

# The Effect of Relaxin on Collagen Metabolism in the Nonpregnant Rat Pubic Symphysis: The Influence of Estrogen and Progesterone in Regulating Relaxin Activity\*

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## ABSTRACT

The aim of this study was to examine the effects of relaxin on collagen content, solubility, and composition in the rat pubic symphysis. Nonpregnant, female Sprague-Dawley rats were bilaterally ovariectomized and either unprimed or primed with estrogen or progesterone alone, or a combination of estrogen and progesterone. One week later these animals were given increasing doses of a synthetic human (gene-2) relaxin (0–100  $\mu\text{g}$ ) before being killed 16 h later. Their pubic symphyseal tissues were then removed and analyzed for collagen content and solubility, whereas collagen composition was determined by SDS-PAGE. Relaxin administration significantly increased the length ( $140 \pm 6\%$ ) and weight ( $170 \pm 9\%$ ) of the interpubic

fibrocartilage in estrogen-primed rats ( $n = 15$ ). At the same time, it decreased the total collagen content by  $68 \pm 6\%$ , without altering the proportions of collagen types, which were predominantly type I (85%) and type II collagen (15%). Relaxin administered alone reduced the total collagen content by  $64 \pm 4\%$  but had no effect on collagen solubility or composition. Progesterone abolished the effects of relaxin in estrogen-primed rats. It is concluded that relaxin has a potent effect on the amount of collagen in the rat pubic symphysis that is enhanced by estrogen and antagonized by progesterone. The changes in the extracellular matrix within the pubic symphysis induced by relaxin may be important in the modifications that this tissue undergoes during pregnancy. (*Endocrinology* 137: 3884–3890, 1996)

THE MOST ABUNDANT proteins of the extracellular matrix of connective tissues are members of the collagen protein family, which form characteristic fiber bundles conferring on the tissue their specific mechanical and physicochemical properties, and providing a scaffolding for cell attachment and migration (1). The precise regulation of collagen synthesis and turnover are thus crucial events in tissue remodeling during growth and in repair processes such as wound healing. Changes in the collagen content, or alterations to the spectrum of collagen types produced, can therefore dramatically alter the properties of tissues. Type I collagen is the most commonly occurring fibril-forming collagen and is the major structural component of tissues such as skin, bone, tendon, and ligament.

The pelvic girdle in many mammalian species is required to undergo numerous structural modifications to enable safe delivery of the young. This process is facilitated by means of hormonal regulation and requires adaptations of the pelvis (2). These adaptations involve an increased flexibility of the pubic symphysis and in some species (including the human,

guinea pig, and mouse), a maturation of the cartilage within the pelvic joint to a flexible and elastic interpubic ligament (3–5). The pubic symphysis is a nonsynovial cartilaginous joint (6) that is movable and connected to the pubic bones by hyaline cartilage. In the nonpregnant adult rat, these bones are stabilized by a strong bar of fibrocartilage, which evolves from the hyaline cartilage and is formed by fibrous connective tissue. Whereas the extracellular matrix of the pubic symphysis of the mouse and guinea pig have been well characterized and reviewed (2), relatively little is known about this joint in the rat. Furthermore, the hormonal influences that bring about the softening and relaxation of the interpubic tissue during pregnancy are poorly understood, although both estrogen and relaxin are thought to be key regulatory components. Although the precise molecular mechanism is unknown, it has been suggested that this process involves a rapid turnover of the extracellular matrix within the reproductive tissues and in particular, an extensive reduction in collagen concentration before parturition (7).

Relaxin is a 6-kDa polypeptide hormone, mainly produced in the corpus luteum and endometrium (8) of the female rat, and is secreted in increased quantities into the peripheral circulation during pregnancy in a number of mammalian species (9, 10). The hormone potentially has many biological functions, including an ability to stimulate a remodeling change in the structure of the connective tissues at its target sites within the reproductive tract (11). These actions and its ability to inhibit myometrial contractions and ripen the uter-

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ine cervix (12) have established relaxin as a hormone that is thought to have an active role in the accommodation of pregnancy and parturition (1).

It has been hypothesized that locally produced relaxin could also act in a paracrine mode in the reproductive tract to cause collagen remodeling during the gestational period (13). Studies on normal human dermal fibroblasts with homologous relaxin *in vitro* have demonstrated that relaxin had several effects on collagen (14). Relaxin was shown to be a potent modulator of these fibroblasts, being able to down-regulate collagen expression by up to 40% and causing a significant increase in collagen turnover. The addition of relaxin to these cells, increased both messenger RNA and protein levels for collagenase and modestly decreased the levels of its inhibitor, the tissue inhibitor of metalloproteinase (TIMP), resulting in a decreased synthesis and secretion of collagen.

Furthermore, while the pubic symphysis has been studied in many species, including the human, mouse, guinea pig, and rat, only very few studies have investigated the effects of relaxin on collagen metabolism in these animals *in vivo*. One such study on the mouse pubic symphysis concluded that relaxin had a distinct effect on collagenolysis: increasing the amount of soluble collagen in relation to the total collagen measured, but decreasing the insoluble and overall collagen content in relation to the dry weight tissue (15). However, these studies and several earlier investigations on the pubic symphysis were completed using crude preparations of relaxin.

The purpose of this study was to determine the effects of purified relaxin (R) on collagen metabolism *in vivo*, and more specifically, on collagen content, solubility, and composition in the rat pubic symphysis. These experiments also investigated the influence that estrogen (E) and progesterone (P), the other main hormones involved in pregnancy, had on relaxin's effects in nonpregnant, ovariectomized animals.

## Materials and Methods

### Animals

Ninety-seven virgin female Sprague-Dawley rats of 200–260 g BW were obtained at 70 days of age from the Department of Zoology Animal Facility, University of Melbourne, and bilaterally ovariectomized under anesthesia (day 1). The animals were then housed in a controlled environment and maintained on a 12-h light, 12-h dark schedule with access to GR2+ laboratory chow (Barastoc Stockfeeds, Pty Ltd., Melbourne, Australia) and water. After surgery, the animals were allocated to the following groups. These experiments were approved by the Institute's Animal Experimental Ethics Committee, which adheres to the Australian Code of Practice for the care and use of animals for scientific purposes.

### Control rats

One week postoperatively, control animals ( $n = 8$ ) received 0.1 ml sesame oil (sc) alone (day 8) and were maintained in a controlled environment for a further 8 days, before being killed under anesthesia with carbon dioxide (at 0830 h, day 16). After this, the pubic symphysis was removed and the pubic symphyseal fibrocartilage (identified as the interpubic tissue that connects the pelvic bones, within the pelvic girdle) dissected out, cleaned from any fat, muscle, fascia and bone, and weighed. Six of these pubic symphyseal tissues were used for the determination of the overall collagen, whereas the other two fibrocartilage samples were used for the determination of soluble and insoluble collagen content and collagen extractability.

### ER rats: estrogen-primed/unprimed rats treated with recombinant human relaxin

One week after surgery, estrogen-primed animals ( $n = 56$ ) received a single sc injection of 5  $\mu\text{g}$  estradiol dibenzoate in 0.1 ml sesame oil, whereas the unprimed rats were injected with 0.1 ml sesame oil alone (at 1630 h, day 8). A further week later, both the estrogen-primed and unprimed animals were subdivided into smaller groups and were given single injections sc of 25  $\mu\text{g}$  ( $n = 12$ ), 50  $\mu\text{g}$  ( $n = 13$ ) or 100  $\mu\text{g}$  ( $n = 17$ ) of a highly purified synthetic human relaxin (Genentech Inc., Los Angeles, CA) in a 10 mM citrate buffer (pH 5.0) or citrate buffer (vehicle) alone ( $n = 14$ ) (at 1630 h, day 15). The relaxin used was chemically synthesized from the human relaxin gene-2 (hRlxG2) sequence (16). Sixteen hours after the administration of relaxin (at 0830 h, day 16), the animals were killed, cleaned, and weighed. The time length used (between the administration of relaxin and the killing of rats) was based on the results, previously published by Frieden and Adams (17), which showed that maximal relaxin activity was obtained after 12 h of administration, with only a 5% drop in this activity after 16 h. Forty-eight animals were used to determine the overall collagen content, whereas a further eight estrogen-primed rats, given 25 ( $n = 2$ ), 50 ( $n = 3$ ) and 100  $\mu\text{g}$  ( $n = 3$ ) of relaxin, were used to determine the amount of soluble, insoluble and extractable collagen.

### EPR rats: estrogen-primed/unprimed rats treated with progesterone and recombinant human relaxin

These rats ( $n = 33$ ) were bilaterally ovariectomized under anesthesia (day 1), housed in a controlled environment for a week and either estrogen-primed or given sesame oil alone (day 8). Two days later, five estrogen-primed and five unprimed animals ( $n = 10$ ) were injected (ip) with 1 mg progesterone in 1 ml sesame oil (day 10); an additional two groups of five rats were given 2 mg progesterone in two separate injections (days 10 and 12); and another ten animals were injected with 1 mg progesterone in sesame oil every 2 days for a total of three injections (days 10, 12, and 14), each dose being given at 0830 h on each day. The doses of progesterone administered (1–3 mg) were based on the findings of Hashimoto and co-workers (18), who found that progesterone production by the rat ovary was maximal on day 14 of pregnancy (1 mg/day). At 1630 h on day 14, all animals were injected (sc) with 50  $\mu\text{g}$  relaxin in 0.1 ml citrate buffer (10 mM, pH 5.0). The animals were then killed 16 h after the administration of relaxin (at 0830 h, day 15), and treated as above. A total of thirty rat pubic symphyseal fibrocartilages were used to analyze total collagen, whereas tissue samples from a further three rats, given progesterone and relaxin, were used for the determination of collagen solubility and extractability.

### Determination of overall collagen content

Each pubic symphyseal fibrocartilage from control and treated groups was trimmed of any excess tissue, blotted, weighed (wet weight), and lyophilized to dry weight. After measurement of the lengths of tissues, the tissues were rehydrated in buffer containing 0.05 M Tris/HCl, pH 7.5, 0.15 M NaCl, and the proteinase inhibitors *N*-ethylmaleimide (10 mM), phenylmethylsulfonyl fluoride (0.1 mM), benzamidine hydrochloride (1 mM) and EDTA (10 mM) for 24 h at 4 C. Samples were then defatted in a mixture containing 2:1 chloroform:methanol for 24 h before being rehydrated again for 60 h at 4 C. The samples were then frozen in liquid nitrogen and chopped finely before the overall collagen content was determined by hydrolyzing the finely diced tissue in 1.0 ml, 6 M HCl at 110 C for 24 h. The hydrolyzates were evaporated to dryness in the presence of sodium hydroxide, and the residues redissolved in 2.5 ml of 0.1 M HCl. Triplicate 10- and 20- $\mu\text{l}$  aliquots from each sample were analyzed for hydroxyproline content, using a scaled down version of the procedure described by Bergman and Loxley (19). Hydroxyproline values were then converted to collagen content by multiplying by a factor of 6.94 (20).

### Determination of the soluble and insoluble collagen

Collagens were serially extracted from the diced fibrocartilagenous samples, as previously described (21). Briefly, the samples were initially extracted with 0.05 M Tris/HCl buffer, pH 7.5, containing 0.15 M NaCl

and proteinase inhibitors for 24 h at 4 C, to extract the newly synthesized collagen (neutral salt soluble fraction). These samples were then centrifuged (at 13,000 rpm, 45 min) and the remaining residue, extracted for a further 24 h with 0.5 M acetic acid at 4 C, to extract the newly cross-linked collagen (acetic acid soluble fraction). Samples were centrifuged as above and the residues freeze-dried, weighed, and subjected twice to limited pepsin digestion (enzyme:substrate ratio, 1:10), for 24 h at 4 C, to extract the mature cross-linked matrix collagens (pepsin soluble fraction) (22). The hydroxyproline content of the extracts were determined and the types of soluble collagen present in the serially extracted fractions determined by PAGE.

#### Cyanogen bromide cleavage of fibrocartilages

The pepsin-digested residues of the pubic symphyseal tissues were dissolved in 100 mM ammonium bicarbonate (NH<sub>4</sub>CO<sub>3</sub>, pH 8.0) to inactivate the pepsin, before being digested in 70% formic acid, containing 50 mg of CNBr; with the cleavage being achieved by the method of Scott & Veis (23), over a period of 4 h at room temperature. The reaction was terminated by drying under vacuum and the residue, redissolved in 0.5 M acetic acid, clarified by centrifugation, and freeze-dried to remove any residual CNBr. The types of insoluble collagen, present in these samples was then determined by electrophoretic analysis by comparison with purified collagen type-specific CNBr peptide standards.

#### SDS-PAGE

The soluble collagen chains were analyzed on 5% (wt/vol) acrylamide gels with a stacking gel of 3.5% (wt/vol) acrylamide. The collagen chains were dissolved in a sample loading buffer containing 0.05 M Tris/HCl, pH 6.8, 2 M urea, 20% (wt/vol) sucrose, 0.1% (wt/vol) SDS, and 0.1% (wt/vol) bromophenol blue, as used by Chan & Cole (24). Interrupted electrophoresis with delayed reduction of the disulfide bonds of type III collagen was used to separate the  $\alpha 1$  (III) chains from the  $\alpha 1$  (I) collagen chains (25).

The insoluble collagen from the CNBr cleavage peptides were analyzed on 12.5% (wt/vol) acrylamide gels with a stacking gel containing 4.5% acrylamide. These peptides were also dissolved in a loading buffer containing 0.05 M Tris/HCl, pH 6.8, 2 M urea, 20% (wt/vol) sucrose, 0.1% (wt/vol) SDS, and 0.1% (w/v) bromophenol blue and run under the same electrophoretic conditions. The gels were stained overnight at 4 C with 0.1% (wt/vol) coomassie brilliant blue R-250 and destained with 30% (vol/vol) methanol containing 7% (vol/vol) acetic acid, before being dried and photographed.

#### Western blot analysis

To confirm the presence of cartilage type II collagen, 15% (vol/vol) of the control and relaxin-treated samples (containing approximately 20–30  $\mu$ g, protein) were electrophoresed as described before, with type-specific CNBr peptide standards. The gel was then electroblotted onto a nitrocellulose membrane for 75 min at 4 C, soaked in BLOTTO (5% skim milk powder) for 60 min and washed in Tris-buffered saline (TBS), containing Tween-20 (TTBS) before being incubated overnight with a type II collagen specific antibody (kindly supplied by Dr. G. Gibson,

Henry Ford Hospital, Detroit, MI) at room temperature (1:600 dilution). After washing the membrane in TTBS, the following day, it was incubated with a second antibody (a goat antirabbit antibody; 1:300 dilution) (Bio-Rad, CA) for 60 min before being washed in TTBS, TBS and treated with a color developer containing p-nitro blue tetrazolium chloride and 5-bromo-4-chloro 3 indolyl phosphate-toluidine salt (Bio-Rad, Richmond, CA), until dark bands were observed.

#### Statistical analysis

Results were analyzed using two-way ANOVA. The effects of estrogen and increasing doses of relaxin (in the ER group)/increasing doses of progesterone (in the EPR group) were tested for significance, with  $P < 0.05$  considered significant.

## Results

#### Determination of length, weight, and water content of fibrocartilages

Table 1 shows the changes in length, weight, and water content of the pubic symphyseal fibrocartilage after various hormonal treatments in both estrogen-primed and unprimed, ovariectomized rats. Relaxin, administered to estrogen-primed animals, significantly increased the length and weight ( $P < 0.05$ ) of the interpubic tissue samples, compared with control and vehicle measurements, whereas no changes were observed in the levels of water with any of the hormonal treatments given. Neither relaxin (at 100  $\mu$ g) alone administered to unprimed animals, nor estrogen alone, had any significant effect on these parameters, indicating that a synergy of the two hormones was required for relaxin to exert its biological effects on these tissues. Conversely, the administration of progesterone (1–3 mg) to both nonprimed and estrogen-primed rats, just before the administration of relaxin (50  $\mu$ g) inhibited the effect induced by relaxin alone ( $P < 0.05$ ), as well as the combined effect induced by relaxin and estrogen, as the length and weight of these samples were comparable to control tissues. However, although it was found that progesterone consistently inhibited the combined effects, caused by estrogen and relaxin, this inhibition was not statistically significant.

#### Fibrocartilage collagen content

Figure 1 shows the total collagen content in pubic symphyseal tissues, treated with increasing doses of relaxin in both unprimed and estrogen-primed animals. These results were also expressed as a percentage of the corresponding dry

**TABLE 1.** Changes in mean ( $\pm$  SE) length, weight, and water content of the rat pubic symphyseal tissue, due to the administration of relaxin alone and in the presence of estrogen and/or progesterone

n	C <sup>a</sup> 6	C <sup>b</sup> 5	E <sup>c</sup> 5	R <sup>d</sup> 5	E <sup>e</sup> R <sup>d</sup> 10	P <sup>f</sup> R <sup>f</sup> 5	E <sup>g</sup> P <sup>f</sup> R <sup>f</sup> 5
Length (mm)	6.6 $\pm$ 0.2	7.0 $\pm$ 0.3	7.8 $\pm$ 0.3	7.6 $\pm$ 0.4	9.1 $\pm$ 0.4 <sup>g</sup>	7.9 $\pm$ 0.3	7.8 $\pm$ 0.3
Wet wt (mg)	29.8 $\pm$ 1.4	33.8 $\pm$ 2.9	40.4 $\pm$ 1.9	33.8 $\pm$ 2.4	50.8 $\pm$ 2.6 <sup>g</sup>	36.2 $\pm$ 2.7	40.3 $\pm$ 3.9
Dry wt (mg)	8.6 $\pm$ 0.7	10.4 $\pm$ 1.1	11.2 $\pm$ 0.5	9.3 $\pm$ 0.3	14.4 $\pm$ 0.9	10.4 $\pm$ 0.8	11.8 $\pm$ 1.4
Water (%)	71.4 $\pm$ 1.4	69.4 $\pm$ 1.5	72.1 $\pm$ 1.3	72.1 $\pm$ 2.6	71.8 $\pm$ 0.7	71.3 $\pm$ 0.6	71.0 $\pm$ 1.5

<sup>a</sup> Sesame oil alone, administered on day 8, after ovariectomy (control).

<sup>b</sup> Sesame oil administered after day 8 and citrate buffer (10 mM, pH 5.0) administered on day 15, after ovariectomy (vehicle).

<sup>c</sup> Five micrograms of estradiol dibenzoate in sesame oil administered on day 8 after ovariectomy.

<sup>d</sup> One hundred micrograms of relaxin administered in citrate buffer (10 mM, pH 5.0), at 1630 h (day 15) to animals killed at 0830 h (day 16).

<sup>e</sup> One milligram of progesterone in sesame oil administered on days 10, 12, and 14.

<sup>f</sup> Fifty micrograms of relaxin administered to animals at 1630 h (day 15) after the administration of progesterone.

<sup>g</sup>  $P < 0.05$  compared with control and vehicle groups.

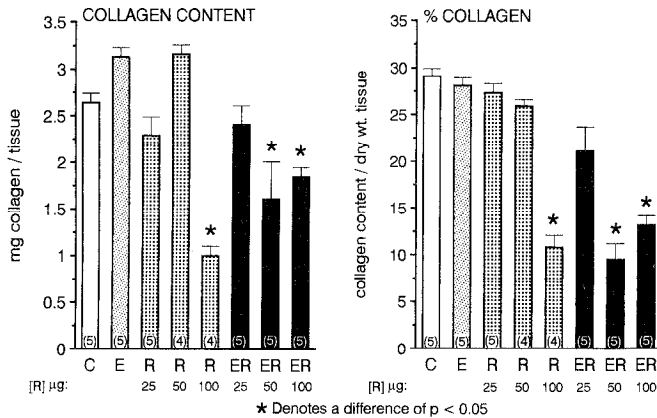


FIG. 1. The total collagen content [which was converted from the hydroxyproline values (20)] from control (C) interpubic tissues and those treated with increasing doses of relaxin (R) and/or estrogen (E); and the results expressed as the corresponding percentage of the dry weight tissue.

weights. The total collagen content of control fibrocartilages was approximately 30% of the dry weight tissue, which represented about 9% of the overall tissue composition. The administration of estrogen or lower doses of relaxin (0–50  $\mu\text{g}$ ) alone to these animals had no significant effect on collagen content; however, the presence of relaxin alone at a higher dose (100  $\mu\text{g}$ ) significantly ( $P < 0.05$ ) reduced collagen content from 30% (in the vehicle alone-treated group) to 10.8%, representing an overall reduction of  $64 \pm 4\%$ . This reduction was achieved using a lower dose of relaxin (50  $\mu\text{g}$ ), when administered to estrogen-primed rats, which decreased the collagen content to 9.6% (an overall reduction of  $68 \pm 6\%$ ), confirming that the presence of estrogen was required for relaxin to have a greater biological effect, on the parameters measured. Although the amount of collagen was reduced to a greater extent in the presence of a higher dose of relaxin alone, this does not take into account the lower cartilaginous weights of the relaxin-alone treated rats, compared to the estrogen-primed animals (Table 1). Hence, when these results were expressed as a percentage of the dry weight tissue (which was greater in the ER treated groups), it was clear that only half the amount of relaxin (50  $\mu\text{g}$ ), administered to estrogen-primed rats, was required to produce the effect seen on collagen reduction as that of 100  $\mu\text{g}$  of the hormone, administered to unprimed animals.

Figure 2 shows the total amount of collagen in fibrocartilage samples, treated with increasing doses of progesterone and a fixed dose of relaxin (50  $\mu\text{g}$ ) in both unprimed and estrogen-primed rats. The dose of relaxin used was based on the results from the previous experiment (refer to Fig. 1), which showed that 50  $\mu\text{g}$  relaxin, administered to estrogen-primed rats, had a similar effect on collagen content as compared to 100  $\mu\text{g}$ . No change in collagen content was observed after the administration of progesterone to both unprimed and estrogen-primed animals, before the administration of relaxin indicating that progesterone had significantly ( $P < 0.05$ ) antagonized the combined effects of estrogen and relaxin.

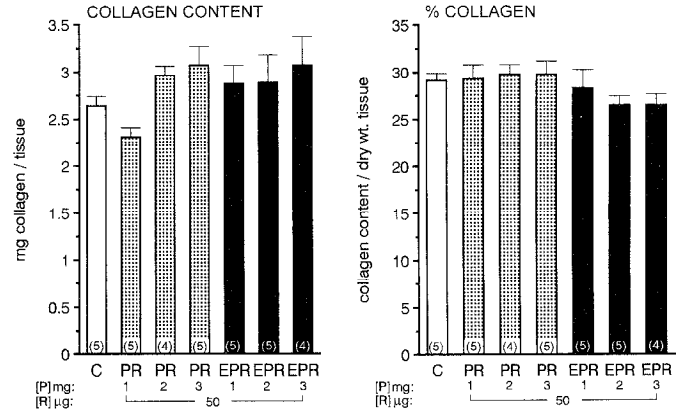


FIG. 2. Total collagen content and the corresponding percentage of dry weight collagen from samples treated with increasing doses of progesterone (P) and a fixed dose of relaxin (R) in both estrogen-primed (219) and unprimed ( $\pm$ ) rats.

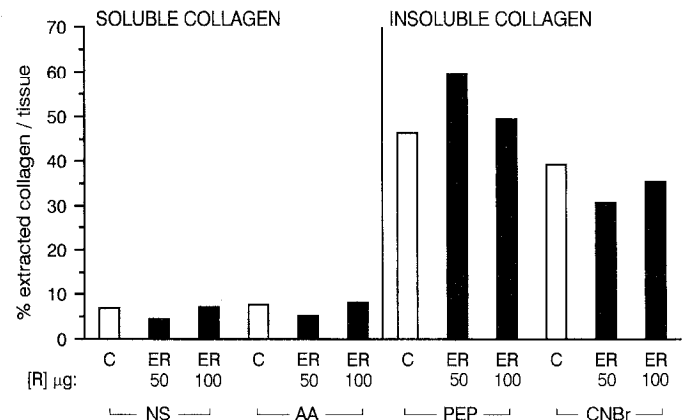


FIG. 3. The amounts of extracted soluble and insoluble collagen from control (C) ( $n = 2$ ) and estrogen-primed (E) samples treated with 50 ( $n = 3$ ) and 100  $\mu\text{g}$  ( $n = 3$ ) relaxin (R), expressed as a percentage of the total extracted collagen in fibrocartilaginous samples. The soluble collagen was extracted with neutral salt (NS), and acetic acid (AA), whereas the insoluble collagen was extracted with limited pepsin (PEP) and cyanogen bromide (CNBr) digestion of the left over soluble collagen residue.

#### Fibrocartilage collagen solubility and extractability

Figure 3 shows the amounts of soluble and insoluble collagen, extracted from the rat interpubic fibrocartilage of control and estrogen-primed animals. Only 15% of the extracted collagen from control rats was soluble, as indicated by extraction with the neutral salt buffer (which extracted the newly synthesized and uncross-linked collagen) and acetic acid (which extracted the newly cross-linked collagen). This indicated that the interpubic fibrocartilage was composed of a mature, cross-linked, insoluble collagen matrix. This collagen required a more extensive extraction by limited pepsin and cyanogen bromide digestion to be quantitated. The administration of increasing doses of relaxin (25, 50 and 100  $\mu\text{g}$ ) to estrogen-primed animals had no effect on collagen solubility.

#### Fibrocartilage collagen composition

Gel electrophoresis of the pepsin-digested collagen from control fibrocartilage samples (Fig. 4, lanes 2–3) demon-

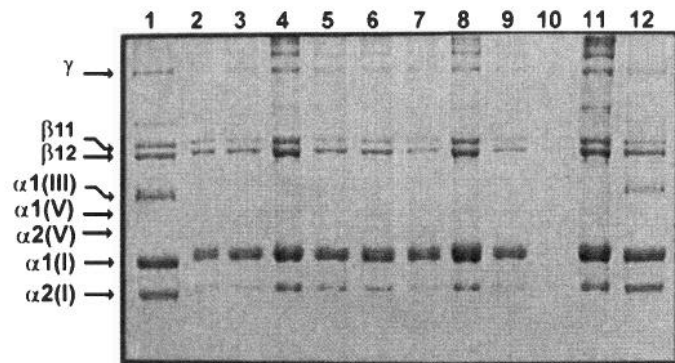


FIG. 4. The pepsin-digested collagen, which represents approximately 60% of the total insoluble collagen, was analyzed by SDS-PAGE, using delayed reduction of the disulfide bonds with 10%  $\beta$ -mercaptoethanol. The samples consist of a type I, III standard (lanes 1, 12), collagen from the two individual pepsin digests from a control (2, 3), samples treated with increasing doses of relaxin (R) (25, 50 and 100  $\mu$ g, respectively) from estrogen-primed (E) rats (4–9) and from a sample treated with progesterone (P) and R (10, 11).

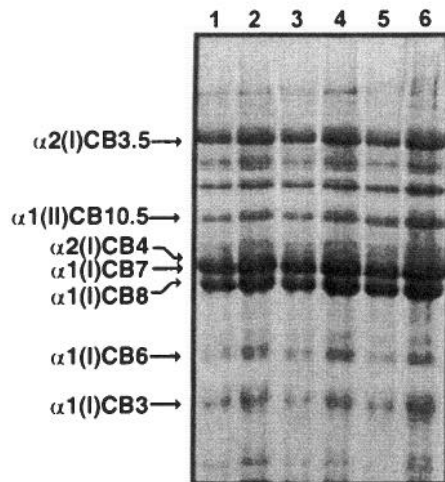


FIG. 5. The type I and type II collagen CNBr cleavage peptides, which were analysed by SDS-PAGE. Samples consist of 10% and 20% (vol/vol) respectively, of the cyanogen bromide treated collagen from control (1, 2) and relaxin (R) treated samples, from estrogen-primed (E) animals, using 50  $\mu$ g (3, 4) and 100  $\mu$ g (5, 6) relaxin.

strated the presence of predominantly type I collagen  $\alpha$ 1(I) and  $\alpha$ 2(I) subunits. The other major species detected, the  $\beta$ 11 and  $\beta$ 12 components, which are covalently cross-linked  $\alpha$ -chain dimers, indicated that this fraction was extensively cross-linked and was consistent with the need for pepsin digestion of the cross-linked domains for efficient extraction. Trace amounts of type V collagen  $\alpha$ 1(V) and  $\alpha$ 2(V) chains were also present, but no type III collagen was detected. Likewise, cyanogen bromide digests of the insoluble residue (Fig. 5, lanes 1–2) confirmed that type I collagen was the major collagenous component of the tissue but further distinguished the presence of the  $\alpha$ 1(II)CB10.5 and  $\alpha$ 1(II)CB11 peptide fragments, providing evidence that type II collagen was also a significant component of the rat interpubic tissue. The presence of type II collagen was confirmed by Western blot analysis (Fig. 6) and quantitative electrophoresis of the collagen types indicated that the type II collagen represented approximately 15% of the total collagen within the tissue

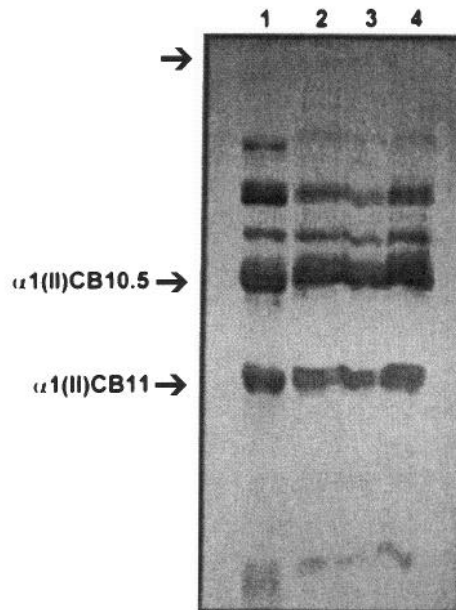


FIG. 6. The presence of type II collagen was confirmed by the identification of the  $\alpha$ 1(II)CB10.5 and  $\alpha$ 1(II)CB11 fragments, by Western blot analysis. Samples consist of a type II collagen CNBr specific standard (1), control (2) and relaxin-treated (R) samples, from estrogen-primed (E) animals, using 50  $\mu$ g (3) and 100  $\mu$ g (4) relaxin.

(data not shown). Furthermore, relaxin treatment of the estrogen-primed samples (Fig. 4, lanes 4–11 and Fig. 5, lanes 3–6) had no effect on the collagen types present in the tissue.

### Discussion

The results indicate that the administration of recombinant purified relaxin has a potent effect on collagen metabolism in the nonpregnant rat pubic symphyseal fibrocartilage, markedly decreasing the overall collagen content in relation to the tissue dry weight, without significantly affecting collagen solubility or composition. Relaxin has been shown to play a similar role in remodelling the connective tissue within the reproductive tract of the pregnant rat (7).

These findings in the rat interpubic fibrocartilage can be compared with previous work completed on the effect of relaxin on collagen metabolism in the mouse (15) and guinea pig (26) interpubic ligament and emphasize the importance of repeating some of the earlier experiments, using purified preparations of relaxin. In both the guinea pig and mouse, the amount of collagen within the tissue increases upon the administration of relaxin to estrogen-primed animals. However, when this amount is expressed as a percentage of the tissue dry weight (which increases to a greater extent), the total collagen concentration actually decreases. In the rat, there is both a marked reduction in both collagen content and concentration. This net effect in all three species, which is a distinct reduction in collagen concentration, suggests that the effect of relaxin on collagen in the interpubic tissue seems to be independent of tissue type, since the effect of relaxin on the nonpregnant fibrocartilage is strikingly similar to its effects on the pregnant interpubic ligament.

The administration of progesterone to both unprimed and estrogen-primed animals, before the administration of relaxin clearly antagonized the combined effects of estrogen and relaxin, as its presence prevented any relaxin-induced

changes in the length and weight or collagen content of the interpubic tissue as compared to control levels. These findings were in agreement with those of Steinetz and co-workers (5), who found that progesterone was able to specifically block estrogen and relaxin induced ligament growth in mice. These studies do not define the mechanism of the action of progesterone; however, two possible mechanisms can be proposed: 1) progesterone may have had an inhibitory effect on estrogen (27, 28), thus indirectly blocking the effects of relaxin; or 2) it had a direct antagonistic effect on relaxin's actions. It appears more likely that the second mechanism, involving a direct inhibition of relaxin by progesterone occurs, based on the finding that progesterone treatment alone, without estrogen priming, significantly inhibited the relaxin effect. Furthermore, progesterone, by blocking the effects of relaxin, may have also stimulated an increase in collagen synthesis. This assumption is in agreement with previous studies (29, 30) which have investigated the effects of progesterone on collagen metabolism, in other tissues of the reproductive tract. Halme & Woessner (30), found that large doses of progesterone (80–150 mg) alone increased collagen synthesis in the rat uterus. Our results highlight the importance that progesterone may have in regulating the interaction between estrogen and relaxin in the pubic symphysis and cervix during pregnancy (31), as it is only during late pregnancy in the rat that the levels of serum progesterone decline, whereas serum estrogen and relaxin levels rise, hence confirming that relaxin's action in remodelling the connective tissues in the rat most likely occurs in later stages of the gestational period.

Analysis of the soluble and insoluble collagen fractions indicated that increasing doses of relaxin had no significant effect on collagen solubility in the rat pubic symphysis. These findings are contrary to those previously described in the mouse pubic symphysis (15), which showed a relaxin-induced increase in collagen solubility within the tissue. However, it should be noted that this reported increase in collagen solubility only occurs during pregnancy (7, 15). Furthermore, these overall results indicated that the effects of relaxin on pubic symphyseal collagen were similar to that on cervical collagen in the pregnant rat, in which it increased collagen solubility, but decreased the overall collagen concentration within the tissue (7).

The absence of any change in collagen solubility after relaxin-treatment of the interpubic tissue and the rapid change (16 h) in total tissue collagen content suggests that a stimulation of collagen proteolysis is likely to be the primary mechanism underlying the relaxin effect. Although relaxin has been shown to reduce fibroblast-induced collagen synthesis *in vitro* (14), the rapid onset and extent of the hormone in this fibrocartilagenous tissue cannot be quantitatively accounted for by a reduced biosynthetic rate of the collagen *in vivo*. This has been confirmed by biosynthetic studies on the nonpregnant interpubic tissue, which indicated that relaxin did not alter the biosynthetic pattern of the collagen (Samuel & Bateman, unpublished results).

Another potential mechanism whereby relaxin could modify tissue structure and biomechanical function is by altering the proportions of the collagen types present in the tissue. Analysis of the various soluble and insoluble collagen

extracts from the nonpregnant interpubic tissue demonstrated that type I collagen was the predominant collagen species (85% of total collagen) along with cartilage-specific type II collagen (15%), which is characteristic of other fibrocartilagenous tissues (32–34).

A notable finding in the CNBr analysis (Fig. 5) was the low recovery of the  $\alpha 1(I)CB6$  fragment, as compared with the  $\alpha 1(I)CB7$  and  $\alpha 1(I)CB8$  fragments, which indicates the significant amount of CB6-derived cross-links in the insoluble residue. This result provided further evidence of the extensive cross-linking in this insoluble fraction, which required CNBr digestion for solubilization. The fact that the  $\alpha 1(I)CB6$  recovery was unaltered by relaxin-treatment, indicated that relaxin had no significant effect on collagen cross-link maturation.

In conclusion, this investigation indicates that relaxin has a marked effect on the overall collagen content in the rat pubic symphyseal fibrocartilage, most likely by stimulating the degradation of collagen. The hormone's effects are significantly enhanced by estrogen and totally antagonized by progesterone. In contrast to previous studies that have used crude preparations of relaxin, the effects of purified relaxin on the rat pubic symphysis are analogous to those previously described in the mouse and human. Furthermore, these studies suggest that relaxin may play a key role in regulating the function of the extracellular matrix and thus the dynamic and mechanical properties of the pubic symphysis during pregnancy.

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