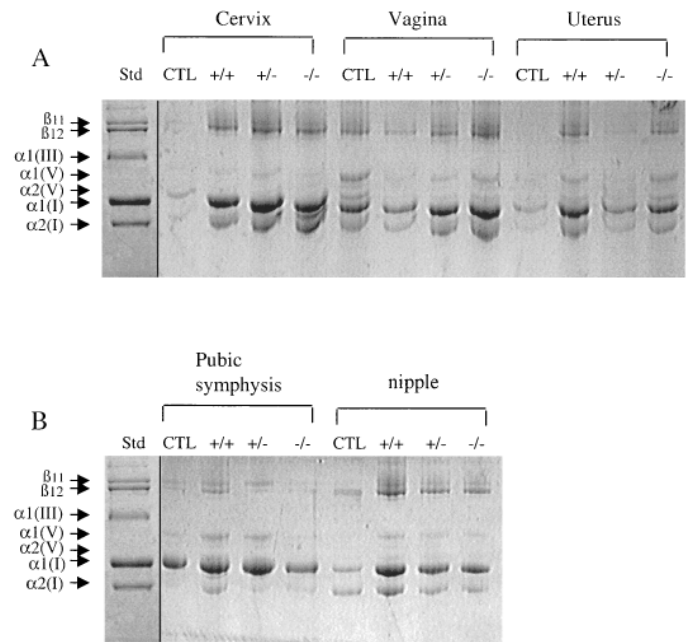


FIG. 2. The collagen types in the nipple, uterus, cervix, vagina, and pubic symphysis. A) Samples from cervix, vagina, and uterus. B) Samples from pubic symphysis and nipple. Std, Collagen standard; CTL, virgin control; +/+, 18.5-day pregnant rlx +/+; ±, 18.5-day pregnant rlx ±; -/-, 18.5-day pregnant rlx -/-.

of the rlx +/+ mice was better developed compared with that of the rlx -/- animals at Day 18.5 of pregnancy. This discrepancy indicates that the effect of rlx on the mammary gland is not necessarily reflected in the weight changes during pregnancy.

In pregnant wild-type mice, the collagen content was lower in the vagina and nipple when compared to similar measures in the wild-type virgin controls. In contrast, there was no collagen content decrease in these tissues in rlx knock-out mice. This indicates that rlx is involved in collagen degradation in the nipple and vagina during pregnancy. The collagen content in the PS, cervix, and mammary gland did not show significant differences between rlx +/+ and rlx -/- pregnant mice. However, histological analysis of these tissues showed that the organization of the collagen fibers in the pregnant knock-out and virgin control mice was more compact when compared to that of the wild-type mice. Furthermore, no collagen type difference was observed in all the tissues when comparing nonpregnant and pregnant mice or rlx +/+ and rlx -/- mice. This indicates that during pregnancy, the collagen changes (either biochemical or histological) induced by rlx are due to collagen degradation or to the reorganization of the collagen fibers rather than to changes in collagen types of the target tissues. It is well known that collagen breakdown, which is induced by matrix metalloproteinases (MMPs), is necessary for normal and pathological tissue growth or expansion [26, 27]. It is possible that the rlx-dependent collagen degradation observed in these tissues (e.g., the nipple) is a prerequisite for adequate nipple growth and for the establishment of successful lactation. Thus, rlx's action may be mediated through the activation of MMPs. It is also possible that the method used in this study is not sensitive enough to detect any minor changes in these tissues during pregnancy.

Water Content—PS

It was suggested long ago that rlx-containing tissue extracts could increase the water content of the rodent uterus

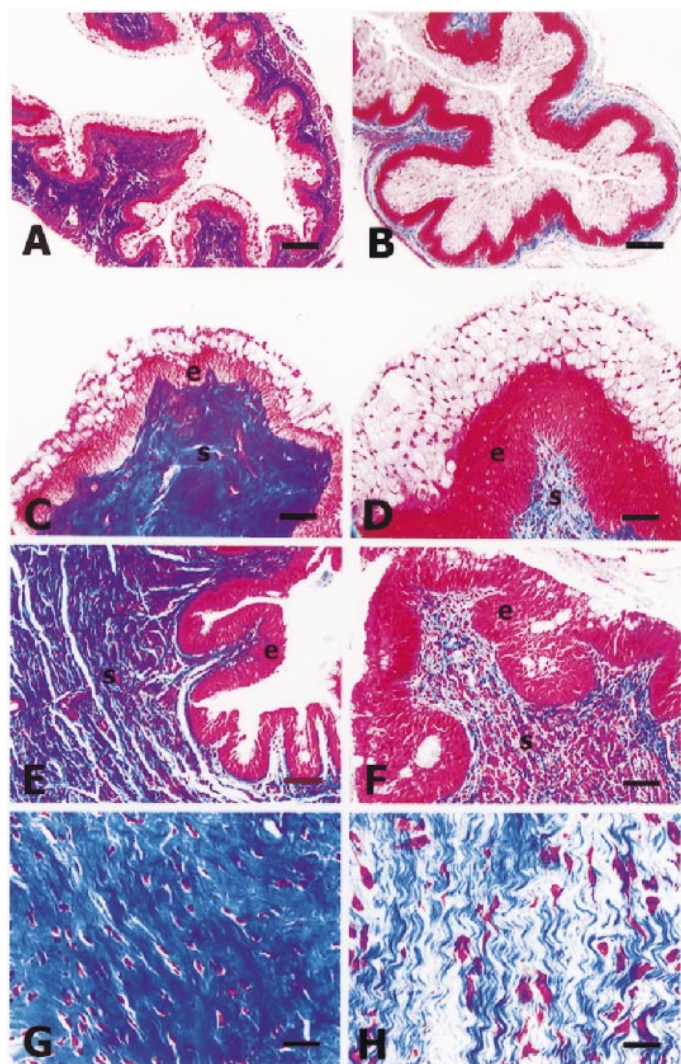


FIG. 3. Sections of 18.5-day pregnant mouse vagina (A–D), cervix (E, F), and pubic symphysis (G, H) stained using the Masson trichrome technique. At low power (A, B), the vaginal wall is seen to be less folded in *rlx* +/+ animals (B) than in their *rlx* -/- counterparts (A). Bar = 200 μ m. At higher power (C, D), the *rlx* -/- mice (C) exhibit denser collagen staining in the vaginal stroma and a thinner overlying epithelium when compared with their *rlx* +/+ (D) counterparts. Bar = 50 μ m. In the cervix, the cervical epithelium is thinner, and cervical stroma collagen stain is denser in the *rlx* -/- (E) mice than in the *rlx* +/+ mice (F). Bar = 50 μ m. The collagen fiber is less organized, and the collagen stain is stronger in *rlx* -/- mice (G) compared with their *rlx* +/+ counterparts (H). Bar = 25 μ m. e, epithelium; s, stroma.

and cervix [28, 29]. Synthetic human *rlx* was also able to increase rat PS water content by 7.3%, which was comparable to the 8% increase seen in pregnant rats [15, 30]. However, in the current study in mice, a much larger increase (>20%) was observed in the water content of the PS of the *rlx* +/+ mouse during pregnancy, but a similar increase was not observed in the *rlx* -/- mouse. The only other tissue to show a substantial change in water content (~10%) was the nipple, but this change was not attributable to the action of *rlx*, as the increase occurred in the pregnant *rlx* -/- animals as well as in the wild-type pregnant mice. The water content does not change much in the mouse cervix during pregnancy.

In the human and the rat, during pregnancy there is an increase in total body water content, an increase that is reflected in a drop in plasma osmolality of approximately

10 mosmol/kg water [31]. This can be reproduced in the ovariectomized rat via treatment with synthetic *rlx* [32]. The pregnant *rlx* -/- mouse has a higher plasma osmolality than does the *rlx* +/+ mouse [8]. Therefore, the *rlx* -/- mouse is supposed to have a lower total body water content compared with the *rlx* +/+ pregnant mouse. However, the overall increase in body water content (~3%) during pregnancy [33] is unlikely to account for the large changes (20%) in the PS water content in the pregnant *rlx* +/+ mice. The magnitude of the changes in this tissue indicates that the extracellular matrix has been modified by *rlx* to contain an increased concentration of a water-holding polysaccharide, such as hyaluronate. One milligram of hyaluronate of the appropriate molecular weight is capable of forming a gel in 1 ml of water [34], and moderate changes in concentration lead to very large changes in osmotic pressure [35]. Hyaluronate is present in the cervix of the rat, increases 17-fold during pregnancy, and is increased in the ovariectomized rat cervix following treatment with *rlx* in addition to estrogen and progesterone [36, 37]. This indicates that the large increase in water content in the *rlx* +/+ mouse—an increase not seen in the *rlx* -/- mouse—could have been attributable to an *rlx*-induced increase in PS hyaluronate content.

Morphological Changes in Vaginal and Cervical Epithelia

During pregnancy, the epithelial lining of the vagina and cervix undergo considerable proliferation, but this does not occur in *rlx* -/- mice. In fact, the epithelia of the *rlx* -/- vagina, at 18.5 days of pregnancy, is only two to three cell layers thick and resembles that of the ovariectomized mouse [38] or the mouse without functional estrogen receptor α [39]. Estrogen (estradiol-17 β) is thought to cause vaginal and uterine epithelial proliferation and differentiation via an action on the stromal cells, which then release a paracrine factor to act on the epithelial cells [38, 40]. This stromal factor might be *rlx*. Relaxin gene expression is detected much more strongly in the pregnant than in the nonpregnant rat uterus [41], though the cell of origin has not yet been convincingly established.

Specific *rlx*-binding sites were detected in the cervical luminal epithelial cells as well as in the smooth muscle cells in pregnant rats [42]. Relaxin has been purported to stimulate the synthesis of prorenin from human decidual cells [43]. The pregnant rats treated with a monoclonal antibody to rat *rlx* showed a moderate decrease in epithelial cell density in the cervix [13]. More recently, it was claimed that *rlx* is responsible for about one half of the cell proliferation that occurs in the rat cervical stroma and epithelium during pregnancy [44]. Thus, the effect of *rlx* as a paracrine factor in the vagina and cervix certainly warrants further investigation.

An alternative hypothesis is that it is systemic *rlx* that acts on the vaginal and cervical epithelia to stimulate proliferation and differentiation. These tissues contain both *rlx*-binding sites and the estrogen receptor α [38, 42]. It has recently been suggested that *rlx* can activate the estrogen receptor [45], as *rlx*-induced uterine stromal hypertrophy in ovariectomized rats can be blocked by pretreatment of the animals with a specific estrogen receptor antagonist. It would be interesting to see if *rlx* could exert effects on vaginal and cervical epithelia in mice without a functional estrogen receptor α .

The results of this experiment confirm the role of *rlx* in terms of the growth of the nipple, vagina, PS, and cervix,

and they also confirm the role of rlx in the decrease in collagen content in the nipple and vagina in mice. These results establish that lactational failure of rlx $-/-$ mothers is due to the great local density of collagen and the lack of growth of the nipple. The unrelaxed PS combined with the densely packed collagen fibers, the limited proliferation of the epithelial cells, and the failure of growth of the vagina and cervix may contribute to a parturition defect in rlx $-/-$ mice, even though the majority of these mice can give birth successfully. These results indicate that rlx acts on its target tissues during pregnancy by regulating collagen breakdown and reorganization, possibly through regulating MMP expression and activation; MMPs are known to be important in normal and pathological tissue growth and expansion [26, 27].

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