Determination of the Source of Relaxin Immunoreactivity during Pregnancy in the Dog

Toshihiko TSUTSUI and Dennis R. STEWART¹⁾

Department of Reproduction, Nippon Veterinary and Animal Science University, 7-1, Kyonan-cho 1-chome, Musashino-shi, Tokyo 180, Japan and ¹⁾Division of Reproductive Biology and Medicine, School of Medicine, The University of California at Davis, Davis, California 95616, U.S.A.

(Received 25 April 1991/Accepted 21 August 1991)

ABSTRACT. We investigated the source of immunoreactive relaxin (IR) in the dog during pregnancy using the following: (1) controls, (2) dogs ovariectomized during pregnancy and maintained on progesterone, (3) dogs hysterectomized during pregnancy or immediately postpartum, (4) corpora lutea, uteri, and placentas collected at various times during pregnancy for determination of IR, and (5) maternal (ovarian and uterine vein) and fetal (cardiac) blood and amniotic and allantoic fluid. Plasma IR patterns remained unchanged in animals subjected to ovariectomy, whereas in the controls IR was first detected at about Day 20 of pregnancy, peaked on Day 35, and was then remained at the same level until parturition. Hysterectomy, on the other hand, resulted in IR values becoming undetectable within 2 to 3 days. The highest tissue concentrations of IR were found in the placenta. These findings indicate that the source of relaxin in the pregnant dog is the placenta.—**KEY WORDS:** dog, placenta, relaxin.

J. Vet. Med. Sci. 53(6): 1025–1029, 1991

Relaxin is a peptide hormone produced during pregnancy in many species. The corpus luteum, placenta, and uterus have been most frequently cited as the source of relaxin. In the pig, relaxin appears to be produced in the corpus luteum [3, 11]. In humans the corpus luteum is the major source of circulating relaxin [22, 23], although small amounts of relaxin have been isolated from term placentas [9, 24] and decidua [6, 7]. In the rat, the corpus luteum is the major source of relaxin [2, 4], although the placenta appears to be needed for corpora lutea to produce relaxin [10]. The placenta has been determined to be the source of relaxin in the rabbit [8], mare [19] and cat [1].

Relaxin immunoreactivity has been recently reported during pregnancy and lactation in the dog [15, 16]. Relaxin is first detectable in plasma in the third or fourth week of pregnancy, with the peak plasma concentration observed 2–3 weeks before delivery. Relaxin is also detectable for up to nine weeks of lactation.

This study was designed to determine the source of immunoreactive relaxin (IR) in the bitch. This was done by determining plasma concentrations of relaxin following ovariectomy or hysterectomy and by analyzing various tissues including the corpus luteum, placenta, uterus, amniotic and allantoic fluid, and maternal uterine and fetal blood.

MATERIALS AND METHODS

Animals : The subjects consisted of 2 to 3-year-old Beagle bitches linebred in the laboratory of one of the authors at Nippon Veterinary and Animal Science University and mixed breed bitches; all animals weighed approximately 10 kg.

The bitches were tested daily for estrus with a male following the first physical signs of proestrus, i.e., vulvar swelling and bleeding. Ovulation was presumed to occur on day 3 of estrus. Mating was permitted only once, on day 5 of estrus, i.e., on the second day after the presumed day of ovulation or the day that formation of the first polar body was expected to occur [20]. The day of mating was regarded Day 0 of pregnancy.

The dogs were randomly divided into a control and four experimental groups. The control group (CON) consisted of 10 animals whose blood was collected throughout pregnancy. The animals in the second group were bilaterally ovariectomized (OVX) on Day 45 (n=1) or Day 50 (n=2) of pregnancy. In a third group of bitches hysterectomy (HYS) was performed on Day 35 and Day 40 of pregnancy and Day 3 postpartum (n=1 per day). The fourth group was used for removal of ovaries, fetoplacental units, and uteri (TISSUE) on Days 21, 23, 35, 47 and 49 of pregnancy (n=3 per day), and placentae were also collected from CON animals on the day of parturition.

A fifth group (UT-OVAR-FET) was used to obtain uterine vein blood, ovarian vein blood, fetal heart blood, amniotic fluid and allantoic fluid on Days 21–60 of pregnancy (n=21). Two bitches in the HYS group and 5 bitches in the TISSUE group were also used in this fifth group. Newborn puppy blood and fetal fluids were also collected from CON animals on the day of parturition.

Blood samples were collected in heparinized syringes by venipuncture of the anterior brachiocephalic vein from CON animals at 3 day intervals from Day 0 of pregnancy until 4 days postpartum. Additional samples were collected on Day 55–Day +4 postpartum to measure anticipated changes in IR concentrations at delivery. In both the OVX and HYS groups, blood was collected immediately before surgery and at 1 to 5 day intervals following surgery. Blood sampling in the TISSUE group was performed during laparotomy under general anesthesia. Fetal blood and fetal fluids were collected from the 4 animals following hysterectomy. Fetal blood samples were collected from the heart. Blood samples were taken from the jugular vein of the newborn puppy on the day of parturition.

Sampling in the UT-OVAR-FET group animals was performed during laparotomy and following hysterectomy.

Surgical procedures: Animals were presurgically treated with a combination of acepromazine and atropine sulfate. Ketamine and valium were administered intramuscularly (IM) to induce anesthesia, which was maintained with 1% halothane and oxygen. The OVEX animals received progesterone (20 mg IM) 24 hr prior to surgery and then daily until Day 61 of pregnancy. In the TISSUE animals, the uterus and ovary were placed on ice until the placentas were removed from the uterus and the CL dissected from the ovary. Tissues were frozen at -20°C until assayed.

Hormone assay: IR was determined using a porcine relaxin radioimmunoassay previously used to measure IR in the mare [17]. Briefly, highly purified porcine relaxin prepared in the author's laboratory was used for standards and iodination. Porcine relaxin lacks tyrosine, so iodination was achieved by first coupling relaxin with the Bolton-Hunter reagent (3-p-hydroxyphenyl-propionyl-N hydroxy-succinimide ester), followed by iodination using the chloramine-T method. Relaxin standards, prepared in male dog plasma, ranged from 0.025 to

10 ng. The rabbit anti-porcine relaxin antibody, #5859, was used at a final dilution of 1/22, 500 which yielded about 35% binding. Plasma samples were assayed in triplicates of 100 μl . The dose-response curve was transformed to logit-coordinates and the best-fit straight line was calculated by weighted least-squares linear regression. Weights were calculated by the method of Rodbar and Lewald [12] using an IBM-AT compatible computer with a computer program written by one of the authors. The least detectable dose was defined as the amount resulting in a response two standard deviations removed from the zero-dose response.

The assay had a least detectable level of 0.27 ng/tube or 2.7 ng/ml plasma. The intra- and inter-assay coefficients of variation were 3.3% and 7.8%, respectively.

Tissue analysis: Tissues were extracted by a modification of the aqueous extraction procedure described by Sherwood [13]. Tissue samples were weighed and homogenized (Tekmar tissue homogenizer) in phosphate-buffered saline (pH 7.5) at 4°C using a ratio of 3 ml/g for placentas, 15 ml/g for uteri and 30 ml/g for corpora lutea. In order to equalize handling losses, different ratios were used so that the total volume of each tissue was nearly the same. Samples were centrifuged at $10,000 \times g$ for 30 min at 4°C , and supernatants were saved for assay. IR was determined in four serial tenfold dilutions of the samples made in PBS 5% BSA in order to obtain IR's within the range of the standard curve. The dilution falling nearest the midpoint of the standard curve was used to calculate concentrations. Values are expressed as ng IR/gm starting tissue. Standards for tissue analysis were also made in this buffer.

RESULTS

IR first became detectable about Day 20 of pregnancy in CON animals when concentrations rose above background (Fig. 1a). IR concentrations increased to 15 to 20 ng/ml by about Day 35 of pregnancy with values, thereafter remaining constant throughout the rest of pregnancy. Following parturition, IR concentrations declined markedly but remained above the level of detection for 4 days postpartum (Fig. 1b).

There were no changes IR concentration throughout pregnancy in the three bitches ovariectomized during pregnancy (Fig. 2), with values declining only after parturition. Hysterectomy, on the other hand,

Fig. 1. Relaxin immunoreactivity ($\pm$ SEM) in 10 dogs during pregnancy. Data are arranged according to gestation date (a) with Day 0 being the day of breeding and (b) according to the day of parturition. The vertical lines indicate the day of breeding or parturition and the horizontal dashed line indicates the limits of detection.

Fig. 2. Plasma relaxin immunoreactivity in dogs ovariectomized (OVX) during pregnancy. No decrease in plasma relaxin immunoreactivity was seen until after delivery. WHELP=parturition

resulted in an immediate decline in plasma IR (Fig. 3) with concentrations becoming undetectable by 48 to 72 hr after surgery.

All tissues yielded low or undetectable levels of relaxin in the Day 21 samples (Fig. 4). The Day 23 placentas had intermediate amounts of IR, while

Fig. 3. Plasma relaxin immunoreactivity in dogs hysterectomized (HYS) during gestation (animals B195 and B201) or after delivery (S-205). Relaxin immunoreactivity fell abruptly following tissue removal and reached baseline within three days.

Fig. 4. Relaxin immunoreactivity in luteal, uterine and placental tissues during various stages of pregnancy in the dog. The number above each placental category is number of individual placentae used to obtain value.

Day 35 through term placentas had the highest amounts. IR remained constant in the corpus luteum and uterus from Days 23 through 49 at concentrations below the Day 23 (and subsequent) placenta concentrations. IR values were higher in placentas than corpora lutea or uteri at all times during pregnancy from Day 35 on.

IR concentrations in uterine and ovarian venous blood, fetal blood and fetal fluids are shown in Fig. 5. The earliest detection of relaxin was in uterine venous blood at Day 23. Concentrations of IR in ovarian venous blood were about 1/3 uterine venous blood levels on Day 30; from Day 40 through term, IR concentrations were similar in ovarian and uterine venous blood. Sufficient fetal blood for IR analysis was not available until Day 45. IR concentrations were always less in fetal blood, ranging from

Fig. 5. Relaxin immunoreactivity in maternal blood obtained from the heart or jugular vein (PE), ovarian vein (OV) and uterine vein (UT), fetal blood obtained from the heart (FE) and fluid obtained from the amniotic (AM) and allantoic (AL) cavities. ND=not determined. *Day of pregnancy. **On the day of parturition.

about 1/4 to 2/3 of maternal blood levels.

DISCUSSION

The IR pattern of dogs ovariectomized during pregnancy whose pregnancies were maintained with exogenous progesterone remained unchanged throughout pregnancy. This suggests that the ovaries do not contribute significantly to plasma IR. IR concentrations declined precipitously in animals which were hysterectomized even though their ovaries remained in place. Taken together, these findings suggest the ovaries do not secrete significant amounts of relaxin in the pregnant dog, and the analysis of corpora lutea for IR content revealed that relaxin is not stored in luteal tissue as in the pig [3, 5].

Hysterectomy caused an immediate decline in IR to undetectable values within 2–3 days following surgery. This indicates that at least one of the components of the fetus, placenta and/or uterus is the source of relaxin secretion in the pregnant dog. In addition, the fact that the ovaries were not removed in these animals indicates that the ovaries are not the source of relaxin during pregnancy in the dog. Based on the tissue analyses, IR was constant in uterine tissue throughout pregnancy, while concentrations in placental tissues increased dramatically between Days 23 and 35; values were 2 to 5 times

higher in placental tissues than the luteal and uterine values for the duration of pregnancy. This implies that placenta is the principal source of relaxin in the dog. Plasma IR levels were well correlated with placental immunoreactivity suggesting that the placenta is actively secretes IR rather than simply serving as storage tissue as in the case of the corpora lutea of the pig [3, 5, 14]. The amount of IR found in luteal and uterine tissues beginning with Day 23 of gestation probably represent hormone contained in the blood within the tissue and/or receptor bound relaxin rather than tissue production.

IR first became detectable in uterine vein blood between Days 21 and 23 of pregnancy. Implantation in a dog mated on the day of formation of the first polar body, i.e., the first possible day of oocyte fertilization, occurs on the 18th day post-copulation [21]. The absence of IR in the blood until a few days following implantation is indirect evidence that the fetoplacental unit is responsible for relaxin secretion. This also indicates that relaxin secretion begins soon after implantation in the dog. It is unclear why IR concentrations were not higher in the uterine vein blood than in the peripheral blood of the bitches at the various times samples were collected during pregnancy. IR concentrations were higher in uterine venous than ovarian venous blood on Day 30, but this was the only time during pregnancy that such a difference existed between uterine venous

and either the peripheral or ovarian venous blood of the bitch.

The IR profile reported here is slightly different from the one reported by Steinetz *et al.* [15] in that maximal concentrations in the current study occurred between weeks 4 and 5 of pregnancy vs weeks 5 and 6. Our finding of an initial increase in IR occurring between Days 20 and 35 is similar to patterns we have observed in the cat [18]. In addition, IR declined from week 6 through term in the Steinetz *et al.* [15] study, whereas values remained constant from week 5 through parturition in our own study. Peak values in the Steinetz *et al.* [15] study were 5 ng/ml and in the current study, 20 ng/ml. The differences are likely to be attributable to the different assay systems employed. Both systems are heterologous assays, but different anti-porcine antibodies were utilized.

Steinetz *et al.* [15] reported that IR can be detected in the blood of lactating Laborador retrievers until 9 weeks postpartum. One explanation could be that a portion of the canine placenta is retained postpartum, and that this tissue may continue to secrete relaxin for sometime. Our study was not designed to rule out an ovarian source during lactation. There is also the possibility that relaxin is produced by the mammary glands.

ACKNOWLEDGEMENT. The authors wish to express their sincere gratitude to George H. Stabenfeldt, D. V. M., Ph. D., U. C. Davis, for his valuable advice.

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