Effects of ovarian steroid hormones and thyroxine on calcitonin secretion in pregnant rats

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In the present study, the roles of ovarian steroid hormones and thyroxine (T4) in regulating the secretion of calcitonin (CT) in pregnant rats were examined. The levels of plasma progesterone, pre- and post-CaCl2 plasma CT, and recovery time of plasma CT and calcium after calcium challenge were greatest in midterm pregnant rats. The levels of basal plasma progesterone, CT, calcium, and recovery time of plasma CT after calcium challenge were less in late pregnant rats, but basal plasma estradiol was highest in late pregnancy. The concentrations of plasma T4 were gradually decreased in rats during pregnancy. Regardless of the presence of estradiol, administration of progesterone in ovariectomized (Ovx) rats resulted in an increase of plasma T4 as well as the basal and calcium-induced secretion of CT. Administration of estradiol alone did not alter the CaCl2-induced levels but decreased the post-CaCl2 levels of plasma calcium in Ovx rats. The basal levels of plasma CT were decreased in Ovx rats treated with T4. These results suggest that the hypercalcitoninemia in midterm pregnant rats is due to an increased secretion of progesterone. Hypocalcitoninemia in late pregnant rats, however, is due in part to lower plasma calcium.

estradiol; progesterone

DURING PREGNANCY, the metabolism of maternal calcium is influenced by fetal requirements (14). This adaptive process depends on the interrelationship between parathyroid hormone (PTH) and 1,25-dihydroxyvitamin D3 [1,25-(OH)2D3], which shows quantitative rather than qualitative changes from the nonpregnant state (14). In addition, the metabolism of calcium is also regulated by calcitonin (CT) and 1,25-(OH)2D3. Changes in the secretion of CT and 1,25-(OH)2D3 in pregnancy are well characterized. For example, the level of CT in rat plasma has been shown to increase up to 19.5 days and to decrease subsequent to 21.5 days of gestation (10). Halloran et al. (13) found that plasma 1,25-(OH)2D3 levels in female rats increased threefold during the latter stages of pregnancy. In humans, plasma levels of CT and 1,25-(OH)2D3 have also been shown to increase during pregnancy (24). A significant increase in plasma vitamin D concentration has been noted in pregnant rats from day 19 to day 21 (17a). A linear reduction of bone density has been observed during the third trimester (17), when the greatest calcium transfer occurs between the mother and the fetus (13). Thus the simultaneous rise in CT and 1,25-(OH)2D3 during pregnancy reduces the bone-resorbing activities at this critical stage and thereby maintains the integrity of the maternal skeleton and protects against osteoporosis (13). However, the mechanism by which CT increases during pregnancy remains unknown.

It is well documented that the level of plasma CT is influenced by gonadal steroid hormones. Androgen deficiency per se has been shown to play an influential role in the pathogenesis of osteoporosis in hypogonadal subjects and may influence bone metabolism by regulating CT secretion (7). Whitehead et al. (23) found that estrogen increases CT secretion in humans. Clinical studies have indicated that postmenopausal estrogen replacement therapy is effective in the prevention of rapid bone loss (1). Because estrogen regulates CT secretion in postmenopausal women, CT may mediate estrogen action on bone (1).

In both humans (20) and rats (11), circulating plasma progesterone concentrations are higher in pregnant subjects than in nonpregnant controls, and estradiol concentrations are increased in pregnant subjects approaching full term. Furthermore, ovariectomy causes a decrease in serum CT and calcium levels (15). In addition, both estradiol and progesterone cause an increase of in vitro CT release from the thyroid C cells of 8-day-old rats (12). These findings suggest that ovarian steroid hormones play a prominent role in regulating the secretion of CT during pregnancy.

In addition to the changes of gonadal steroid hormones, the concentration of plasma thyroxine (T4) is gradually decreased during pregnancy (8). Compared with the hormonal profile of early pregnancy, the concentration of plasma 3,5,3′-triiodothyronine (or T3) is lower and that of plasma thyrotropin is higher in late gestation (8). Because the skeletal density (4) and the concentration of plasma CT (2) decrease in hypothyroid patients, CT secretion may also be regulated by T4.

This investigation was designed to study the role of ovarian steroid hormones and that of T4 in regulating CT secretion in rats during pregnancy. To study the effects of steroid hormones and T4 on CT release, ovariectomized (Ovx) rats were treated with ovarian steroid hormones or T4 to characterize the hormonal effects on CT secretion.

MATERIALS AND METHODS

Animals. Female Sprague-Dawley rats weighing 220–270 g were housed in a temperature-controlled (22 ± 1°C) room...
with 14 h of artificial illumination daily (0600–2000) and were given food and water ad libitum. Vaginal smears were taken every morning after overnight pairing of proestrus rats with males. The day of a positive smear was designated as day 1 of pregnancy (day 7 n = 8–9, day 14 n = 9, day 21 n = 18). Diestrous rats were used as control animals (n = 14). To study the effects of steroid hormone on CT secretion, Ovx rats were used. Castrations were performed under ether anesthesia. Two weeks after castration, the Ovx rats were injected subcutaneously with estradiol benzoate (EB, 25 µg/kg body wt, n = 7–8), progesterone (50 mg/kg, n = 7–8), EB and progesterone (n = 7–8), or saline (n = 7–9) once daily for 3 days. EB and progesterone were dissolved in sesame oil at concentrations of 25 µg/ml and 50 mg/ml, respectively (22). To study the effects of T4 on CT secretion, other Ovx rats were injected subcutaneously with T4 (20 µg/kg, n = 8) or saline (n = 8) once daily for 2 wk. T4 was dissolved in alkaline saline (pH 9.55) at concentrations of 20 µg/ml.

Experiments. Rats were catheterized via the right jugular vein and left femoral vein under ether anesthesia (22). Twenty hours later, CaCl2 (10 mg/ml) was infused (1 ml/30 min) via the femoral catheter connected to a peristaltic pump (21). Blood samples (0.6 ml each) were collected from the right jugular vein at 0, 30, 60, and 120 min after CaCl2 challenge (21).

Plasma was separated by centrifugation at 10,000 g for 1 min and stored at −20°C for radioimmunoassay (RIA) of CT. Plasma calcium concentration was determined by an automatic calcium analyzer (Calcette; Precision Systems, Natick, MA). Rats were decapitated, and blood samples were collected for measurement of CT, T4, estradiol, and progesterone levels in plasma by RIAs.

RIA of CT. Concentrations of plasma and medium CT were measured by a human CT RIA kit purchased from Nichols Institute Diagnostics (San Juan Capistrano, CA) (21). Inhibition curves of rat thyroid medium, rat plasma, and human plasma paralleled those of human CT standards (21). The antisera for CT showed negligible or no cross-reactivity against bovine PTH-(1–84), human PTH-(1–34), insulin, prolactin, human growth hormone, thyroid-stimulating hormone, and adrenocorticotropic hormone. Salmon CT up to 40 nM did not cross-react with the antisera. The sensitivity of the RIA was 4 pg/ml. The recovery of CT from human serum pools was 86–94%. Intra- and interassay coefficients of variation were 6.7% (n = 10) and 8.3% (n = 10), respectively.

RIAs of estradiol and progesterone. Concentrations of plasma progesterone were determined by RIA as described previously (16). With antiprogesterone serum no. W5, the sensitivity of the progesterone RIA was 5 pg/assay tube. Intra- and interassay coefficients of variation were 4.8% (n = 5) and 9.5% (n = 4), respectively.

The antiserum against estradiol (no. W1) was generated by immunizing the rabbit with 1,3,5(10)-estratrien-3,17β-diol-6-one 6-carboxymethylxime/bovine serum albumin (BSA) conjugate (Steraloids). For the RIA system, a known amount of unlabeled estradiol or a heterologous steroid or an aliquot of rat plasma extract, adjusted to a total volume of 0.3 ml by a buffer solution [0.1% gelatin-phosphate-buffered saline (PBS), pH 7.5], was incubated with 0.1 ml of estradiol-BSA antiserum (1:4,000) diluted with 0.1% gelatin-PBS and 100 µl [3H]estradiol (8–0,000 counts/min; Amersham International, Buckinghamshire, UK) at 4°C for 24 h. Triplicate standards, with 4–5 points ranging from 1–300 pg, were incubated in each assay while unknown samples were assayed in duplicate. An adequate amount of dextran-coated charcoal (0.25%) was added and further incubated in an ice bath for 15 min. After incubation, the assay tubes were centrifuged at 1,000 g for 15 min. The supernatant was mixed with 1 ml liquid scintillation fluid (Ready Safe, Beckman) before the radioactivity was counted in an automatic beta counter (Wallac 1409, LKB, Pharmacia, Turku, Finland). With antiestradiol no. W1, the sensitivity of the estradiol RIA was 1 pg/assay tube. The inhibition curves produced by estradiol and other-extracted rat plasma were parallel. Cross-reactivities were 9% with estrone; 0.5% with estriol, testosterone, 5α-dihydrotestosterone, androstenediol, or corticosterone; and <0.3% with 17α-estradiol, progesterone, pregnenolone, cortisol, hydrocortisone, or cholesterol. Intra- and interassay coefficients of variation were 6.0% (n = 5) and 5.9% (n = 5), respectively.

RIA of T4. The concentration of total T4 in plasma and media samples was measured by an Amerlex-M T4 RIA kit provided from Johnson & Johnson Clinical Diagnostics (Amersham International). The sensitivity of this assay was 3 ng/ml, and the intra- and interassay coefficients of variation were 3.3% (n = 10) and 4.7% (n = 10), respectively.

Statistical analysis. Treatment means were tested for homogeneity with analysis of variance, and the difference between specific means was tested for significance by use of Duncan's multiple range test (19). P < 0.05 was taken to indicate statistical significance.

RESULTS

Concentrations of plasma total T4, estradiol, progesterone, calcium, and CT in rats during pregnancy. Plasma levels of total T4 on days 7, 14, and 21 of gestation were lower than T4 levels in diestrous rats (Fig. 1, top). The plasma total T4 was negatively correlated with the day of gestation (correlation coefficient = −0.87, P < 0.01) and decreased gradually during gestation.

Concentrations of plasma estradiol were comparable in diestrous rats and days 7 and 14 of gestation. By day 21 of gestation, estradiol levels increased significantly (P < 0.01, Fig. 1, 2nd from top). The level of plasma estradiol was not different among diestrous rats and pregnant rats at days 7 and 14 of gestation. In contrast, concentrations of plasma progesterone were greater (P < 0.01) in pregnant rats on days 7 and 14 of gestation than in diestrous rats and pregnant rats of day 21 of gestation (Fig. 1, middle). The greatest level of plasma progesterone was found in pregnant rats on day 14 of gestation. The level of plasma progesterone was not different between pregnant rats at day 21 of gestation and diestrous rats.

Basal levels of plasma calcium were similar in diestrous and early (day 7) and midterm (day 14) pregnant rats but were lower in pregnant rats at 21 days of gestation (Fig. 1, 2nd from bottom). The level of plasma calcium was not different between days 7 and 21 of gestation. The greatest and lowest levels of plasma CT were found in pregnant rats on days 14 and 21 of gestation, respectively (Fig. 1, bottom). The concentration of plasma CT was not different between diestrous rats and pregnant rats at day 7 of gestation or between days 7 and 21 of gestation.

Response of CT to CaCl2 challenges in pregnant rats. The percent changes of plasma calcium and CT levels in response to intravenous infusion of CaCl2 are illustrated in Fig. 2. After 30 min of CaCl2 infusion, plasma calcium levels increased in all groups (Fig. 2, top).
Thereafter (at 60 and 120 min), calcium levels either returned to basal levels (diestrous, and days 7 and 14 of gestation) or were further reduced in 21-day-pregnant rats (60 min; Fig. 2, top). In addition, plasma calcium levels were lower on day 21 of gestation than for other groups at 60 min. Before and after CaCl$_2$ challenge, the calcium levels were not different among diestrous rats and pregnant rats at days 7 and 14 of gestation at 0–60 min. Ninety minutes after termination of CaCl$_2$ challenge, plasma calcium levels were lower on day 7 of gestation than for diestrous rats and pregnant rats at day 14 of gestation.

Infusion of CaCl$_2$ for 30 min increased plasma concentration of CT in all rats (Fig. 2, bottom). The post-CaCl$_2$ levels (at 60 and 120 min) of plasma CT in pregnant rats on day 14 were significantly higher ($P < 0.05$) than those in diestrous rats. In pregnant rats on day 21, the CaCl$_2$-induced levels of plasma CT were lower ($P < 0.01$) than those in pregnant rats on day 7, and the post-CaCl$_2$ levels (at 60 min) of plasma CT were lower ($P < 0.01$) than in other groups. Ninety minutes after termination of CaCl$_2$ challenge, rat plasma CT concentrations were restored to levels not significantly different from pre-CaCl$_2$ levels. In addition, rat plasma CT was lower on day 21 than on days 7 and 14 of gestation at 120 min.

Concentrations of plasma T$_4$, calcium, and CT in steroid-treated Ovx rats. Concentrations of plasma estradiol and progesterone in EB- and/or progesterone-injected Ovx rats ranged from 17 to 30 pg/ml and from 19 to 47 ng/ml, respectively (data not shown).
The concentration of plasma total \( T_4 \) was higher in Ovx rats treated with progesterone \((P < 0.01)\) or with EB plus progesterone \((P < 0.05)\) than in Ovx rats treated with oil (Fig. 3, top). The levels of plasma total \( T_4 \) in Ovx rats were not different between oil and EB treatments, between EB and EB plus progesterone treatments, or between progesterone and EB plus progesterone treatments.

Basal levels of plasma calcium in Ovx rats remained unaltered by the treatments of ovarian steroid hormones (Fig. 3, middle).

Compared with oil-injected animals, administration of progesterone or EB plus progesterone in Ovx rats increased the basal level of plasma CT by 67\% \((P < 0.01)\) and 44\% \((P < 0.05)\), respectively (Fig. 3, bottom). The levels of plasma CT in Ovx rats were not different between oil- and EB-treated, between EB- and EB plus progesterone-treated, or between progesterone- and EB plus progesterone-treated animals.

Response of CT to CaCl\(_2\) challenge in Ovx rats treated without or with ovarian steroids. Replacement of EB or progesterone resulted in a lower \((P < 0.01\) and \(P < 0.05\), respectively) plasma calcium at 60 min and a lowest \((P < 0.01)\) plasma calcium at 120 min after CaCl\(_2\) infusion in EB-treated Ovx rats (Fig. 4, top). Meanwhile, the levels of plasma calcium at 60 min in Ovx rats were different between those EB and oil treated, or between those progesterone and oil treated. At 120 min, the levels of plasma calcium in Ovx rats were different between EB- and oil-treated, between progesterone- and EB-treated, or between EB plus progesterone- and EB-treated animals.

Compared with oil-injected animals, administration of progesterone or EB plus progesterone in Ovx rats increased the CaCl\(_2\)-induced levels of plasma CT by 68\% \((P < 0.05)\) and 80\% \((P < 0.05)\), respectively (Fig. 4, bottom). In addition, compared with EB-injected animals, administration of progesterone or EB plus progesterone in Ovx rats increased the CaCl\(_2\)-induced levels of plasma CT by 53\% \((P < 0.05)\) and 64\% \((P < 0.05)\), respectively (Fig. 4, bottom). Thirty and 60 min after termination of CaCl\(_2\) infusion, no difference in plasma CT concentration was found between oil- and hormone-treated animals. The maximal increase of plasma CT in response to CaCl\(_2\) infusion from 0 to 30 min was greater (68–92\%, \(P < 0.01\)) in progesterone- and EB plus progesterone-treated than in oil-injected Ovx rats (Fig. 4, bottom).

Concentrations of plasma \( T_4 \), calcium, and CT in \( T_4 \)-treated Ovx rats. The concentration of plasma total \( T_4 \) was higher in Ovx rats treated with \( T_4 \) \((P < 0.01)\) than in Ovx rats treated with saline (Fig. 5, top). Basal levels of plasma calcium in Ovx rats remained unaltered by the treatments of \( T_4 \) (Fig. 5, middle).
In the present study we found that 1) the midterm pregnant rats exhibited highest levels of plasma progesterone and CT and a long recovery time of plasma CT after calcium challenge; 2) the late pregnant rats exhibited lower basal plasma calcium and CT and a short recovery time of plasma calcium and CT after calcium challenge; 3) regardless of the presence of estradiol, administration of progesterone increased the levels of plasma CT and T4; 4) administration of progesterone alone or progesterone plus estradiol increased the basal level of plasma CT and the response of CT to calcium; 5) administration of estradiol increased the clearance of plasma calcium; and 6) administration of T4 decreased the basal levels of plasma CT but did not alter CT secretion induced by calcium challenge.

A previous study has shown that the circulating progesterone concentration is higher in rats during pregnancy, except at term, than in virgin controls (11). However, the level of plasma estradiol markedly increases during late pregnancy in rats (11). These profiles of ovarian steroid hormone levels correspond with our observations in the pregnant rats. In this study, both the basal and calcium-induced levels of plasma CT and plasma progesterone are markedly higher in midterm pregnant rats. Apparently, a higher secretion of CT is correlated with progesterone production.

Garel and Jullienne (10) found that the levels of CT in the plasma after 17.5 days of gestation were already higher than the level in control rats, increased further up to 19.5 days, and subsequently decreased at 21.5 days. Moreover, an increased plasma CT level during pregnancy has also been reported in humans (5, 25). Collectively, these data suggest that the midterm enhancement of circulating CT may be due in part to the higher secretion of progesterone. This is supported by our observation that progesterone alone increased the basal and calcium-induced plasma CT in Ovx rats. Because no increase of plasma estradiol level was observed in midterm pregnant rats, and neither basal...
nor calcium-induced plasma CT levels were enhanced by the administration of estradiol in Ovx rats, we suggest that the greater secretion of CT in midterm pregnant rats is due to the stimulatory effect of progesterone rather than the invalid effect of estradiol.

Despite the increase in CT secretion, plasma calcium levels were not altered in midterm pregnant rats compared with nonpregnant rats. These results suggest that hypercalcitoninemia in pregnant rats is calcium independent; they compare favorably with the data reported by Quan-Sheng and Miller (17a). However, in addition to the reduction of CT secretion, this study shows that hypocalcemia always occurs in rats during late pregnancy (i.e., day 21). Administration of estradiol did not affect basal, calcium-induced, and postcalcium levels of plasma CT but decreased postcalcium levels of plasma calcium in Ovx rats. The reason for lower post-CaCl2 levels of plasma calcium caused by estradiol remains unknown but may be an overcompensation as the body returns plasma calcium levels to normal. Because estrogen depresses serum calcium levels in postmenopausal women (23), the decreased basal level of plasma calcium and short recovery time of plasma calcium after CaCl2 challenge in late pregnancy might be due to a high level of plasma estradiol.

On the basis of our results of a lower basal level of plasma calcium and a short recovery time of plasma calcium after CaCl2 challenge, hypocalcemia may be one of the main factors for the occurrence of hypocalcitoninemia in late pregnancy. The observation that hypocalcemia occurs in late pregnancy has been noted not only in rats (10, 17a) but also in women (5) and has been suggested to result from the increased demand of fetal growth (17a). Because administration of progesterone alone or progesterone plus estradiol increased the levels of plasma T4 in Ovx rats (Fig. 3), the decrease of plasma T4 levels in rats during pregnancy was independent of the levels of plasma progesterone and/or estradiol but might be due to the increased metabolic clearance rate of T4 in pregnant rats (9). Although it has been shown that CT deficiency is present in primary hypothyroidism (2), we found that administration of T4 decreased the basal levels of plasma CT in Ovx rats. Because we found a lower level of plasma T4 in midterm pregnant rats but a higher level of plasma T4 in progesterone-treated Ovx rats, there seems to be no correlation between T4 and CT levels in the plasma of female rats. There is no evidence at the present time to indicate that T4 is a physiological regulator for CT secretion in pregnant rats.

In women, the occurrence of osteoporosis is due either to menopause or to aging (3, 18). Administration of estrogen and/or CT is facilitative in relieving the syndromes of osteoporosis (1). Our observations in Ovx rats indicate that progesterone alone or in combination with estradiol increases the secretion of CT. However, more studies are needed before progesterone can be considered as a promising therapeutic reagent for treating osteoporosis.

In summary, results of this study demonstrate that abundant production of progesterone is associated with the increase of both spontaneous and calcium-induced levels of plasma CT in midterm pregnant rats. Because administration of progesterone increased rather than decreased the levels of plasma T4 and CT in Ovx rats, the hypercalcitonemia in midterm pregnant rats is dependent on progesterone rather than T4. The hyposecretion of CT in late pregnant rats is at least partially due to the hypocalcemia.

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