Evidence That Relaxin’s Effects on Growth and Softening of the Cervix Are Not Mediated through Prostaglandins in the Rat*

O. DAVID SHERWOOD, EMILY S. JUNGHEIM, JAIME L. MASFERRER, AND JOYCE M. CRAMER

Department of Molecular and Integrative Physiology (O.D.S.), University of Illinois at Urbana-Champaign, Urbana, Illinois 61801; and Department of Inflammatory Disease Research (J.L.M.), G.D. Searle & Company, St. Louis, Missouri 63198

ABSTRACT
Relaxin plays a major role in promoting the growth and softening of the cervix that occurs during the second half of pregnancy in the rat. There is limited evidence that prostaglandins play a role in cervical softening in mammalian species. Accordingly, this study was conducted to determine if prostaglandins mediate relaxin’s effects on the rat cervix. To attain that objective, indomethacin was used to inhibit cyclooxygenase, the key enzyme in the conversion of arachidonic acid to prostaglandins.

Twenty-six nonpregnant female rats were ovariectomized when they were 78 days old (day 1 of treatment). At ovariectomy (O), each rat was fitted with silicon tubing implants containing progesterone (P) and estrogen (E) in doses that provided blood levels similar to those during late pregnancy in rats. Rats were randomly assigned to three treatment groups. Group OPE controls (n = 8 rats) received 2 ml indomethacin vehicle (0.5% methyl cellulose, 0.025 Tween 80 in water) gavage at 0900 h on days 8 and 9 and 0.5 ml relaxin vehicle (0.9% NaCl) sc at 6-h intervals from 1200 h on day 8 through 0600 h on day 10. Group OPER (n = 9 rats) was treated as group OPE except that 20 μg highly purified porcine relaxin was administered. Group OPERI (n = 9 rats) was treated as group OPER except that indomethacin was administered at a dose (20 mg/kg BW) that reduced cervical PGE2 levels by more than 90%. Between 0800 h and 1000 h on day 10, the cervices were removed, trimmed of fat, weighed, and placed in ice-cold Krebs-Ringer bicarbonate buffer, pH 7.5. Cervical extensibility (degree of softening) was determined within 4 h of tissue collection.

In conclusion, this study provides evidence that relaxin’s effects on cervical growth and softening in the rat are not mediated through prostaglandins. (Endocrinology 139: 867–873, 1998)

RELAXIN is produced and secreted by the corpora lutea during the second half of pregnancy in the rat (1). One of relaxin’s vital physiological roles in this species is to promote the growth and softening (ripening) of the cervix that are required for rapid and safe delivery (2, 3).

Neither the mechanisms associated with cervical growth and softening nor their hormonal control are well understood. It has been suggested that cervical ripening be considered an inflammatory reaction (4, 5), a process that is characterized by vasodilation, fluid accumulation in the extracellular compartment, migration of leukocytes through the blood vessels, and remodeling of collagen fibrils. Consistent with this view, relaxin-induced cervical softening in the rat is characterized by increased cross-sectional area of blood vessels, increased tissue water content, and reduced density and organization of collagen fiber bundles (6–8).

Eicosanoids such as prostaglandin E2(PGE2), PGF2α, 6-keto-PGF1α, PGD2, and thromboxane A2 serve as important autocrine and paracrine hormones that mediate many cellular functions (9, 10). There is recent evidence that PGE2 plays a major role in the inflammatory process in the rat. Selective neutralization of PGE2 with a monoclonal antibody for PGE2 blocked inflammation in carrageenin-induced rat paw inflammation (11). There is also evidence that cervical softening may be mediated, at least in some species, by local synthesis of eicosanoids and most notably PGE2. It was reported that cervical PGE2 production increases at delivery in sheep (12). Additionally, PGE2 administration was reported to promote softening of the cervix in rats (13), sheep (12, 14, 15), and humans (16–18). Intracervical or intravaginal administration of PGE2 is currently the chemotherapeutic approach used most frequently to promote ripening of the human cervix before delivery (5, 19, 20).

Cyclooxygenase (COX) is a key enzyme in the synthesis of eicosanoids. The nucleotide and amino acid sequences of two forms of COX (COX-1 and COX-2) have been determined in rats (21–23) and other mammalian species (24–28). COX-1 is constitutively expressed in tissues such as gut and kidney that require prostaglandins for normal physiological processes (29). COX-2 is an inducible form of the enzyme that is induced rapidly in response to inflammatory (30–33) and hormonal (34, 35) stimuli.

In view of the evidence that (a) the inflammatory process and relaxin-induced cervical softening share common characteristics, and (b) the eicosanoid PGE2 promotes inflammation and may also promote cervical softening, we hypothe-

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Address all correspondence and requests for reprints to: Dr. O. D. Sherwood, Department of Molecular and Integrative Physiology, University of Illinois at Urbana-Champaign, 524 Burrell Hall, 407 South Goodwin Avenue, Urbana, Illinois 61801. E-mail: od-sherw@uiuc.edu.
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sized that relaxin’s effects on the rat cervix are mediated through increased production of prostaglandins. The present study examined that hypothesis by blocking eicosanoid synthesis in relaxin-treated rats with the nonsteroidal antiinflammatory drug indomethacin, which is a potent nonselective inhibitor of both COX-1 and COX-2 (11, 30, 31, 36).

Materials and Methods

Animals

Relaxin-induced growth and softening of the cervix does not normally occur in nonpregnant rats. Nevertheless, nonpregnant rats were used for the present study for several reasons. First, it is possible to provide uniform doses of progesterone, estrogen, and relaxin and thereby limit variation within treatment groups. Second, it is possible to administer acutely a high dose of porcine relaxin that provides a marked increase in cervical wet weight and softening within 48 h of the initial relaxin injection (Whaley, J. E., and O. D. Sherwood, unpublished data). This short duration of relaxin treatment permits the use of a high dose of indomethacin that likely would be too toxic for a treatment period of 3 days or longer. Finally, by employing nonpregnant rats, it is possible to avoid the confounding effects that the high dose of indomethacin may cause through detrimental effects on the fetuses and placenta. Also, justifying the use of nonpregnant rats is the fact that relaxin’s effects on the rat cervix, uterus, and vagina have either been discovered or demonstrated in nonpregnant rats (1, 37).

Nonpregnant female Sprague-Dawley-derived rats were obtained at approximately 70 days of age from Harlan Sprague-Dawley, Inc. (Indianapolis, IN). Rats were housed in individual hanging wire cages (20 × 25 × 17 cm) in a temperature- (23–25 C) and light-controlled room, with an alternating 14 h of light (2100 h–1100 h) and 10 h of darkness. Rats were provided with free access to water and Teklad 6% Mouse/Rat Diet (Harlan/Teklad; Madison, WI). The animal experimentation described in this report was approved by the University of Illinois at Urbana-Champaign Laboratory Animal Care Advisory Committee.

Exp 1: determination of a suitable dose of indomethacin

The release of PGE₂ from the kidneys into the urine during a 24-h period was used to determine a dose of indomethacin that effectively blocks prostaglandin synthesis (32). Four groups of 86-day-old female rats (n = 3 or 4/group; 206 ± 3 g BW) were transferred to metabolism cages (Hoeltge Inc., Cincinnati, OH). At 0800 h, rats were administered 5, 10, or 20 mg/kg indomethacin (Sigma Chemical Co., St. Louis, MO) or indomethacin vehicle [2 ml of 0.5% methyl cellulose (Sigma) and 0.025% Tween 80 (Sigma) in water] via gavage. To stimulate urination, 6 ml of physiological saline was administered sc at the beginning of both periods of urine collection. Urine was collected from 0900 h–1600 h and again 8 h later from 0000 h–0800 h to determine the effectiveness with which indomethacin reduced kidney PGE₂ synthesis throughout the 24 h following its administration. The volume of urine was recorded for each animal. PGE₂ levels were determined by a previously described competitive enzyme immunoassay for PGE₂ (31, 38, 39). This enzyme immunoassay is based on competition between an acetylcholinesterase-PGE₂ linked tracer and unlabeled PGE₂ for a limited number of specific antibody binding sites. The procedure followed the instructions provided by the source of the competitive immunoassay kit and reagents (Cayman Chemicals, Ann Arbor, MI).

Exp 2: influence of indomethacin on relaxin’s biological effects—treatments

Twenty-six 78-day-old rats were anesthetized with ether and bilaterally ovariectomized via two dorsal muscle incisions (40). Rats were randomly assigned to one of three treatment groups: 1) ovariectomized (O), progesterone (P), and estrogen (E)-treated control group (group OPE, n = 8); 2) ovariectomized, progesterone, estrogen, and relaxin (R)-treated group (group OPER, n = 9); and 3) ovariectomized, progesterone, estrogen, relaxin, and indomethacin (I)-treated group (group OPERI, n = 9). See Fig. 1.

Progesterone and estrogen were administered to all 26 animals throughout the 10-day treatment period by following the procedure described by Burger and Sherwood (41). Because relaxin’s effects on the rat cervix are estrogen dependent (1), treatment with estrogen was necessary. Moreover, it was considered advantageous to use a procedure that provides serum levels of both progesterone and estrogen that are similar to those during the second half of rat pregnancy. In brief, progesterone capsules were constructed from 52 mm lengths of SILASTIC brand silicon tubing (Dow Corning, Midland, MI; id 1.5 mm, od 1.9 mm).

![Diagram of the experimental design. See Materials and Methods for details.](image-url)

**Fig. 1.** Diagram of the experimental design. See Materials and Methods for details.

**Fig. 2.** Mean (+SE) rate of release of PGE₂ into the urine (A) 2–8 h and (B) 16–24 h following the administration of indomethacin. Mean (+SE) volumes of urine collected for periods A and B were 4.39 ± 0.63 ml and 4.74 ± 0.10 ml, respectively. Asterisks (*, P < 0.05; **, P < 0.01) indicate values that differ from the indomethacin vehicle control. n = 3 or 4 rats per group.
Fig. 3. Effects of indomethacin on physiological determinants other than the reproductive tract. A, Rate of release of PGE$_2$ into the urine before (B, 0900 h–1600 h on day 7) and after (A, 0900 h–1600 h on day 9) the administration of indomethacin and/or relaxin. Mean (±SE) volumes of urine collected for the periods A and B were 9.03 ± 0.72 ml and 9.73 ± 0.97 ml, respectively. Asterisks (**, P < 0.01) indicate a difference from the before indomethacin control. B, Hematocrit. Asterisks (**, P < 0.01) indicate differences from the OPE and OPER groups that were treated with indomethacin vehicle. C, Body weight. All values are means (+SE). The number of rats per group is indicated at the base of each bar.

The tubing was sealed at one end with a 1-mm SILASTIC glue plug (Dow Corning), filled with 60 mg crystalline progesterone (Sigma), and the open end was closed with a glue plug. Progesterone implants were rinsed twice in 100% ethanol and stored at room temperature. On the day before ovariectomy, progesterone implants were soaked in PBS (0.14 μM NaCl, 0.01 M Na$_2$PO$_4$, pH 7.0) at 25 C overnight. Immediately after ovariectomy on day 1, two capsules were inserted sc over each flank with slight variations (44). In brief, each cervix was suspended between two metal hooks (1.3-mm diameter stainless steel), with the lower hook fixed in position and the upper mobile hook connected to a Grass FT03 force displacement transducer (Grass Instruments, Quincy, MA). The cervix, vagina, and uterus were removed, trimmed of fat and connective tissue, and weighed on an electronic analytical balance (H110; Sartorius Corp., Bohemia, NY). Cervices were placed in Krebs-Ringer bicarbonate buffer, pH 7.5, and maintained at 4 C until their extensibilities were determined immediately after tissue collection. The wet weights of the vagina and uterus were determined because they, like the cervix, are target tissues for relaxin in the rat (1, 37, 41, 43).

Cervical extensibility was determined as previously described with slight variations (44). In brief, each cervix was suspended between two metal hooks (1.3-mm diameter stainless steel), with the lower hook fixed in position and the upper mobile hook connected to a Grass FT03 force displacement transducer (Grass Instruments, Quincy, MA). The cervix was placed in a 60-ml organ bath containing Krebs-Ringer bicarbonate buffer, pH 7.5, which was oxygenated with 95% O$_2$–5% CO$_2$. The temperature was maintained at 37 C by circulating water through the outer chamber of the organ bath. Transducers were calibrated in grams before use, and tension generated within cervices were expressed in grams. Outputs from the transducers were recorded on a MacLab/4 data acquisition system. For each cervix, the distance between the two hooks was gradually increased until approximately 5 g tension was recorded. Tension generated within the cervical tissue was recorded continuously. Tension that developed at extension (initial extensibility) and 20 min after extension (final extensibility) was determined. Linear regressions of grams of tension per millimeters of extension were used to compare the effects of treatment on the tensile properties of the cervix.

Exp 2a: influence of indomethacin on cervical and vaginal PGE$_2$ levels

Because cervices obtained with Exp 2 were used to determine their extensibility, they could not be used to determine the effectiveness with which indomethacin lowered cervical PGE$_2$ levels. Accordingly, 25 non-pregnant rats were treated as described for groups OPE, OPER, and OPERI in Exp 2 except that cervical and vaginal tissue was collected 8 h after the initial injection of relaxin on day 8 of treatment. The cervix and vagina were removed 8 h following the initiation of relaxin administration because previous studies demonstrated that inflammatory me-
Dihydroxy-1,25-vitamin D₃ elevates PGE₂ as well as COX-1 and COX-2 messenger RNA levels maximally 5–12 h following an inflammatory insult in rats (9, 28, 29, 31). Moreover, we found that relaxin induces a significant increase in cervical wet weight within 8 h of its administration to nonpregnant rats (Whaley, J. E., and O. D. Sherwood, unpublished data).

Immediately after their removal, the cervix and vagina were weighed and frozen in liquid nitrogen. The tissues were stored in liquid nitrogen until they were processed for PGE₂ determinations. To extract PGE₂ from the tissues, each cervix and vagina was pulverized individually in a stainless steel chamber that was maintained at −195°C with liquid nitrogen. The pulverized tissue was transferred to 15 ml thick-walled Corex centrifuge tubes (Corning, Inc., Corning, NY), and 70% ethanol was added at a 20:1 (vol/wt) ratio. The tissue was sonicated for 20 sec (Vibra Cell; Sonics Materials, Danbury, CT) and then agitated for 1 h at 4°C on a shaker (Eberbach Corp., Ann Arbor, MI). The extract was separated from the tissue residue by centrifugation at 17,000 × g for 30 min at 4°C. The extract was transferred to 12 × 75 mm disposable culture tubes (Fisher Scientific) and evaporated under a stream of nitrogen at 45°C with a Multivap analytical evaporator (Organomation Associates Inc., South Berlin, MA). The extract was dissolved in 200 µl of PBS and frozen until PGE₂ levels were determined.

Statistical analysis

Differences among groups were compared by ANOVA and Tukey's test (45).

Results

Exp 1: determination of a suitable dose of indomethacin

Figure 2, A and B, shows the influence of increasing doses of indomethacin on the release of PGE₂ from the kidney into the urine 2–8 h and 16–24 h following indomethacin treatment, respectively. The administration of 10 and 20 mg indomethacin/kg BW markedly reduced PGE₂ release (P < 0.05) during both urine collection periods. A decision was made to administer 20 mg indomethacin per kg body weight once per day because this dose of indomethacin inhibited the release of PGE₂ from the kidneys most effectively.
**Exp 2: influence of indomethacin on urine PGE₂ and animal health**

Within groups OPE and OPER, there was no significant difference in the rate of release of kidney PGE₂ before (day 7) and after (day 9) the administration of indomethacin vehicle (Fig. 3A). The rate of release of PGE₂ was markedly reduced \( (P < 0.01) \) following the administration of indomethacin to group OPERI. The administration of 20 mg/kg dose of indomethacin had deleterious effects on the rats. Group OPERI animals had intestinal lesions, gastrointestinal bleeding, and a dramatically reduced hematocrit \( (P < 0.01; \) Fig. 3B). The body weights of the three treatment groups did not differ on day 10 of treatment (Fig. 3C).

**Exp 2: influence of indomethacin on cervical growth and softening**

The mean wet weight of cervices in relaxin-treated group OPER rats was nearly twice \( (P < 0.01) \) that in the relaxin-deficient group OPE control rats, but it did not differ from the mean wet weight of cervices in group OPERI rats, which were treated with indomethacin as well as relaxin (Fig. 4A). Consistent with those findings, the mean slope of tension generated with extension in cervices obtained from group OPER rats was approximately half \( (P < 0.01) \) that in cervices from group OPER rats, and it did not differ from that in cervices obtained from group OPERI rats both at extension (Fig. 4B) and 20 min after extension (Fig. 4C).

Results obtained with both the vagina and uterus are consistent with those obtained with the cervix. The mean wet weights of both the vagina (Fig. 5A) and the uterus (Fig. 5B) were greater in group OPER than in group OPE \( (P < 0.01) \), but they did not differ from those in group OPERI.

**Exp 2a: influence of indomethacin on cervical and vaginal PGE₂ levels**

Consistent with its effects on urine PGE₂ (Fig. 3A), administration of 20 mg indomethacin per kg body weight inhibited both cervical (Fig. 6A) and vaginal (Fig. 6B) PGE₂ by more than 90%. Also consistent with Exp 2 (Figs. 4A and 5A), relaxin-induced increases in the wet weight of the cervix (Fig. 6C) and vagina (Fig. 6D) were not inhibited by indomethacin. The mean wet weights of the cervix (Fig. 6C) and vagina (Fig. 6D) in groups OPER and OPERI did not differ.

**Discussion**

This report provides evidence that relaxin-induced growth and softening of the rat cervix are not mediated through prostaglandins. Because relaxin plays a major role in promoting the cervical softening that occurs during late pregnancy, the present findings indicate that cervical softening in...
the rat is not an inflammatory process mediated by prostaglandins.

The present study also provides evidence that relaxin-induced growth of the vagina and uterus are not mediated through eicosanoids. There is evidence that relaxin-binding sites (putative relaxin receptors) are found associated with the same cell type in the cervix, vagina, uterus, and mammary nipples in rats and pigs (43, 46–48), and we have postulated that relaxin brings about its effects in different target tissues, at least in part, by a common mechanism (46–48). The findings in this study are consistent with that hypothesis.

The conclusion that relaxin does not mediate its effects through prostaglandins is dependent upon indomethacin having effectively inhibited cyclooxygenase activity in this study. That was the case. The high dose of 20 mg/kg BW inhibited urinary and tissue (cervix and vagina) PGE2 by more than 90%. Moreover, after 2 days of treatment with indomethacin, we found intestinal lesions, gastrointestinal bleeding, and a markedly reduced hematocrit; all typical side effects caused by the inhibition of COX-1 in the gastrointestinal tract. In spite of the fact that prostaglandin synthesis was nearly completely inhibited, we failed to detect any changes in relaxin-induced cervical growth or softening.

It is established that relaxin plays a major role in promoting the growth and softening of the cervix that occurs during the second half of pregnancy in rats (2, 8, 41). Whereas this study provides evidence that relaxin’s effects on the rat cervix are not mediated through prostaglandins, there is reason to avoid extrapolation of the present findings to other species. It remains to be demonstrated that relaxin plays a major role in promoting cervical softening during late pregnancy in species such as the sheep, cow, goat, and human being. It is possible that cervical softening in these and other species is mediated, at least in part, by hormones other than relaxin and by eicosanoids.

Further investigation is needed to understand the molecular mechanism(s) underlying cervical softening in the rat and other species. Relaxin may mediate its effects on cervical softening in the rat through induction of the enzyme nitric oxide synthase (NOS) and subsequent increased production of nitric oxide (NO). It was recently reported that cervical NOS levels increase during labor in the rat, and inhibition of NOS with the inhibitor L-nitro-arginine methyl ester (L-NAME) prolongs delivery and reduces cervical extensibility (49). Bani Sacchi and co-workers reported that relaxin’s effects on coronary blood flow (50) and mast cells (51) in the physiological and therapeutic significance of separate pathways for prostaglandin synthesis. In: Folco GC, Samuelsson B, Maclous L, Velo GP (eds) Eicosanoids: From Biotechnology to Therapeutic Applications. Plenum Press, New York, pp 3–36.

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