Inhibition of salmon calcitonin on secretion of progesterone and GnRH-stimulated pituitary luteinizing hormone

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Tsai, Shiow-Chwen, Chien-Chen Lu, Jiaann-Jong Chen, Yu-Chung Chiao, Shyi-Wu Wang, Jjuan-Juuan Hwang, and Paulus S. Wang. Inhibition of salmon calcitonin on secretion of progesterone and GnRH-stimulated pituitary luteinizing hormone. Am. J. Physiol. 277 (Endocrinol. Metab. 40): E49–E55, 1999.—The effects of salmon calcitonin (sCT) on the production of progesterone and secretion of luteinizing hormone (LH) were examined in female rats. Diestrous rats were intravenously injected with saline, sCT, human chorionic gonadotropin (hCG), or hCG plus sCT. Ovariectomized (Ovx) rats were injected with saline or sCT. In the in vitro experiments, granulosa cells and anterior pituitary glands (APs) were incubated with the tested drugs. Plasma LH levels of Ovx rats were reduced by sCT injection. Administration of sCT decreased the basal and hCG-stimulated progesterone release in vivo and in vitro. 8-Bromo-cAMP dose dependently increased progesterone production but did not alter the inhibitory effect of sCT. H-89 did not potentiate the inhibitory effect of sCT. Higher doses of 25-hydroxycholesterol and pregnenolone stimulated progesterone production and diminished the inhibitory effects of sCT. sCT did not decrease basal release of LH by APs, but pretreatment of sCT decreased gonadotropin-releasing hormone (GnRH)-stimulated LH secretion. These results suggested that sCT inhibits progesterone production in rats by preventing the stimulatory effect of GnRH on LH release in APs and acting directly on ovarian granulosa cells to decrease the activities of post-cAMP pathway and steroidogenic enzymes.

cytochrome P-450 side-chain cleavage; 3β-hydroxysteroid dehydrogenase

Both basal release and thyrotropin-releasing hormone (TRH)-stimulated release of prolactin in isolated rat pituitary cells are inhibited by salmon CT (sCT; Ref. 37). We found that CT peptides, including human CT (hCT), sCT, and CT gene-related peptide (CGRP), inhibit the spontaneous and gonadotropin-stimulated secretion of testosterone by acting directly at testes and reducing the release of pituitary luteinizing hormone (LH) through a mechanism involving an increase in cAMP production (44). In rats, CT exhibited a peak concentration in plasma on the day of diestrus and dropped to the lowest on the day of estrus (8). These observations indicate an endocrine role of sCT at ovarian level.

It has been well-known that the LH-increased productions of progesterone (11, 22, 25) are correlated with the increased generation of cAMP (11, 22). An increased expression of the cytochrome P-450 side-chain cleavage (P-450scc; Refs. 22, 23) and 3β-hydroxysteroid dehydrogenase (3β-HSD; Ref. 17) has also been demonstrated. The conversion of cholesterol to pregnenolone is the rate-limiting step in the final formation of progesterone, and this step is regulated by mitochondria enzyme 3β-HSD. Progesterone is the main secretory product of granulosa cells and diffuses into theca cells to serve as a substrate for biosynthesis of androgens (19, 25). The theca cells provide androgens, whereas granulosa cells convert androgens to estrogens by 17β-HSD and cytochrome P-450 aromatase.

In the present study, sCT was employed instead of rat CT, because the ultimobranchial CT is biologically more active and stable than mammalian CT (18, 32). The effects of sCT on the basal and human chorionic gonadotropin (hCG)-stimulated release of progesterone in rats and the release of LH from anterior pituitary glands (APs) were examined. We found that sCT inhibits production of progesterone both in vivo and in vitro through the mechanisms involving decreased gonadotropin-releasing hormone (GnRH)-stimulated pituitary LH release and the activities of P-450scc and 3β-HSD in granulosa cells. Furthermore, we suggested that the inhibitory effect of sCT on progesterone release may be related to the post-cAMP pathway.

MATERIALS AND METHODS

Animals. Mature diestrous female (250–300 g) and immature female (30–40 g) Spraque-Dawley rats were housed in a temperature-controlled room (22 ± 1°C) with 14 h of artificial light.
Effect of sCT on progesterone release in vivo. Diestrous rats were catheterized via the right jugular vein (44). Twenty hours later, they were injected with saline (1 ml/kg), sCT (3.4 ng·ml⁻¹·kg⁻¹), hCG (5 IU·ml⁻¹·kg⁻¹), or hCG plus sCT via the jugular catheter. Blood samples (0.5 ml each) were collected at 0, 30, 60, and 120 min after the challenge. Plasma was separated by centrifugation of blood samples at 10,000 g for 1 min. The concentration of progesterone in plasma was measured by RIA (6). The activities of sCT on LH release in vivo. Some rats were ovariectomized (Ovx) 2 wk before being catheterized and were injected with saline or sCT (3.4 ng·ml⁻¹·kg⁻¹) via the jugular catheter. Blood samples were collected at 0, 15, 30, 60, and 120 min after the challenge. Plasma was separated by centrifugation at 10,000 g for 1 min. The concentration of LH in plasma was measured by RIA (44).

Dispersion and preparation of rat granulosa cells. The preparation of granulosa cells was modified from the method described by Too et al. (42). The immature female rats at 25–27 days of age were subcutaneously injected with pregnant mares’ serum gonadotropin (PMSG; 15 IU/rat). Forty-eight hours later, rats were killed by cervical dislocation. Ovaries were excised and transferred into the sterile DMEM/Ham’s F-12 (1:1) medium, containing 0.1% bovine serum albumin (BSA, Sigma, St. Louis, MO), 20 mM HEPES, 100 U/ml penicillin-G, and 50 µg/ml streptomycin sulfate. After the fat and connective tissues were trimmed free, the large and medium-sized follicles were punctured with a 26-gauge needle to release granulosa cells. The harvested cells were pelleted and resuspended in growth medium (DMEM/Ham’s F-12 containing 10% fetal calf serum, 2 µg/ml insulin, 100 IU/ml penicillin, and 50 µg/ml streptomycin sulfate). Cell viability was >90% as determined with a hemacytometer and the trypan blue method. Granulosa cells were plated in 24-well plates at ~1 × 10⁶ viable cells per well and were incubated at 37°C with 5% CO₂-95% air for 2 days. Morphologically, the cultured granulosa cells appeared nearly round (or polygonal) in shape, not like fibroblasts, throughout our culture conditions. Total cell proteins were determined by the method of Lowry et al. (26).

Incubation of granulosa cells with hCG and/or sCT. The granulosa cells were washed twice by serum-free BSA-M199 medium (medium 199, 0.3% BSA, 100 IU/ml penicillin, 50 µg/ml streptomycin sulfate) and then incubated with sCT (0–10⁻⁸ M), hCG (0.5 IU/ml), or hCG plus sCT for 2 h. To further evaluate the role of intracellular cAMP in regulation of progesterone release by sCT, the effects of 8-bromo-cAMP, a cAMP analog to mimic increase of intracellular cAMP, 10⁻⁴ or 10⁻³ M and H-89 (an inhibitor of protein kinase A catalytic subunit, 5 × 10⁻⁴ or 5 × 10⁻³ M; Refs. 10, 30, 34) on the action of sCT in granulosa cells were examined. After incubation at 37°C with 5% CO₂-95% air for 2 h, samples of medium were then collected, cleared by centrifugation, and stored at −20°C until analyzed for progesterone by RIA.

Effect of sCT on steroidogenesis. The activities of P-450sc and 3β-HSD were determined by measuring the conversion of [³H]pregnenolone to 17α-[³H]hydroxyprogesterone and [³H]progesterone as described previously (43). Granulosa cells were incubated with [³H]pregnenolone (10,000 counts/min, 0.2 pmol) in the absence or presence of sCT (10⁻⁸ or 10⁻⁶ M) for 2 h. The medium was collected and extracted by vigorous agitation in 1 ml diethyl ether and then quick-frozen in a mixture of acetone and dry ice. The diethyl ether layer was collected, dried, and reconstituted in 100 µl of 100% ethanol containing 5 µg of each of the unlabeled carriers, including pregnenolone, progesterone, and 17α-hydroxyprogesterone. Aliquots (50 µl) were applied to a TLC plate and developed in a mixture of carbon tetrachloride and acetone (4:1, vol/vol). The sheets were dried, and the location of steroid-containing spots was indicated under ultraviolet light. The migration rates (Rf) were 0.55 for pregnenolone, 0.71 for progesterone, and 0.50 for 17α-hydroxyprogesterone. The spots were cut off and transferred into vials containing 1 ml of liquid scintillation fluid (Ready Safe, Beckman, Fullerton, CA) before the radioactivity was counted in an automatic beta counter (Wallac 1409, Pharmacia, Turku, Finland).

Effect of sCT on LH release in vivo. The diestrous rats were killed by decapitation. The anterior pituitary glands (APs) were excised, bisected, preincubated, and then incubated for 30 min with Locke’s medium containing 10 mM glucose, 0.003% bacitracin, and 0.05% HEPES at 37°C. One hemi-AP was assigned to a flask containing 1 ml of medium. APs were then incubated with sCT (0, 10⁻¹², 10⁻¹⁰, 10⁻⁸ M) for 30 min before being incubated with GnRH (10⁻⁹ M). After further incubation of APs with GnRH, AP tissues were weighed. The media were collected and stored at −20°C until analyzed for LH by RIA (44). The effect of sCT on GnRH-stimulated LH release was compared with that of controls (non-sCT pretreated).

RIA of progesterone. The concentration of progesterone in plasma and media was determined by RIA as previously described (6). The sensitivity of progesterone RIA was 5 pg/assay tube. The intra- and interassay coefficients of variability were 4.8% (n = 5) and 9.5% (n = 4), respectively.

RIA of LH. The concentration of LH in plasma and media was determined by RIA as previously described (44). The rat LH-I-6 used for iodination and rat LH-RP-3 serving as standard preparation were provided by the National Hormone and Pituitary Program, the National Institute of Diabetes and Digestive and Kidney Diseases, the National Institute of Child Health and Human Development, and the United States Department of Agriculture. The sensitivity was 0.1 ng for LH. The intra- and interassay coefficients of variability were 3.8% (n = 4) and 6.6% (n = 5), respectively.

Statistical analysis. All data were expressed as means ± SE. Treatment means were tested for homogeneity with ANOVA, and the differences between the specific means were tested for significance by means of Duncan’s multiple range test or Student’s t-test (39). The level of significance chosen was P < 0.05.

RESULTS

Plasma calcium and progesterone in response to hCG and/or sCT. The mean levels of plasma calcium of diestrous rats at all time points were 10.28 ± 0.14 mg/dl for the saline-injected group, 10.21 ± 0.11 mg/dl for the hCG-injected group, 10.66 ± 0.12 mg/dl for the sCT-injected group, and 10.30 ± 0.12 mg/dl for the animals injected with both hCG and sCT. There were no significant differences in plasma calcium levels.
among these four groups. The mean levels of plasma calcium of Ovx rats at all time points were 10.0 ± 0.16 mg/dl.

Intravenous injection of saline did not alter the level of plasma progesterone (Fig. 1, top). Thirty to 120 min after sCT injection, the mean concentrations of plasma progesterone dropped by 37–49%. Injection of hCG plus sCT resulted in a significantly lower level of plasma progesterone at 30 min after challenge compared with that induced by hCG alone (P < 0.01).

Plasma LH in response to sCT. Intravenous injection of saline did not alter the level of plasma LH (Fig. 2), but a single injection of sCT decreased plasma LH at 15, 30, and 60 min after challenge (P < 0.05). A maximal reduction in the plasma LH level was observed at 1 h after injection of sCT. After 2 h, the plasma LH concentration rose to the basal level.

Effect of sCT on progesterone in granulosa cells in vitro. The effect of sCT ranging from 10^{-12} to 10^{-8} M on progesterone release by granulosa cells of PMSG-primed rats is illustrated in Fig. 3. During a 2-h incubation, sCT at 10^{-8} M caused a significant inhibition of progesterone release (P < 0.05). Incubation with hCG for 2 h caused a significant increase of progesterone release from granulosa cells (P < 0.01). The hCG-induced release of progesterone was significantly (P < 0.05 or P < 0.01) inhibited by sCT ranging from 10^{-10} to 10^{-8} M.

8-BrcAMP at 10^{-3} M stimulated the release of progesterone both in the absence or presence of sCT (P < 0.01), but 8-BrcAMP could not fully reverse the inhibitory effect of sCT (Fig. 4). Administration of hCG at 10^{-8} M of H-89 resulted in a decrease in the release of progesterone (Fig. 5). In the presence of H-89, no further inhibition of progesterone release by sCT was observed.

Effect of sCT on the activities of P-450sc and 3β-HSD. With unlabeled steroids as carriers, [3H]progesterone, 17α-[3H]hydroxyprogesterone, and [3H]androstenedione (<100 counts/min, data not shown) were produced after incubation of granulosa cells with [3H]pregnenolone (Fig. 6). sCT did not alter the accumulation of [3H]pregnenolone and production of 17α-[3H]hydroxyprogesterone but decreased the production of [3H]progesterone.
gesterone, indicating that the 3\(\beta\)-HSD activity (conversion of [\(3^H\)]pregnenolone to [\(3^H\)]progesterone) was inhibited.

Administration of 25-OH-cholesterol (10\(^{-7}\)-10\(^{-5}\) M; Fig. 7) and pregnenolone (10\(^{-9}\)-10\(^{-5}\) M; Fig. 8) increased progesterone release (\(P<0.05\) or \(P<0.01\)). sCT at 10\(^{-8}\) M decreased not only the basal release of progesterone but also the progesterone response to the addition of lower doses of 25-OH-cholesterol (10\(^{-7}\) M, \(P<0.05\); Fig. 7) or pregnenolone (10\(^{-9}\)-10\(^{-7}\) M, \(P<0.05\); Fig. 8). 25-OH-cholesterol and pregnenolone at 10\(^{-5}\) M reversed the inhibitory effect of sCT on progesterone release (Figs. 7 and 8).

Effect of sCT on LH release in vitro. The mean pre-sCT levels of LH release from AP ranged between 11.1 and 11.8 ng/mg of AP (Fig. 9). Incubation of sCT did not alter the release of LH. Although GnRH (10\(^{-9}\) M) increased LH release significantly (Fig. 9, \(P<0.01\)), pretreatment with sCT dose dependently diminished the stimulatory effect of GnRH on LH release (Figs. 9 and 10).

DISCUSSION

In the present study, we found that administration of sCT in rats significantly 1) inhibited the spontaneous and hCG-stimulated secretion of progesterone in vivo and in vitro, 2) inhibited the GnRH-stimulated release of LH by rat anterior pituitary glands, and 3) decreased the activities of P-450scc and 3\(\beta\)-HSD. Furthermore, we suggested that the inhibitory effect of sCT on progesterone release was through the post-cAMP pathway in granulosa cells.
The presence of CT in the brain and pituitary glands has been demonstrated in many mammalian species, including humans (7, 27). In humans, intravenous infusion of sCT caused a serum calcium-independent reduction in thyrotropin and LH secretion in response to hypothalamic-releasing hormone (24). Administration of CT inhibits the release of rat growth hormone (28), thyrotropin (29), LH (44), and prolactin (9), as well as the secretion of LH from APs (44), the secretion of prolactin in isolated rat pituitary cells (37), and the rise of rat prolactin after stress (9). The CT receptors designated C1a and C1b receptors have been identified in rat brain (1, 35). Both C1a and C1b-transfected cells responded to increasing concentrations of sCT with increases of cAMP (1, 36).

The present data provide evidence that sCT diminishes the GnRH-stimulated release of rat LH by acting directly on the APs (Figs. 9, 10). Because of the sample size and RIA sensitivity, we measured the concentration plasma LH in Ovx rather than in intact rats. After injection of sCT, the levels of plasma calcium were not altered (data not shown), but the release of plasma progesterone and LH was significantly decreased (Figs. 1 and 2). We concluded that the decreases of progesterone and LH were independent of the calcium-decreased effect of sCT. After pretreatment with sCT, the reduction of LH secretion in response to GnRH in the anterior pituitary glands (Fig. 10) might be one of the reasons for diminishing the level of plasma LH and progesterone after a single injection of sCT. According to our in vitro observation, 10^{-8} M sCT peptides are effective in reducing both spontaneous and hCG-stimulated release of progesterone. Another reason for sCT-induced diminution in plasma progesterone is that sCT inhibits progesterone production by acting directly on granulosa cells in ovarian follicles of PMSG-treated immature female rats. At the present time, the inhibitory effect of sCT on progesterone release was in part due to 1) decreased progesterone release directly, 2) decreased progesterone release in response to gonadotropin (LH or hCG), and 3) decreased LH release in response to GnRH. A link between sexual hormones and CT in female rat CT had been suggested because of an increased level of plasma CT in the afternoon during diestrus (8). Because the secretion of both LH and progesterone is decreased during diestrus in rats, it seems that CT plays a significant physiological role in regulating the production of pituitary gonadotropins and ovarian steroid hormones.

It has been well established that hCG stimulates progesterone secretion both in vivo (5) and in vitro (38) and increases granulosa cAMP content (38). In the present study, we found that both the stimulatory effects of hCG on plasma progesterone and the progesterone production in vitro were diminished by sCT. The
increased production of CAMP caused by CT has been demonstrated in perfused rat bone (40), rat osteoblast-like cell line (20), rat osteoclasts (33), atra (45), and aortic smooth muscle cells (21). An increase of the conversion of \(^{3}H\)-CAMP in TM3 mouse testis cell line by sCT has been described (31). We have found that sCT, hCT, or CGRP induces an increase of cAMP production in both testicular tissues and anterior pituitary glands in rats (44). These observations reflect the presence of a different post-CAMP event between hCG and CT actions.

Our results indicated that administration of \(8\text{-BrcAMP}\) did not alter the inhibitory effect of sCT and that H-89 did not potentiate the inhibitory effect of sCT. We suggested that sCT-inhibited progesterone release might be through a post-CAMP pathway, and the interaction between sCT and hCG on the signal transduction in rat granulosa cells is still open to elucidate.

The accumulation of \(^{3}H\)-pregnenolone was not altered and the amount of \(^{3}H\)-progesterone was decreased by sCT, indicating that the activity of \(3\beta\text{-HSD}\) was decreased by administration of sCT. It has been demonstrated that 25-OH-cholesterol at \(2.5 \times 10^{-9}\) M stimulates testosterone release by Leydig cells in rat testes (4). In the adrenocortical cell culture, a significant effect of 25-OH-cholesterol (1.86 \(\times 10^{-9}\) M) on progesterone release has been observed (13). In the present study, the attenuation of a higher dose of 25-OH-cholesterol on the decrease of progesterone release by sCT suggests an inhibition of sCT on the activity of \(P-450_{\text{sec}}\), the rate-limiting enzyme in progesterone biosynthesis, for the conversion of cholesterol to pregnenolone. The final step in progesterone biosynthesis is the conversion of pregnenolone to progesterone under the activity of the microsomal enzyme \(3\beta\text{-HSD}\). Administration of pregnenolone produced an increase in the release of progesterone and attenuated the inhibitory effect of sCT. Although the inhibitory effects caused by sCT might be mediated by decreasing the expression of steroidogenic enzymes and/or decreasing the cholesterol transport, the inhibition of the activity of \(P-450_{\text{sec}}\) and \(3\beta\text{-HSD}\) by sCT may account for the reduction of progesterone secretion in rat granulosa cells.

In summary, our present findings suggest that sCT inhibits progesterone production in rats by 1) acting directly on granulosa cells in the ovary, 2) inhibiting LH release and preventing LH response to GnRH, and 3) preventing progesterone response to gonadotropin, without altering plasma calcium level. The inhibitory effect of sCT on progesterone release is associated with a decrease of \(P-450_{\text{sec}}\) and \(3\beta\text{-HSD}\) activities in granulosa cells. The effective dose of sCT in reducing progesterone and LH secretion in vitro is 1–10 nM, which is greater than the normal level of rat plasma CT (<100 pg/ml, data not shown). We therefore conclude that CT expresses mainly a pharmacological rather than a physiological effect on progesterone and LH secretion. The inhibitory effects of CT on LH and progesterone secretion may be interesting in the therapy of hypogonadotropic or hypergonadotropic hypergonadism.

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