Gestational changes in calbindin-D9k in rat uterus, yolk sac, and placenta: Implications for maternal–fetal calcium transport and uterine muscle function

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ABSTRACT Calbindin-D9k was quantified and its cellular location was defined in uterus, yolk sac, and placenta. In late gestation (days 17 to term) coordinated induction of calbindin-D9k was seen in uterine epithelial lining cells and the juxtaposed yolk sac visceral epithelium as well as the intraplacental yolk sac epithelium. The induction of calbindin-D9k in these cells coincided with the time of exponential fetal bone growth and maximal fetal accumulation of calcium, suggesting a role of the protein in these epithelial layers in maternal–fetal calcium transport. Dynamic changes also occurred in the calbindin-D9k contents of the two layers of uterine smooth muscle (outer longitudinal and inner circular) during mid- and late gestation. During early pregnancy (days 0–4), calbindin-D9k was present in the two smooth muscle layers. By midgestation (day 10), calbindin-D9k had decreased by a factor of 10 in these tissue layers. During late gestation calbindin-D9k rebounded in the inner circular smooth muscle layer. These uterine changes of early and midgestation were reproduced by the endocrine changes of pseudopregnancy. Progesterone appeared to be a good candidate for controlling the midgestational decrease of uterine muscle calbindin-D9k as it blunted estrogen's induction of the protein in the muscle layers and stroma in a dose-dependent manner. Changes in myometrial calbindin-D9k may reflect variations in muscular calcium storage, thereby representing alterations in potential for contraction.

After weaning, the 9-kDa calcium binding protein (calbindin-D9k) is found in high concentrations in the calcium transporting epithelia of the mouse and rat duodenal mucosa and the distal convoluted tubule of mouse kidney (1–6). The presence of this cytoplasmic protein is a marker for active transcellular calcium transport, and the expression of calbindin-D9k in these epithelial tissues is dependent on the hormonal action of 1,25-dihydroxyvitamin D₃ [1,25(OH)₂D₃] (7).

Calbindin-D9k is also found in uterine myometrium and endometrial stroma of the mature nonpregnant female where both estrogen and progesterone action can control its abundance (8, 9). The function of calbindin in uterine muscle is unknown; thus far myometrium is the only muscle found to contain calbindin-D9k.

Before birth, calbindin-D9k is found in transporting epithelia of the yolk sac and intraplacental yolk sac (IPYS) and in the luminal epithelium of the maternal uterus (9–13). By analogy to the intestinal and renal epithelia, calbindin-D9k in these epithelial cells is thought to play a role in transcellular calcium movement (9). The hormonal control of calbindin-D9k expression in these epithelial tissues is complex. Studies in thyroparathyroidectomized animals and in vitro experiments have shown effects of 1,25(OH)₂D₃ on calbindin-D9k content in fetoplacental tissue (11, 14). The key steroid hormones estrogen and progesterone regulate the expression of calbindin-D9k in the uterine myometrium and stromal cells, but they do not induce calbindin-D9k in the uterine epithelial cells (8, 9). In the epithelial cells, a pregnancy-specific hormonal state may be critical as recent data using immunochemical techniques (9) and cDNA probes (13) suggest that calbindin-D9k is expressed in these cells only during pregnancy.

We recently proposed a model of maternal–fetal calcium transfer in rats (9). Reminiscent of egg shell formation, our working hypothesis is that calcium is transported across the uterine epithelial cells of lower mammals into the uterine lumen where it is available to the yolk sac and IPYS. Calbindin-D9k in the yolk sac and IPYS epithelium subserves transcellular movement of calcium from the uterine lumen to the fetal blood vessels. In lower mammals, this uterine luminal pathway is important for other nutrients such as immunoglobulins, amino acids, transferrin, and vitamin B₁₂ (15–17).

The present studies were undertaken to characterize the gestational changes of epithelial calbindin-D9k in the rat uterus, placenta, and yolk sac and its control by maternal factors. An understanding of the gestational appearance of calbindin-D9k in uterine epithelial cells provides clues to (i) factors involved in triggering this expression and (ii) the protein's functions in nutrient transport and uterine muscle function. To dissect the specific role of maternal factors in control of uterine calbindin-D9k content, we used a pseudopregnant rat model that mimics the early maternal hormonal changes of pregnancy without the presence of a fetoplacental unit (18–21).

MATERIALS AND METHODS

Animals. Timed pregnant rats (Sprague–Dawley) were obtained from Dominion Laboratories (Dublin, VA). The day after mating was considered day 1 of gestation. Pseudopregnancy was achieved in nonpregnant rats by applying electrical stimulation to the cervix followed 4 days later by an endometrial scratch (18–21). The day after the uterine scratch was called day 1 of pseudopregnancy. Effects of progesterone were studied in 21-day-old weanling female rats. Estradiol-17β (Research Plus Steroid Laboratories, Denville, NJ) and progesterone (Sigma) were injected subcutaneously (9). Progesterone (or vehicle) was administered once daily for 3 days at the doses indicated. On the last 2 days of treatment, rats were given once-daily injections of estradiol (0.1 μg/
day). The uterus was excised ≈18 hr after the last dose of hormone(s).

**Tissue Fixation and Immunohistochemical Staining.** Tissues were fixed by freeze substitution (22) and embedded in paraffin. The streptavidin/biotin technique was used as described (9). The primary rabbit antiserum (to rat intestinal calbindin-D<sub>9k</sub>) was diluted 1:3000 in Tris-buffered saline. Two types of controls were used: slides incubated with normal rabbit serum (1:3000), and slides incubated with antiserum that contained rat calbindin-D<sub>9k</sub> (3.3 µg/ml). Immunohistochemical staining intensity was scored as follows: 0, no staining; 1, mild or spotty staining; 2, moderate staining; 3, heavy staining. Sections to be compared with each other were stained in parallel. Scoring of changes during gestation was confirmed independently by a second observer; the observers' (M.E.B. and C.L.M.) means result were highly correlated (r = 0.91; n = 44 for 4 tissue layers at 11 time points; see Fig. 2) and the grand means (±SD) agreed within 0.3 (0.5 ± 0.7 vs. 0.8 ± 0.8).

**Quantitative Immunooassays.** Tissue samples, obtained under ether anesthesia, were homogenized as described (9) in buffer that contained 2 mM phenylmethylsulfonyl fluoride and aprotinin (0.2 trypsin inhibitor units/ml). The 40,000 × g (20 min) supernatants were used for assays of total protein (23) and of immunoreactive calbindin-D<sub>9k</sub> (9). The antiserum was prepared against rat intestinal calbindin-D<sub>9k</sub> purified as described earlier but with an additional preparative gel electrophoresis step using EDTA in the electrophoresis buffer (24). The interassay coefficient of variation was 7–9% in the range of concentrations to which samples were diluted prior to analysis (17–170 µg/ml).

**Other Methods.** The general linear model program of SAS (SAS Institute, Cary, NC) was used. The significance of changes with gestational age, or with dose of hormone, was tested by analysis of variance. The t test for unpaired samples with Bonferroni correction was used to assess the significance (at the P = 0.05 level) of differences between mean values. Data are presented as means ± SEM.

**RESULTS**

**Gestational Changes of Calbindin-D<sub>9k</sub> in the Utero–Placental Unit.** The calbindin-D<sub>9k</sub> concentrations in uterus, yolk sac, and placenta changed markedly and significantly (P < 0.0001–0.005) during normal gestation (Fig. 1). The uterus was the most dynamic and complex of the tissues studied and showed a significant (P < 0.0001) biphasic response. The uterine content of calbindin-D<sub>9k</sub> was high before mating and during early gestation, was significantly lower by day 7 of gestation, and increased significantly in late gestation between days 15 and 21 (Fig. 1). During late gestation, yolk sac calbindin-D<sub>9k</sub> also increased markedly and placental calbindin-D<sub>9k</sub> increased from undetectable to >0.5 µg per mg of placental protein (Fig. 1).

For insights into the function and hormonal control of utero–placental calbindin-D<sub>9k</sub>, we determined the cellular localization of the protein during gestation (Figs. 2 and 3). The concentration of uterine calbindin-D<sub>9k</sub> measured by quantitative immunoassay (Fig. 1 Upper) correlated closely with the immunohistochemical staining intensity (Fig. 2). Nonpregnant age-matched uteri contained a high concentration of calbindin-D<sub>9k</sub> (2.7 ± 0.4 µg per mg of protein; n = 11) and displayed intense staining that was localized to the myometrium (both inner circular and outer longitudinal muscle layers) and stroma. The luminal epithelial cells were negative in all nonpregnant animals. By day 7 of gestation, uterine calbindin-D<sub>9k</sub> content had decreased significantly (vs. control or day 4) to 0.6 ± 0.3 µg per mg of protein (n = 6) and reached a nadir of 0.2 ± 0.1 µg per mg of protein (n = 6) at day 11 of gestation. This decrease by a factor of 10 in uterine calbindin-D<sub>9k</sub> noted in early and midgestation corresponded to a decrease in the immunohistochemical calbindin-D<sub>9k</sub> signal in both the inner circular and outer longitudinal muscle layers and in the endometrial stroma (Figs. 2 and 3). By the end of pregnancy (21 days of gestation), when uterine calbindin-D<sub>9k</sub> had increased (significantly) almost 10-fold from midgestation levels to 2.0 ± 0.2 µg per mg of protein (n = 4), the protein reappeared in only one muscle layer, the circular myometrium, and appeared for the first time in high amounts of luminal epithelial cells (Figs. 1 and 2). These results are consistent with a role for calbindin-D<sub>9k</sub> in the regulation of smooth muscle cell activity during pregnancy.

![Fig. 1. Calbindin-D<sub>9k</sub> during normal gestation in rats. The number of samples in each group was 4–11. Day 0 represents age-matched nonpregnant females.](image1)

![Fig. 2. Immunohistochemical staining intensity of uterine calbindin-D<sub>9k</sub> during rat pregnancy. The number of pregnant animals for each determination was 3 except on day 19 (1 observation). Controls (day 0) represent determinations on 11 age-matched nonpregnant female rats.](image2)
columnar epithelial cells first became positive at day 18 of gestation (Fig. 2) and remained positive through parturition. Thus the studies revealed two characteristics of the late-pregnant state, the appearance of calbindin-D$_{9k}$ in uterine epithelium and the differential appearance of the protein in the circular but not longitudinal muscle (days 18–21).

A third characteristic of the late-pregnant state (days 17–18) was the significant ($P < 0.005$) increase of placental calbindin-D$_{9k}$ (Fig. 1) in the IPYS lining the sinuses of Duval (12). At day 15, no placental calbindin-D$_{9k}$ was detected; by day 17, the protein was observed in total placental extracts. By day 21, a statistically significant induction of placental calbindin-D$_{9k}$, ~2-fold, was seen (Fig. 1), and the protein was localized within the IPYS (Fig. 4). This immunohistochemical localization of rat calbindin-D$_{9k}$ in the IPYS during late gestation (Fig. 4) agreed with our previous detailed placental localization studies of calbindin-D$_{9k}$ in mouse placenta (12). The high concentration of placental immunolabeling (Fig. 4) was confined to columnar epithelial cells of the IPYS, which form one side of the endodermal sinuses adjacent to fetal vessels. In late gestation, these placental epithelial cells containing calbindin-D$_{9k}$ are in direct contact with the uterine lumen on one side and face fetal vessels on the other.

Coordinated with the induction of calbindin-D$_{9k}$ in uterine epithelial cells and placental IPYS was the further ($P < 0.005$) induction of visceral yolk sac calbindin-D$_{9k}$ (Fig. 1). Immunohistochemical localization studies indicated that the protein was present in the visceral epithelial cells of the yolk sac (Fig. 4). This agrees with previous studies of the mouse (12) and rat (10) yolk sac. In mice (11, 25), calbindin-D$_{9k}$ appears in midgestation (days 10–11) in the visceral epithelial yolk sac. The present study in rats (Fig. 1) shows a statistically
significant increase in visceral yolk sac calbindin-D$_{9k}$ between days 15 and 21. All the immunoreactivity was localized to the epithelial cells as shown in Fig. 4. These cells, like the IPYS, are in direct contact with the uterine lumen on one side and fetal blood spaces on the opposite side.

Specific Localization of Calbindin-D$_{9k}$ in Uterine But Not Vascular Smooth Muscle. Immunolocalization (Fig. 3) demonstrated calbindin-D$_{9k}$ in the two muscle layers (outer longitudinal and inner circular) of the uterus. Fig. 5 demonstrates the specificity of this staining for uterine smooth muscle, as vascular smooth muscle, between the stained outer longitudinal muscle layer and the stained inner circular muscle layer, was unstained.

Endocrine Control of Uterine Calbindin-D$_{9k}$. To dissect maternal and fetal endocrine factors involved in controlling uterine calbindin-D$_{9k}$, we use the pseudopregnant rat model. On day 4 of pseudopregnancy (Fig. 6), the calbindin-D$_{9k}$ content was decreased significantly to $\frac{1}{6}$ of control values and remained significantly decreased through the end of the study (day 9). Immunolocalization showed that calbindin-D$_{9k}$ content decreased to trace levels in the uterine layers (outer longitudinal and inner circular) and also in the stromal layer. Calbindin-D$_{9k}$ was not present in the uterine epithelium. Thus, pseudopregnancy completely mimicked the changes in uterine calbindin-D$_{9k}$ seen during normal early to midpregnancy.

High levels of progesterone are observed during pseudopregnancy and may be an endocrine factor in decreasing calbindin-D$_{9k}$ content (19). Our previous studies indicated that estradiol produced a dose-dependent increase of myometrial calbindin-D$_{9k}$ in immature rats (9). We therefore studied the dose dependency of progesterone's ability to block the effect of estradiol treatment in immature rats. Fig. 7 summarizes this experiment and demonstrates that progesterone produced a dose-dependent ($P < 0.0001$) inhibition of estradiol induction of uterine calbindin-D$_{9k}$ (Fig. 6). Statistically significant inhibition was observed at doses of 1.0 and 5.0 mg of progesterone per day administered for 3 consecutive days.

**DISCUSSION**

Endometrial Calbindin-D$_{9k}$. The present studies establish that calbindin-D$_{9k}$ appears in the uterine epithelium only during late pregnancy at a time that is critical for growth of the fetal skeleton. Our earlier studies showed that calbindin-D$_{9k}$ was present in term pregnant rat uterine epithelium but not in mature nonpregnant rat uterine epithelium (9). This induction during pregnancy of uterine calbindin-D$_{9k}$ suggests to us that, as in birds, large amounts of calcium are transported to the rat fetus via the uterine lumen and yolk sac (placenta). The induction of uterine epithelial calbindin-D$_{9k}$ between day 18 and term, coincided with the appearance of calbindin-D$_{9k}$ in the intraplacental yolk sac (Fig. 1) at day 17 through term. Although visceral yolk sac calbindin-D$_{9k}$ appeared earlier (by day 10–11) (11, 25), a marked increase occurred between day 17 and term. The coordinated increases of calbindin-D$_{9k}$ in these three epithelial layers (endometrial, visceral yolk sac, and IPYS) suggest that a common hormonal signal modulates calbindin-D$_{9k}$ expression in these juxtaposed layers during late pregnancy (9). As shown earlier by Comar (26), day 17 through term is a period of exponential fetal growth. These last 5 days of pregnancy account for $>99\%$ of fetal calcium deposition. Thus, epithelial calbindin-D$_{9k}$ induction in the total utero–placental unit (uterus, yolk sac, and placenta) coincides with the time of maximum fetal calcium demands for bone growth.

**FIG. 6.** Calbindin-D$_{9k}$ in pseudopregnant rat uterus. (Upper) Total immunoreactive calbindin-D$_{9k}$ in uterus of the pseudopregnant rat. The number of control age-matched nonpregnant rats was 14. Each value for the pseudopregnant rats represents the mean of four to five animals. (Lower) Immunochemical staining intensity of uterine calbindin-D$_{9k}$ during pseudopregnancy in both layers of myometrium (circular and longitudinal). The numbers of animals on days 4, 6, and 9 were 4, 3, and 2; 11 age-matched females were used for day 0.

<table>
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<tr>
<th>Days after Endometrial Treatment</th>
<th>Pseudopregnant Uterus</th>
<th>Pseudopregnant Myometrium</th>
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<tr>
<td>1</td>
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**FIG. 7.** Dose dependency of progesterone effect on total immunoreactive calbindin-D$_{9k}$ in the immature uterus. Weanling rats were given progesterone doses as indicated daily for 3 days. For the last 2 days, once-daily injections of estradiol (0.1 μg/day) were added. Approximately 18 hr after the last dose, the uterus was excised. The number of animals in each group was seven or eight.
A key question is whether vitamin D controls or modulates the expression of calbindin-D9k in the juxtaposed epithelial layers of the uterus, yolk sac, and placenta. It is clear that sex steroids can control calbindin-D9k in the myometrium and endometrial stroma but these sex steroids alone cannot induce calbindin-D9k in uterine epithelium (9). Several experiments suggest that vitamin D directly or indirectly affects fetoplacental calbindin-D9k (11, 14). In vitro studies of yolk sac in organ culture showed a direct influence of 1,25(OH)₂D₃ on calbindin-D9k expression (11). The presence of 1,25(OH)₂D₃ receptors in placenta, yolk sac, and uterus (27-31) also suggests that vitamin D action is part of these tissues' biochemistry. On the other hand, the work of Halloran and DeLuca (32) and Brommage and DeLuca (33) suggested that vitamin D was not needed for fetal calcification and placental calcium transfer.

Myometrial Calbindin-D9k. The finding in this study of a biphasic fluctuation in myometrial calbindin-D9k during pregnancy was unexpected. The changes in calbindin-D9k may reflect changes in muscle function related to the cell's overall calcium storage and ability to contract. Before mating and during early pregnancy, high levels of myometrial calbindin-D9k are correlated with times when uterine contractions both propel sperm and ensure even spacing of embryos (34). The decrease of myometrial calbindin-D9k during midgestation coincides with relaxation and stretching of the uterus to accommodate fetal growth. Near term, reappearance of calbindin-D9k in the inner myometrium layers corresponds with the time of uterine contractions in preparation for parturition. These temporal associations are consistent with a role for calbindin-D9k in uterine contraction.

Endocrine Controls of Myometrial Calbindin-D9k. To investigate endocrine controls of uterine calbindin-D9k, we used the pseudopregnant rat. By inducing a pseudopregnant state, high levels of endogenous progesterone and prolactin can be achieved (19-21). Although the changes during pseudopregnancy are not identical to those of true pregnancy, the corpus luteum continues to function and, despite the lack of a fetal placental unit, the maternal endocrine state is similar to that of true pregnancy, lasting up to 14 days (19-21). As shown in Fig. 5, calbindin-D9k decreased markedly in both muscle layers of myometrium and in the endometrial stroma, and no calbindin-D9k appeared in the epithelial lining cells. These changes were similar to the normal decrease in calbindin-D9k seen during the 1st 2 weeks of true pregnancy. We conclude that the maternal endocrine state is sufficient to cause this pregnancy-induced uterine modulation.

Progesterone is a hormonal candidate for controlling the decreased expression of calbindin during early and midgestation. In our previous work on the immature uterus, estrogen was shown to stimulate calbindin-D9k appearance in the myometrium and stroma in a dose-dependent manner, and progesterone was found to blunt this estrogen response (9). Fig. 6 indicates that the effect of progesterone is dose dependent. Since progesterone reaches high levels during early and midpregnancy, the present studies and our earlier findings (9) suggest a role for progesterone in controlling uterine calbindin-D9k during early to midgestation. The smooth muscle excitability in the uterus (34) follows the temporal and endocrine patterns that we have observed for myometrial calbindin-D9k. Smooth muscle excitability increases dramatically in the estrogen-dominated uterus at estrus and at parturition (term). In the progesterone-dominated uterus during early to midpregnancy (before term) the uterus is in a nonexcitable quiet state (35).

Conclusion. During late pregnancy, in association with maternal-fetal calcium transport and uterine muscle contraction, calbindin reappears in uterine muscle, as well as the three epithelial transporting layers of the uterus, yolk sac, and placenta. Which endocrine systems control these late calbindin inductions is unknown. Previous work has indicated that 1,25(OH)₂D₃ receptors appear in the rat uterus after estrogen treatment (29-30). In addition, 1,25(OH)₂D₃ receptors have been found in yolk sac and placenta (27, 28). The study of calbindin-D9k appearance and localization in the uterus-yolk sac-placenta relationship provides an attractive model system to study the genetic interactions of the sex steroid hormones and 1,25(OH)₂D₃. Additional studies are required to examine the function of calbindin in the myometrium and its role in normal and premature labor.

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