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Role of Corticotropin-Releasing Hormone in Ovarian Steroidogenesis

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The physiologic role of corticotropin-releasing hormone (CRH) was examined in the ovary. We investigated the effects of CRH on steroidogenesis in rat and human granulosa cells in vitro as well as the direct effects of CRH on the ovary in vivo. We further examined the gene expression of CRH in human granulosa cells. CRH significantly inhibited the production of estradiol (E_2) and progesterone (P_4) in rat and human granulosa cells in vitro. These inhibitory effects were completely abolished by α-helical CRH, a CRH receptor antagonist. Forskolin-induced increase in E_2 and P_4 production was attenuated by CRH. On the other hand, CRH significantly increased serum concentrations of E_2 and corticosterone in vivo in hypophysectomized rats, but this increase was completely blocked by adrenalectomy. It is probable that these effects did not result from a direct action on the ovary but were an indirect effect via the adrenal gland. Finally, by reverse transcriptase polymerase chain reaction we demonstrated that CRH mRNA was expressed in human granulosa cells. Our findings indicate that CRH exerts inhibitory effects on steroidogenesis in rat and human granulosa cells, acting through the CRH receptor. These effects are attributed to cellular events downstream of cyclic adenosine monophosphate generation. CRH seems to modulate steroidogenesis via autocrine or paracrine actions in the ovary.

Key Words: Corticotropin-releasing hormone; ovary; steroidogenesis; α-helical CRH; forskolin.

Introduction

Corticotropin-releasing hormone (CRH) is a 41 amino acid polypeptide hormone and plays a major role in the hypothalamic control of the pituitary-adrenal axis.

This CRH and its binding sites are widely distributed in many peripheral sites, such as the adrenal gland, placenta *(1)*, testis *(2,3)*, human lymphocytes *(4)*, and active inflammatory sites *(5)*. It is therefore suggested that CRH is importantly involved in regulating various functions of peripheral tissues. In inflammatory sites, e.g., CRH participates in proinflammatory regulation *(5)* in an autocrine/paracrine fashion. In the rat testis, CRH seems to play an inhibitory role in testosterone biosynthesis and secretion in an autocrine fashion *(2,3)*.

Recently, CRH receptor was found in the rat and human ovary *(6,7)*, and CRH receptor mRNA was also detected in the human ovary *(8)*. The CRH action in the ovary has therefore attracted considerable attention. For example, it was reported that CRH suppresses estrogen production induced by follicle-stimulating hormone (FSH) in human and rat granulosa cells *(9)*. The effect of CRH has been suggested to be linked to the inhibition of aromatase activity and to be independent of cyclic adenosine monophosphate (cAMP) generation. Also suggested was that the effects of CRH on steroidogenesis in the ovary are interleukin-1 receptor mediated *(10)*. It is thus possible that CRH, synthesized in the ovary, modifies steroidogenesis in the ovary via an autocrine or paracrine action.

In the present study, we first examined whether CRH affected the forskolin-induced increase in estradiol and progesterone in rat and human granulosa cells, since it has been suggested that CRH inhibits human chorionic gonadotropin (hCG)–induced androgen production in Leydig cells through cAMP-dependent pathways. Then we examined the direct effects of CRH on the ovary in vivo.

Results

Effects of CRH on Steroidogenesis in Granulosa Cells In Vitro

Baseline E_2 and P_4 production by pregnant mare's serum gonadotropin (PMSG)–stimulated granulosa cells of rats was 4478.9 ± 746.0 pg/mL and 1.78 ± 0.18 ng/mL (mean ± SEM) respectively. CRH (10^{-9} to 10^{-7} *M*) significantly ($p < 0.05$) inhibited E_2 and P_4 production (Fig. 1). With CRH treatment, E_2 production was equivalent to 86–95% of control values, and P_4 production was equivalent to 82–93% of control values.

CRH (10^{-8} *M*)-induced inhibition of E_2 and P_4 production was completely abolished by CRH receptor antagonist

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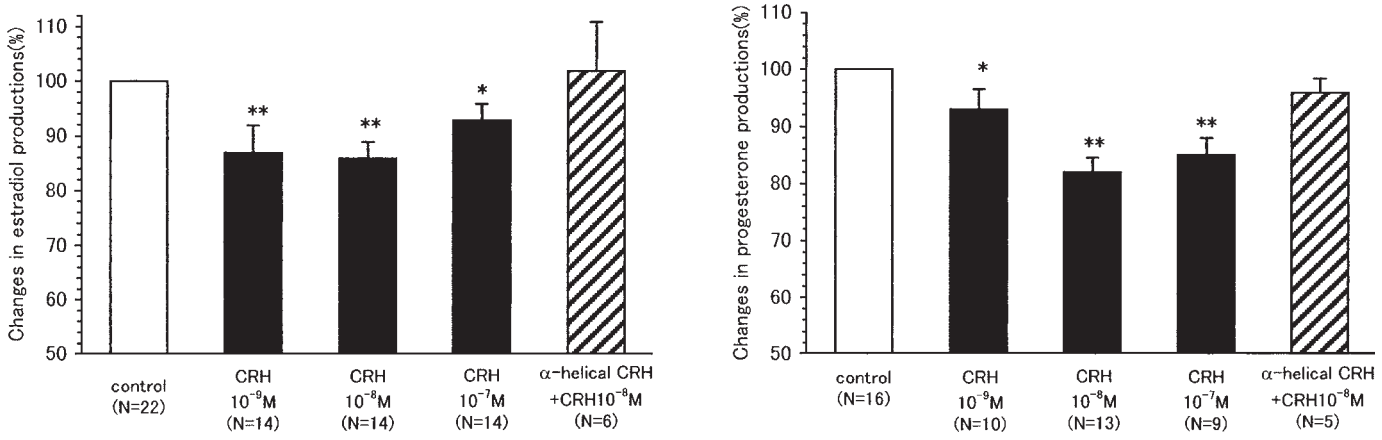

Fig. 1. Effects of CRH (10^{-9}–10^{-7} *M*) in vitro on estradiol production (**A**) and progesterone production (**B**) in rat PMSG-stimulated granulosa cells (mean ± SEM). α-helical CRH, α-helical CRH (10^{-6} *M*); *$p < 0.05$ vs control; **$p < 0.01$ vs control.

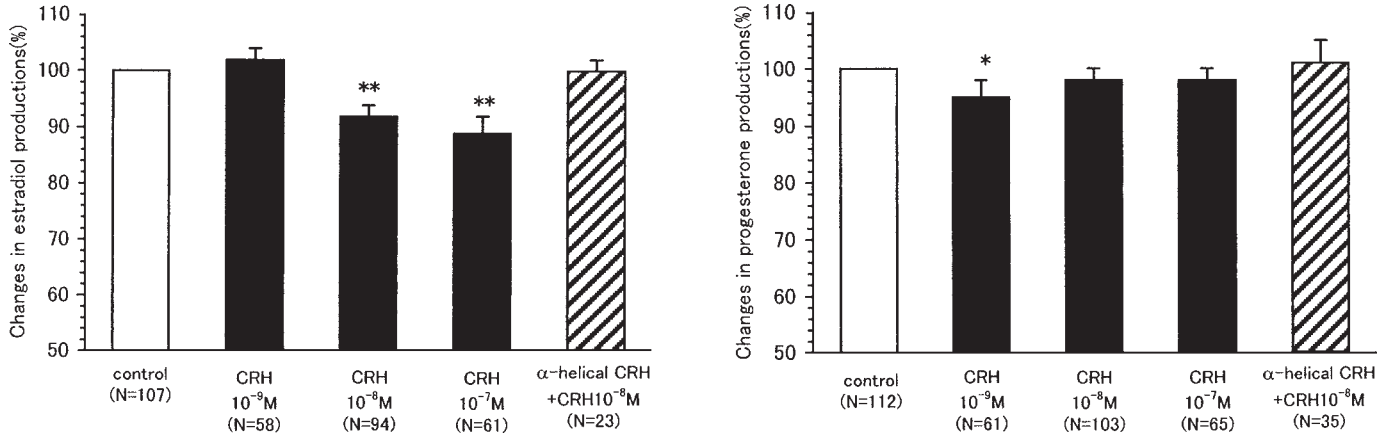

Fig. 2. Effects of CRH (10^{-9}–10^{-7} *M*) in vitro on estradiol production (**A**) and progesterone production (**B**) in hMG/hCG-stimulated granulosa cells (mean ± SEM). α-helical CRH, α-helical CRH (10^{-6} *M*); *$p < 0.05$ vs control; **$p < 0.01$ vs control.

α-helical CRH (10^{-6} *M*). α-Helical CRH (10^{-6} *M*) had no effect on basal E_2 and P_4 production.

Baseline E_2 and P_4 production by human menopausal gonadotropin (hMG)/hCG-stimulated human granulosa cells was 1521.85 ± 183.30 pg/mL and 97.76 ± 9.95 ng/mL (mean ± SEM), respectively. CRH (10^{-8}–10^{-7} *M*) significantly inhibited E_2 production ($p < 0.05$) in a concentration-dependent manner. P_4 production was significantly inhibited ($p < 0.05$) only at the 10^{-9} *M* concentration of CRH. CRH-induced inhibition of E_2 production was significantly antagonized by α-helical CRH (10^{-6} *M*) (Fig. 2).

In both rat and human granulosa cells, forskolin (10^{-5} *M*), an adenylate cyclase activator, significantly increased E_2 and P_4 production. The forskolin-induced increases in E_2 and P_4 production were significantly attenuated by CRH (10^{-9}–10^{-7} *M*) (Figs. 3 and 4).

Effects of CRH on Ovary In Vivo

Hypophysectomy significantly reduced ovarian weight, as well as serum E_2 and corticosterone concentrations. In hypophysectomized rats, on the other hand, CRH did not change ovarian weight but significantly increased serum E_2 and corticosterone concentrations ($p < 0.05$). Adrenalectomy combined with hypophysectomy completely blocked CRH-induced increases in E_2 and corticosterone production (Table 1).

Detection of CRH mRNA in Human Granulosa Cells

As shown in Fig. 5, a 360-bp CRH PCR product was observed in RNA from human granulosa cells, by reverse transcriptase polymerase chain reaction (RT-PCR). The DNA sequences of this PCR product were consistent with those of CRH cDNA previously reported *(11)*.

Discussion

The present study demonstrates that CRH inhibits PMSG-induced E_2 and P_4 production in both rat and human granulosa cells. Furthermore, this inhibitory effect of CRH on steroidogenesis in the ovary may at least in part be medi-

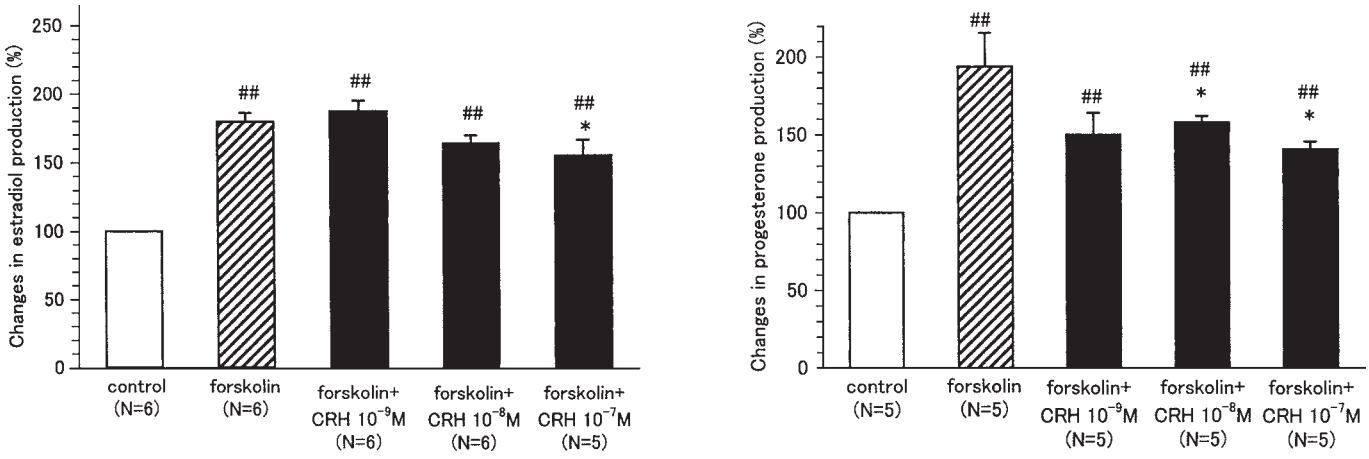

Fig. 3. Effects of CRH on forskolin-induced estradiol production (**A**) and progesterone production (**B**) in rat granulosa cells (mean ± SEM). forskolin, forskolin 10^{-5} *M*; ##$p < 0.01$ vs control; *$p < 0.05$ vs forskolin.

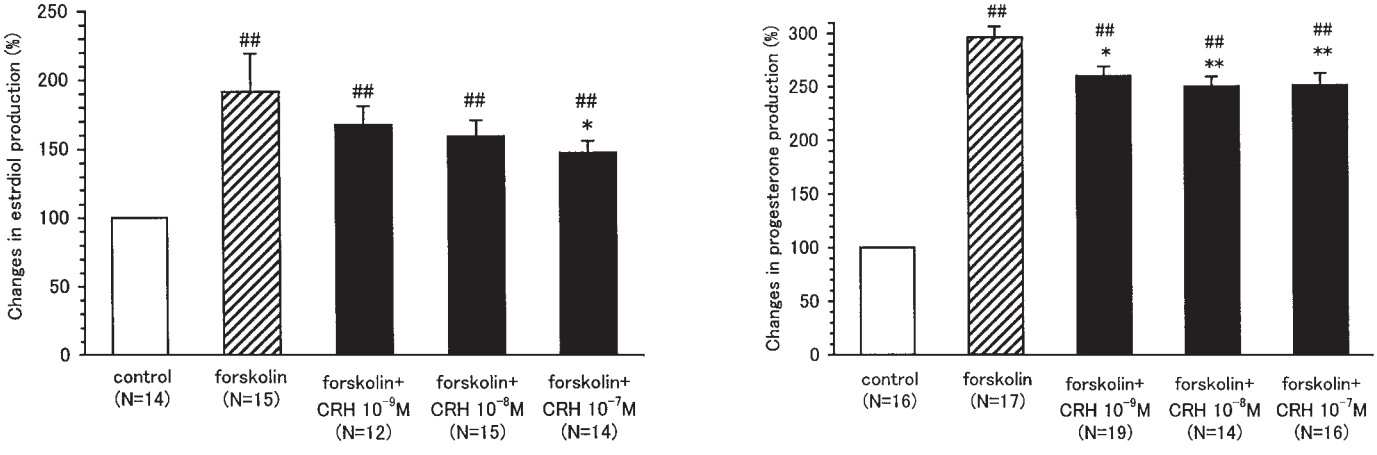

Fig. 4. Effects of CRH on forskolin-induced estradiol production (**A**) and progesterone production (**B**) in human granulosa cells (mean ± SEM). forskolin, forskolin 10^{-5} *M*; ##$p < 0.01$ vs control; *$p < 0.05$ vs forskolin; **$p < 0.01$ vs forskolin.

ated through a cAMP-dependent pathway. The results of our in vivo study indicate that exogenously applied CRH does not decrease plasma concentrations of E_2 and suggest that CRH inhibits steroidogenesis in the ovary via an autocrine or paracrine fashion.

Our results showing that CRH inhibited PMSG-induced steroidogenesis is in good accord with previous reports *(9, 10)*. In addition, the results suggest that the mechanism for the action of CRH at least in part involves a cAMP pathway, since forskolin, an adenylate cyclase activator, increased E_2 and P_4 production, and CRH partially attenuated this forskolin-induced E_2 and P_4 production. In support of this finding, it has been reported that an inhibitory effect of CRH on steroidogenesis in Leydig cells is mediated by cAMP production *(2)*. However, it has also been reported that CRH inhibits the FSH-induced increase in steroidogenesis of the ovary without affecting the level of cAMP *(9)*. Our results, together with these reports, indicate that the inhibitory effects of CRH on steroidogenesis in the ovary may relate to cellular events downstream of cAMP production.

In our study, the culture medium contained androstenedione as the substrate for E_2 production, as described by Ghizzoni et al. *(10)*, but did not contain the substrate for P_4. The responses of progesterone to CRH medium might therefore be diminished. Moreover, human granulosa cells were harvested after hCG injection, but rat granulosa cells were harvested without hCG administration. Priming by hCG might cause a different response to CRH, since a small amount of CRH immunostaining was seen in granulosa cells, but intense positive immunostaining for CRH was detected in large granulosa-derived luteinized cells *(6,7)*.

Our results showed that the suppressive effects of 10^{-8} *M* CRH on both E_2 and P_4 production were completely blocked by α-helical CRH (10^{-6} *M*). CRH therefore appears to regulate steroidogenesis in granulosa cells via CRH receptors. In support of this view, it has been shown that CRH receptor

Table 1
Effects of CRH In Vivo on Serum Concentration of Estradiol and Corticosterone in Hypophysectomized Rats[a]

	Estradiol (pg/mL)	Corticosterone (ng/mL)
Control (n = 3)	170.3 ± 78.95	94.9 ± 30.6
Hypophysectomy (n = 6)	17.03 ± 3.66[b]	1.63 ± 0.25[d]
Hypox + 1CRH (n = 7)	38.22 ± 13.18[b]	3.38 ± 1.21[c,d]
Hypox + 3CRH (n = 6)	142.40 ± 56.15[c]	2.79 ± 0.48[d]
Hypox + adrex + 3CRH (n = 3)	10.77 ± 2.08[b]	0.15 ± 0.03[d]

[a] Data are expressed as mean ± SEM. Hypox, hypophysectomy; adrex, adrenalectomy; 1CRH, 10 μg of CRH intraperitoneally 30 min before decapitation; 3CRH, 10 μg of CRH intraperitoneally for 3 d.
[b] $p < 0.05$ vs control.
[c] $p < 0.05$ vs hypophysectomy.
[d] $p < 0.01$ vs control.

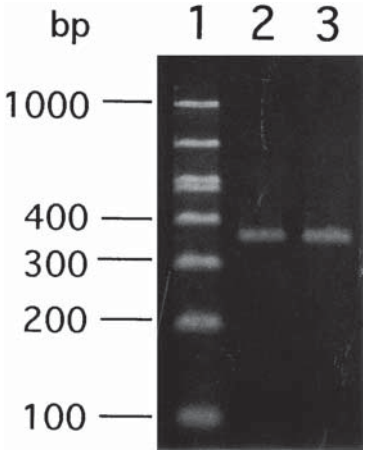

Fig. 5. RT-PCR analysis of CRH in human granulosa cells and placental tissues. PCR products were resolved on 1.5% agarose gel and stained with SYBR green I nucleic acid gel stains. The 360-bp PCR product is found in RNA extracted from human granulosa cells and placental tissues. Lane 1, size markers; lane 2, RNA-extracted placental tissues; lane 3, RNA extracted from human granulosa cells.

mRNA is expressed in human ovary *(8)*. In addition, an autoradiographic study showed that CRH binds to stromal and thecal cells surrounding follicles and to the cumulus oophorus in rat ovary *(6)*.

There are no published reports regarding the effects of CRH on steroidogenesis in the ovary of hypophysectomized rats. In the present study, we found that CRH significantly increased serum E_2 concentrations in hypophysectomized rats, but that this increase was completely blocked by adrenalectomy. These findings suggest that CRH can modulate E_2 production via actions on the adrenal gland.

Fehm et al. *(12)* reported that CRH increases corticosterone in hypophysectomized rats and suggested that CRH-induced cortisol secretion was mediated by extrapituitary adrenocorticotropic hormone (ACTH) because an increase in plasma ACTH was observed after CRH in ACTH-deficient patients. Adreis et al. *(13)* also reported that the CRH direct secretory effect on corticosterone was probably mediated by local ACTH production, because the corticosterone response of adrenal slices to CRH is completely blunted by corticotropin inhibiting peptide (CIP), a well-known competitive inhibitor of ACTH.

Administration of ACTH can increase estrogen concentrations in nonpregnant rats *(14)* and pregnant humans *(15)*. There is evidence that administration of ACTH increases adrenal estrogen and androgen release. Furthermore, it is known that androgens are metabolized to estrogens *(16)*.

Recently, Mastorakos et al. *(17)* reported that stress from immobilization decreased ovarian CRH content in rats and that ovarian CRH is acutely down regulated by a massive increase in glucocorticoids, such as that detected after immobilization.

Regarding the increase in estrogen after administration of CRH in hypophysectomized rats, the aforementioned data suggest that CRH stimulates local ACTH production, which, in turn, increases estrogen concentrations. Another possibility is that CRH-stimulated corticoids down regulate ovarian CRH, which inhibits CRH-induced suppression of ovarian steroidogenesis.

Our results suggest that the source of CRH, which affects ovarian steroidogenesis, is probably not from the hypothalamus but from the ovary itself. In support of this assumption, in vivo CRH treatment in hypophysectomized and adrenalectomized rats decreased the serum E_2 level as shown in the present study, although this decrease did not reach statistical significance.

CRH is widely distributed outside as well as inside the hypothalamus, including peripheral inflammatory sites *(5)*, Leydig cells *(2)*, and placenta *(1)*. We confirmed gene expression of CRH in human granulosa cells. Asakura et al. *(8)* also dem-onstrated gene expression of CRH in the thecal-stromal compartments of human ovary. Mastorakos et al. showed that rat *(6)* and human ovaries *(7)* contain immunoreactive CRH in thecal cells surrounding follicles, as well as in stromal cells and in large granulosa-derived luteinized cells. Again, these findings support the concept that CRH produced in the ovary acts on ovarian steroidogenesis in a paracrine or autocrine fashion.

Materials and Methods

Rat Granulosa Cell Culture

Female immature Sprague-Dawley rats (28 d old) were given an sc injection of 20 IU of PMSG (Teikoku Zouki, Kawasaki, Japan). Forty-eight hours later, the rats were killed

by cervical dislocation, and then the ovaries were removed for collection of granulosa cells. The ovaries were decapsulated, and the follicles were punctured with a 27-gage hypodermic needle. Granulosa cells were separated from red cells by Lymphoprep™ (Nycomed, Oslo, Norway). The granulosa cells were released into HTF medium (Irvine, Santa Ana, CA) containing 1% bovine serum albumin and 25 m*M* α-modified Eagle's medium, recovered by centrifugating for 5 min at 400*g*, washed once, and suspended in HTF medium. The incubation was performed in triplicate at 37ºC with approx 1×10^6 cells/mL, each in a final volume of 1 mL of HTF medium supplemented with 1.25 µg/mL of insulin, in an atmosphere of 95% O_2 and 5% CO_2. HTF medium contained 10^{-6} *M* androstenedione as a substrate for E_2 production as described by Ghizzoni et al. *(10)*. Viability (80–90%) was assessed by observing the exclusion of trypan blue.

Granulosa cells were cultured with graded concentrations of human CRH (Sigma, St. Louis, MO) for 5 d. At the end of the incubation period, media were collected and stored at –20ºC until assays for E_2 and P_4. In additional experiments, we added the CRH receptor antagonist α-helical CRH (Sigma) or forskolin (Sigma) to the incubation medium at the same time. P_4, E_2, and corticosterone were measured by radioimmunoassay (RIA) with a P_4 RIA kit, E_2 RIA kit (Daiichi, Tokyo, Japan), and a rat corticosterone [^{125}I] assay system with magnetic separation (Amersham, Buckinghamshire, England).

Human Granulosa Cell Culture

Human granulosa cells were harvested by aspiration of follicles during oocyte retrieval for in vitro fertilization. Informed consent was obtained from all subjects before the procedure. This study was approved by our institutional review board. Follicle growth was stimulated by hMG (Pergonal; Teikoku Zouki) after suppressing ovarian function by intranasal administration of a gonadotropin-releasing hormone agonist (buserelin, 900 µg daily; Nippon Hoechst Marion Roussel, Tokyo, Japan). A dose of 10,000 IU of hCG (Teikoku Zouki) was given intramuscularly when two or more follicles had reached a mean diameter of more than 16 mm. Oocyte retrieval was performed under ultrasound guidance 36 hours after hCG treatment.

Granulosa cells were cultured for 24 h and studied as just described.

In Vivo Studies

Immature Sprague-Dawley rats (28 d old) were hypophysectomized with the exception of the controls (sham operated). The completeness of hypophysectomy was confirmed by physical inspection of the sella turcica. Hypophysectomized rats were divided into four groups. The first group was treated with 20 IU of PMSG subcutaneously 5 d after operation (d 5). The second group was given PMSG as in the first group and also received an ip injection of 10 µg of CRH 30 min before decapitation. Administration of 10 µg of CRH to hypophysectomized rats was shown to increase significantly serum corticosterone concentration *(18)*. The third group was given PMSG as in the first group and also received ip injections of 10 µg of CRH for 3 d (d 4–6) as described by Hoheisel et al. *(19)*. The fourth group underwent adrenalectomy and was treated as in the third group. The control group was given PMSG alone.

Seven days after the operation, all rats were killed by deep anesthesia. Blood was collected for measurement of serum E_2 and corticosterone concentrations and the ovaries were removed and weighed.

mRNA Levels of CRH

CRH mRNA was examined in human granulosa cells by RT-PCR, using specific primers for CRH. Total RNA was extracted using an RNeasy Mini Kit (Qiagen, Hilden, Germany). Total RNA of human term placenta, which was obtained after informed consent, was used as a positive control. The total RNA was reverse transcribed by murine leukemia virus RT using random hexamers in a Gene Amp RNA PCR Core Kit (Perkin-Elmer, Norwalk, CT). cDNA was amplified by PCR using a specific set of primers. The sense primer was 5'-CAA CTT TTT CCG CGT GTT GCT-3', and the antisense primer was 5'-ATG GCA TAA GAG CAG CGC TAT-3'. This set of primers was reported to generate a 360-bp PCR product as part of CRH cDNA from human granulosa cells *(8)*.

Amplification was performed with Takara Extaq (Takara Shuzo, Kyoto, Japan) for 30 cycles under the following conditions: 2.0 µL of cDNA, 0.4 µL (10 pmol/µL) of each primer, 1.6 µL of deoxynucleotide triphosphates (2.5 mmol/L), and 0.5 U of *Taq* polymerase in a total volume of 20 µL. Each PCR amplification consisted of an initial denaturing reaction (2 min, 95ºC); 30 cycles of denaturing (1 min, 95ºC), annealing (1 min, 61ºC), and elongation (1 min, 72ºC) reactions; and a final elongation (7 min, 72ºC) reaction. PCR products were visualized by ultraviolet illumination (312 nm) of 1.5% agarose gels (agarose-1000; Life Technologies, Rockville, MD) stained with ethidium bromide. The identity of the PCR product was confirmed by direct sequencing.

Statistical Analyses

Significant differences between groups were tested by analysis of variance with Scheffe post hoc test. Statistical analysis was done with StatView software (Abacus, Berkeley, CA). A p value of <0.05 was considered statistically significant. Data are expressed as the mean ± SEM.

Acknowledgments

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