The Effect of CRH and Its Inhibitor, Antalarmin, on *in Vitro* Growth of Preantral Mouse Follicles, Early Embryo Development, and Steroidogenesis

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In vitro growth systems of preantral follicles allow studying the effect of various endocrine, paracrine, and autocrine factors on follicular growth and oocyte maturation. CRH is a 41-amino-acid neuropeptide responsible for endocrine, autonomic, immunological, and behavioral responses of mammals to stress and has two receptors, CRH receptor type 1 (CRH-R1) and CRH-R2. Antalarmin, a CRH-R1 antagonist, has been used to elucidate the role of CRH in stress, inflammation, and reproduction. The present study describes *in vitro* growth of mouse preantral follicles, early embryo development, and steroidogenesis in the presence of CRH and its antagonist antalarmin. We cultured 732 follicles in control media, 1306 in CRH 10^{-7} mol/liter, and 1202 in CRH 10^{-7} plus antalarmin 10^{-6} mol/liter. The culture medium was assayed on alternate days for 17β-estradiol, progesterone, and β-human chorionic gonadotropin. Total RNA was extracted from preantral follicles as well as early preimplantation embryos and was assessed by real-time RT-PCR for the expression of *CRH-R1* and *CRH-R2* mRNAs. Hormone analysis showed that the CRH group had lower levels of 17β-estradiol, progesterone, and β-human chorionic gonadotropin as the culture progressed, in comparison with the other two groups. RT-PCR demonstrated the presence of CRH-R1 and CRH-R2 in all stages of preantral follicle culture. Morula/blastocyst-stage embryos expressed only CRH-R1. In conclusion, CRH has an inhibitory effect on *in vitro* fertilized oocytes, resulting from cultured preantral follicles at all stages of preimplantation embryo development. Furthermore, the presence of CRH in the culture medium inhibits steroidogenesis by preantral mouse follicles cultured *in vitro*. **(*Endocrinology* 154: 222–231, 2013)**

The *in vitro* culture and growth of preantral ovarian follicles currently represents one of the most important tools of investigation in the field of assisted reproduction. In all mammalian species, follicle and oocyte development follows a distinctive sequence of events that ends with the ovulation of a mature metaphase II oocyte. *In vitro* growth of preantral follicles is a lengthy process, during which they attain the competence to complete the germinal vesicle (GV) to metaphase II (polar body) transition. Each preantral follicle consists of an oocyte arrested in prophase I and is enveloped by various layers of granulosa and theca cells. Folliculogenesis is regulated by a plethora of factors such as epidermal growth factor (EGF), basic fibroblast growth factor, IGFs, and bone morphogenetic proteins (1–4), some of which are secreted by the follicle itself (5).

CRH is a 41-amino-acid neuropeptide responsible for endocrine, autonomic, immunological, and behavioral responses of mammalian organisms to stress (6). This neuropeptide is the major regulator of the hypothalamic-pituitary-adrenal axis and along with its receptors CRH receptor type 1 (CRH-R1) and CRH-R2 has been identified in most female reproductive tissues, including uterus

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Abbreviations: CRH-R1, CRH receptor type 1; EGF, epidermal growth factor; GV, germinal vesicle; β-hCG, β-human chorionic gonadotropin; IVG, *in vitro* follicle growth.

(7), ovary (8), and endometrium (9). With regard to the ovaries, CRH is localized in luteinized cells of the stroma, in theca cells, in various cells of the developing corpora lutea, and in the oocytes of antral follicles (8, 10). The two different receptors of CRH, CRH-R1 and CRH-R2, which mediate the biological effects of CRH, are found in stroma and theca cells surrounding the follicles and in the cumulus oophorus (11). During folliculogenesis, CRH is not expressed in primordial follicles but is expressed in mature follicles. This fact indicates a possible autocrine/paracrine role of CRH in follicular maturation (8, 10).

In vitro experiments have shown that CRH has a significant regulatory role on ovarian steroidogenesis by inhibiting steroid biosynthesis via IL-1 receptor (12). Especially in the rat, CRH suppresses estrogen production from granulosa cells *in vitro* (13). On the other hand, the abundant availability of CRH-R antagonists has led to extensive research focusing on the stress axis and the diseases that may be associated with stress (14). Antalarmin, a synthetic micromolecular CRH-R1 antagonist, has been used to elucidate the role of CRH in stress, inflammation, and reproduction (15).

The present study describes *in vitro* growth of mouse preantral mouse follicles in the presence of CRH and its antagonist antalarmin. Our previous study demonstrated that CRH inhibits *in vitro* oocyte maturation in mice at the concentration of 10^{-7} mol/liter, and antalarmin reverses this effect at the concentration of 10^{-6} mol/liter (16). In addition, the concentration of CRH 10^{-7} mol/liter is capable of suppressing FSH-stimulated (1 IU/liter) estrogen release by about 30% (17). To enrich these data, we examined the effect of CRH at this specific concentration on steroidogenesis and early embryo development. Furthermore, we examined the expression of *CRH-R1* and *CRH-R2* mRNA in both preantral follicles and preimplantation embryos using real-time quantitative RT-PCR.

Materials and Methods

Animals

All female and male mice used in this study were C57BL/6 female × CBA male F1 hybrids raised and cared for at the Pasteur Institute (Athens, Greece). This study was reviewed and approved by the University Hospital Ethics Committee and the Animal Care and Use Committee of the Pasteur Institute.

Experimental groups

To assess the effect of CRH and its antagonist antalarmin on *in vitro* growth of preantral mouse follicles and on early embryo development, three experimental groups were studied: 1) control group consisting of preantral follicles cultured *in vitro* in the basic culture medium, 2) CRH 10^{-7} mol/liter group consisting of preantral follicles cultured *in vitro* in the basic culture medium supplemented with CRH (CRH, human, rat; Sigma-Aldrich, St. Louis, MO) at a concentration of 10^{-7} mol/liter, and 3) CRH 10^{-7}/antalarmin 10^{-6} mol/liter group consisting of preantral follicles cultured *in vitro* in the basic culture medium supplemented with CRH at a concentration of 10^{-7} mol/liter and antalarmin in a concentration of 10^{-6} mol/liter (kindly provided by Dr. E. Zoumakis), diluted first in absolute ethanol at a concentration of 10^{-3} mol/liter and stored at 4 C.

Follicle collection for *in vitro* growth

Prepubertal mice (aged 12–14 d) were euthanized via cervical translocation, and their ovaries were aseptically removed and placed in isolating medium consisting of Dulbecco's PBS (Life Technologies, Paisley, UK) with 10% BSA (bovine serum fraction V; Sigma-Aldrich). The ovaries were mechanically dissected using hypodermic needles (26 gauge) and preantral follicles measuring 100–130 nm in diameter with centrally located oocyte and at least one granulosa cell layer were selected for culture. This technique yielded at least 40 good quality early preantral follicles per ovary.

In total, 3240 preantral follicles were collected and randomly distributed in the three experimental groups. In particular, 732, 1306, and 1202 follicles were assigned to control, CRH 10^{-7} mol/liter, and CRH 10^{-7}/antalarmin 10^{-6} mol/liter groups, respectively.

Follicle culture for *in vitro* growth

After two washing steps in isolating medium and one wash in culture medium, the preantral follicles were cultured in groups of 20–25 follicles in 60 × 15 mm petri dishes (BD Falcon, Franklin Lakes, NJ) under 1 ml mineral oil. The culture medium consisted of 1 ml α-MEM (Invitrogen, Life Technologies, Paisley, UK) supplemented with 5% fetal bovine serum (Invitrogen); 10 μl insulin-transferrin-selenium (Invitrogen) equivalent to 5 μg/ml insulin, 5 μg/ml transferrin, and 5 ng/ml selenium; 100 mIU/ml recombinant FSH (Puregon; Merck & Co., Whitehouse Station, NJ); and 10 mIU/ml recombinant LH (Luveris; Merck-Serono, Merck KGaA, Darmstadt, Germany). Culture conditions were adjusted to 37 C, 5% CO_2 in air and 95% humidity. Half of the medium was renewed on alternate days.

Sampling of conditioned medium for evaluation of steroidogenesis and β-human chorionic gonadotropin (β-hCG) measurements

Samples of 500 μl of spent medium were collected sequentially on culture d 3, 5, 7, 9, and 11. In addition, samples of culture medium were also collected on d 1 for baseline analysis. Two steroids, estradiol-17β and progesterone, and one glycoprotein hormone, β-hCG, were determined in all samples through electrochemiluminescence immunoassay. It should be noted that only petri dishes containing 20 preantral follicles were sampled to achieve comparable results in hormone determination among the study groups.

Ovulation and *in vitro* fertilization of the *in vitro* grown oocytes

After 10 d of culture, ovulation induction was carried out by adding 1.5 IU/ml hCG (Pregnyl; Organon, Oss, The Nether-

lands) and 5 ng/ml EGF (Recombin; Invitrogen) to the preantral follicles culture (18). At 16–18 h later, the *in vitro* released mucified cumulus-oocyte complexes were observed. The 3- to 6-month-old male mice were killed through cervical translocation, their epididymides were freed, and each of them was placed in droplets of 100 μl Ham's F10 medium without hypoxanthine (Life Technologies) supplemented with 5 mg/ml BSA under mineral oil. Epididymal contents were carefully squeezed out to release spermatozoa. The suspensions were allowed to capacitate for 2 h in the incubator (37 C, 5% CO_2 in air, 95% humidity) and the spermatozoa were transferred to the cumulus-oocyte complexes for insemination at a final motile sperm concentration of $1–2 \times 10^6$/ml. Three hours later, oocytes were washed and freed from cumulus cells and the attached sperm and cultured in 1 ml Ham's medium with 5 mg/ml BSA, 1.5 U/ml recombinant LH and 100 mIU/m recombinant FSH under mineral oil for 5 d to evaluate early embryo development.

RNA extraction and cDNA preparation

Total RNA was extracted from preantral follicles obtained sequentially on culture d 3, 5, 7, 9, and 11 as well as morula and blastocyst stage development. A total RNA extraction kit (RNeasy micro kit) was obtained from QIAGEN (Valencia, CA), and the extraction was performed according to the manufacturer's protocol.

Aliquots (500 ng/ml) of total RNA extracted were reversed transcribed using 0.5 mM dNTP mix (Ambion, Austin, TX), 5 μM oligo-deoxythymidine primer (Ambion), 80 U ribonuclease inhibitor (Invitrogen), 1 × First Standard Buffer (Invitrogen), 1600 U MML-V reverse transcriptase (Invitrogen) and up to 40 μl total volume of reaction nuclease-free water. The reactions were performed at 80 C for 3 min, at 42 C for 60 min, and at 92 C for 10 min on a Mastercycler (Eppendorf, Hamburg, Germany).

Real-time PCR

The expression of *CRH-R1* and *CRH-R2* genes in preantral follicles as well as morula and blastocyst stage development were assessed by real-time RT-PCR using primers and probes pairs particularly synthesized by TIB-MOLBIOL (Berlin, Germany) for this study (Table 1).

The specific primers and probes were used at a concentration of 20 pmol/μl in each reaction. Quantitative real-time PCR was performed in a final reaction volume of 20 μl in a LightCycler 480 white 96-multiwell plate (Roche Diagnostics, Mannhein, Germany). All samples were run in duplicate, and no-template controls were included in all runs to exclude possible DNA contamination. The RT-PCR mixture contained 5× LightCycler 480 Genotyping Master (Roche), 0.5 μM for each primer, 0.2 μM for each probe, up to 20 μl total volume of reaction H_2O of LightCycler 480 Genotyping Master (Roche), and 5 μl cDNA. For the expression of *CRH-R1* and *CRH-R2*, *muG6PD* was used as a reference gene. Real-time PCR was performed on LightCycler 480 II (Roche) with the following parameters: one cycle at 95 C for 10 min for preincubation, 40 cycles for amplification (95 C for 10 min, 56 C for *CRH-R1* and 58 C for *CRH-R2* for 20 min, and 72 C for 10 min), and one cycle at 4 C for cooling.

Statistics

As far as the results of the early embryo development are concerned, they were analyzed by Fisher's exact test. The results of the hormones were analyzed by employing ANOVA followed by *post hoc* tests (Dunnett's test for comparisons with a control, Bonferroni correction for multiple comparisons). To check the validity of the results obtained using a parametric ANOVA test, we additionally performed a Kruskal-Wallis test. RT-PCR results were analyzed by employing a paired *t* test. To check the validity of the results obtained using the parametric test, we additionally performed a Wilcoxon signed rank test. The obtained results did not differ from the results of the parametric tests employed. Statistical significance was set at $P < 0.05$.

Results

Evaluation of CRH and its inhibitor, antalarmin, on *in vitro* growth of preantral follicles and early embryo development

Among the 3240 preantral follicles collected, 732 were assigned to the control group, 1306 to the CRH 10^{-7}

TABLE 1. Primers sense and antisense and probes fluorescein and LightCycler of *CRH-R1, CRH-R2,* and *muG6PD*

	Sequence	Identifier	Melting temperature (°C)
CRH-R1		NM_007762	
CRH-R1 S	CAACACCACAAACAATggC TA	422–442	55.9
CRH-R1 A	TgATgACggCAATgTggTAgT	555–535	57.1
CRH-R1 FL	CTgggCAgCCCgTgTgAA-FL	467–484	63.4
CRH-R1 LC	640- TATTCTgAgTgCCAggAgATTCTCAACgA p	486–517	64.6
CRH-R2		NM_009953	
CRH-R2 S	CCAgATCgggACCTgCTg	326–343	58.8
CRH-R2 A	AggCACTCTCTgTAggCATTCC	442–421	58.6
CRH-R2 FL	AgTAgAgAgACCgTgCCCCgAg-FL	368–389	63.1
CRH-R2 LC	640-ACTTCAATggCATCAAgTACAACACgACC p	391–419	64.4
muG6PD		NM_009953	
G6PD S	CCAgATCgggACCTgCTg	326–343	58.8
G6PD A	AggCACTCTCTgTAggCATTCC	442–421	58.6
G6PD FL	AgTAgAgAgACCgTgCCCCgAg-FL	368–389	63.1
G6PD LC	640-ACTTCAATggCATCAAgTACAACACgACC p	391–419	64.4

S, Sense; A, antisense; FL, fluorescein; LC, LightCycler.

TABLE 2. The numbers and percentages of mature mouse preantral follicles that reached the two-, four-, and eight-cell and morula/blastocyst embryo stages

	Preantral (n)	Maturation [n (%)]	2-cell [n (%)]	*P* value	4-cell [n (%)]	*P* value	8-cell [n (%)]	*P* value	M/B [n (%)]	*P* value
Control	732	246/33.6	51/20.73		43/17.47		31/12.60		22/8.94	
CRH 10^{-7}	1306	342/26.18	34/9.94	<0.001	26/7.60	<0.001	13/3.80	<0.001	10/2.92	0.003
CRH 10^{-7}/antalarmin 10^{-6}	1202	346/28.78	72/20.80	NS	59/17.05	NS	44/12.71	NS	34/9.82	NS
CRH 10^{-7} *vs.* CRH 10^{-7}/antalarmin 10^{-6}				<0.001		<0.001		<0.001		<0.001

P values refer to the comparison of CRH 10^{-7} and CRH 10^{-7}/antalarmin 10^{-6} *vs.* the control group, whereas a comparison of CRH 10^{-7} *vs.* CRH 10^{-7}/antalarmin 10^{-6} is also shown (*P* value <0.05 is considered statistically significant). M/B, Morula/blastocyst stage; NS, not significant.

mol/liter group, and 1202 to the CRH 10^{-7}/antalarmin 10^{-6} mol/liter group. To evaluate the effect of CRH and its inhibitor antalarmin on early embryo development, we studied three distinct early embryo stages, *i.e.* two-cell, four-cell, and eight-cell stage, and two advanced embryo stages, *i.e.* morula and blastocyst stage, which were grouped for analysis (morula/blastocyst stage). The extraction of the first polar body by metaphase II oocytes helped us count how many of these follicles matured. In particular, 246 mature follicles were counted in the control group, 342 in the CRH 10^{-7} mol/liter group, and 346 in the CRH 10^{-7}/antalarmin 10^{-6} mol/liter group. The percentages of mature preantral follicles in control, CRH 10^{-7} mol/liter, and CRH 10^{-7}/antalarmin 10^{-6} mol/liter groups that reached the two-cell stage were 20.73, 9.94, and 20.80%, respectively, whereas the corresponding percentages for the four-cell stage were 17.47, 7.60, and 17.05%. Likewise, the eight-cell stage was attained by 12.60, 3.80, and 12.71% of mature preantral follicles of the three groups and the morula/blastocyst stage by 8.94, 2.92, and 9.82% of them, respectively (Table 2).

At all embryo stages, significant differences were noted between the CRH 10^{-7} and control groups as well as between the CRH 10^{-7} and CRH 10^{-7}/antalarmin 10^{-6} groups, with the rates being lower in the former. In particular, significant differences were observed at the two-, four-, and eight-cell stages, where the rates in the CRH 10^{-7} group were lower compared with the control group ($P < 0.001$) and lower compared with the CRH 10^{-7}/antalarmin 10^{-6} group ($P < 0.001$). With regard to the morula/blastocyst stage, the rates in the CRH 10^{-7} group were lower compared with the control ($P = 0.003$) and lower compared with the CRH 10^{-7}/antalarmin 10^{-6} group ($P < 0.001$) (Table 2).

Evaluation of estradiol production

Baseline estradiol levels in the medium used to culture preantral follicles was 31.9 ± 2.703 pg/ml (mean ± SEM) on d 1. In the control group, the production of estradiol increased on subsequent days from d 5 of culture onward (d 5, 7, and 9). On d 11, a small decrease was noticed. On the other hand, exposure of preantral follicles to CRH resulted in a significant decrease of the amount of estradiol released into the incubation medium on d 5 ($P = 0.045$), d 7 ($P = 0.014$), d 9 ($P < 0.001$), and d 11 ($P = 0.001$). Interestingly, the addition of a 10-fold excess of the CRH-R1 antagonist, antalarmin, overcame the negative effect of CRH on estradiol release (d 5, $P = 0.009$; d 7, $P = 0.010$; d 9, $P < 0.001$; and d 11, $P = 0.002$), indicating that the suppressive effect of CRH was receptor mediated (Fig. 1).

Evaluation of progesterone production

Baseline progesterone levels in the medium used to culture preantral follicles was 0.030 ± 0.000 ng/ml (mean ± SEM) on d 1. On subsequent days (day 3, 5, 7, 9), the

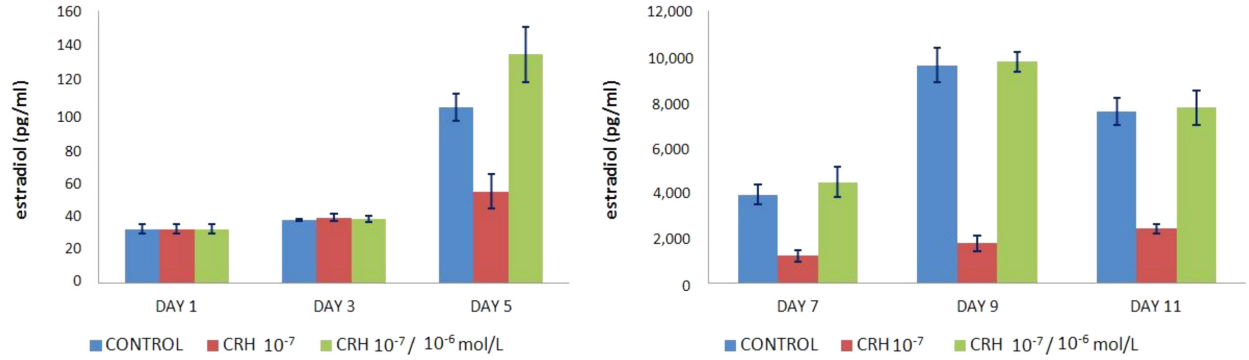

FIG. 1. A, Estradiol levels in the culture media of preantral mouse follicles on d 1, 3, and 5 in the three experimental groups: 1) control, 2) CRH 10^{-7} mol/liter, and 3) CRH 10^{-7} plus antalarmin (ANT) CRH 10^{-6} mol/liter; B, estradiol levels in the culture media of preantral mouse follicles on d 7, 9, and 11 in the three experimental groups: 1) control, 2) CRH 10^{-7} mol/liter, and 3) CRH 10^{-7} plus antalarmin (ANT) CRH 10^{-6} mol/liter.

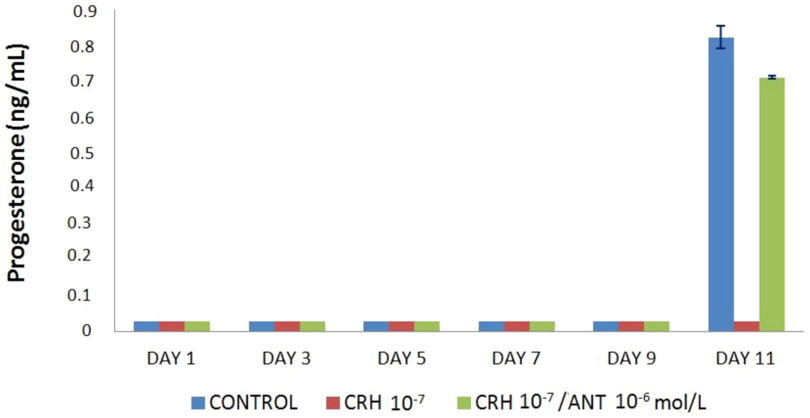

FIG. 2. Progesterone levels in the culture media of preantral mouse follicles on d 1, 3, 5, 7, 9, and 11 in the three experimental groups: 1) control, 2) CRH 10^{-7} mol/liter, and 3) CRH 10^{-7} plus antalarmin (ANT) CRH 10^{-6} mol/liter.

production of progesterone in Control group did not increase. On d 11, 1 d after triggering ovulation, an increase of 0.824 ± 0.032 ng/ml was recorded. Notably, CRH did not affect the amount of progesterone released into the incubation medium up to d 9, whereas it suppressed progesterone levels on d 11 (d 11 progesterone 0.030 ± 0.000 ng/ml, $P < 0.001$). The addition of a 10-fold excess of the CRH-R1 antagonist antalarmin reversed the negative effect of CRH on progesterone release (d 11 progesterone 0.712 ± 0.004 ng/ml, $P < 0.001$), suggesting that the suppressive effect of CRH was receptor mediated (Fig. 2).

Evaluation of β-hCG production

Baseline β-hCG levels of medium used to culture the follicles was 0.100 ± 0.000 mIU/ml (mean ± SEM) on d 1. On alternate days, from d 3–9, the production of β-hCG marginally increased in the control group. After the addition of 1.5 IU/ml hCG and 5 ng/ml EGF for triggering ovulation, an increase of β-hCG at 174.6 ± 3.480 mIU/ml was noticed. In the CRH 10^{-7} mol/liter group, the amount of β-hCG measured in the culture medium was elevated but to a lesser extent compared with the control group (d 11 β-hCG 6.05 ± 0.077 mIU/ml, $P < 0.001$). The addition of the CRH-R1 antagonist antalarmin in a 10-fold higher concentration compared with that of CRH overcame the effect of CRH on β-hCG release (d 11 β-hCG 185.6 ± 6.087 mIU/ml, $P < 0.001$) indicating that the suppressive effect of CRH was receptor mediated (Fig. 3).

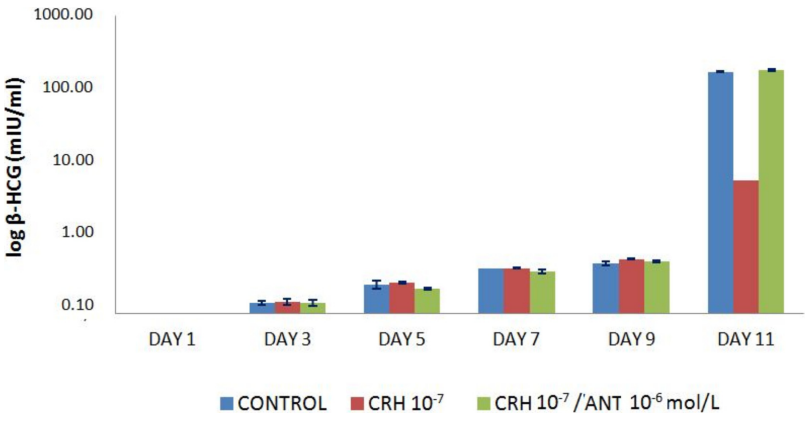

FIG. 3. Log β-hCG levels in the culture media of preantral mouse follicles on d 1, 3, 5, 7, 9, and 11 in the three experimental groups: 1) control, 2) CRH 10^{-7} mol/liter, and 3) CRH 10^{-7} plus antalarmin (ANT) CRH 10^{-6} mol/liter.

The expression of *CRH-R1* and *CRH-R2* genes in preantral follicles

CRH-R1 mRNA and *CRH-R2* mRNA were found in mouse preantral follicles on d 3, 5, 7, 9, and 11 of follicle cultures in the presence (CRH 10^{-7} mol/liter group) or absence (control group) of CRH and in the presence of both CRH and antalarmin (CRH 10^{-7}/antalarmin 10^{-6} mol/liter group). The experiments were performed three times with different groups of preantral follicles, and the results were concordant. Roche Quantification Software was used to determine *CRH-R1*, *CRH-R2*, and *muG6PD* transcript concentrations and to calculate the *CRH-R1*/*muG6PD* and the *CRH-R2*/*muG6PD* ratios. According to our results, the expression of *CRH-R1* was significantly higher ($P = 0.010$) than the expression of *CRH-R2* during *in vitro* growth of the mouse preantral follicles (Table 3).

The expression of *CRH-R1* and *CRH-R2* genes in the morula/ blastocyst stage

CRH-R1 mRNA was found in mouse morula and blastocysts derived from *in vitro* fertilization of preantral follicles of all three study groups (Fig. 4). On the other hand, *CRH-R2* mRNA was not found in mouse morula and blastocysts (data not shown). The experiments were done in triplicate using different groups of mouse embryos, and the results were in agreement.

Discussion

We have recently shown that 10^{-9}, 10^{-7}, and 10^{-6} mol/liter CRH added to long-term early preantral follicle cultures inhibits *in vitro* oocyte maturation, most probably at the GV and/or GV breakdown stage, suggesting that CRH contributes to arrest of nuclear maturation of mouse oocytes at the early stages of this process (16). With

TABLE 3. Statistical analysis of expression of *CRH-R1* and *CRH-R2*

Ratios	n	Mean ± SEM	*P* value
CRH-R1/muG6PD	15	0.1160674 ± 0.0403604	0.010[a]
CRH-R2/muG6PD	15	0.01274133 ± 0.002833	

[a] Wilcoxon signed rank test (nonparametric test).

regards to steroidogenesis, Calogero and colleagues (17) have previously found that the presence of CRH in a concentration of 10^{-7} and 10^{-8} mol/liter is capable of suppressing FSH-stimulated (1 IU/liter) estrogen release by about 30% in rat granulosa cell cultures. In the same study, the lowest significant effect in estrogen production by human granulosa-lutein cells was achieved with a CRH concentration of 10^{-10} mol/liter, which is 100- to 1000-fold lower compared with rats. Furthermore, Ghizzoni and colleagues (12) working on human granulosa cells obtained from women undergoing *in vitro* fertilization have shown that graded doses of CRH (10^{-10} to 10^{-6} mol/liter) added in the culture media significantly decreased estradiol and progesterone levels. Motivated by this background, in this study, we examined the effect of 10^{-7} mol/liter CRH and the combination of 10^{-7} mol/liter CRH plus 10^{-6} mol/liter of its inhibitor antalarmin on *in vitro* growth of preantral mouse follicles, early embryo development after fertilization, and steroidogenesis.

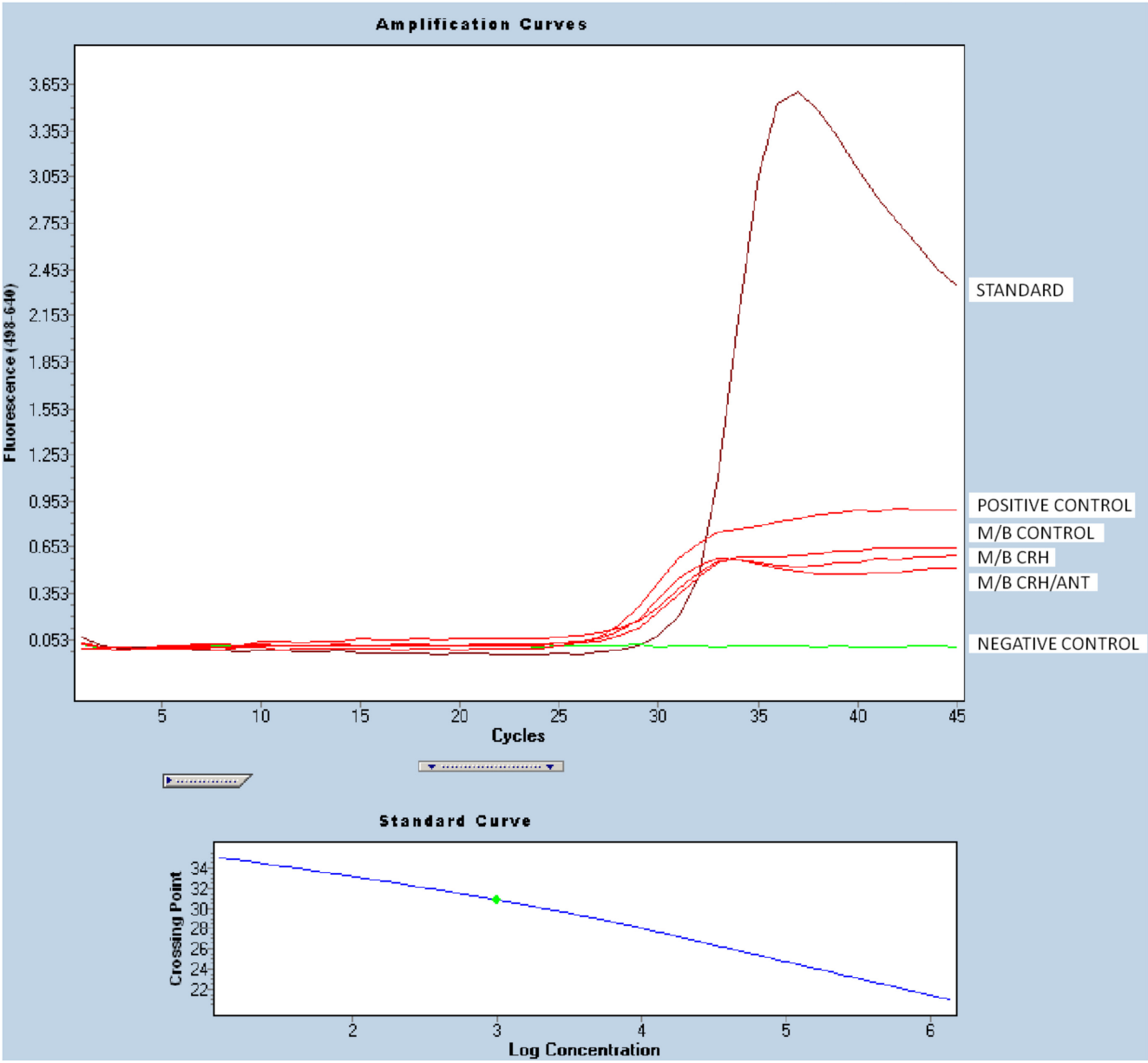

FIG. 4. Expression of CRH-R1 using real-time RT-PCR in the morula/blastocyst (M/B) control group, the M/B CRH 10^{-7} mol/liter group, and the M/B CRH 10^{-7} plus antalarmin 10^{-6} mol/liter (ANT) group.

A previous study measured immunoreactive CRH levels in the follicular fluid collected from hormonally stimulated human ovaries through a long GnRH-agonist protocol. The levels varied between 2.26×10^{-11} and 8.09×10^{-11} mol/liter (mean ± SEM $4.76 \pm 0.97 \times 10^{-11}$ mol/liter) (12). Small amounts of immunoreactive CRH were also measured within media of granulosa-lutein cell cultures (mean ± SEM $1.31 \pm 0.29 \times 10^{-11}$ mol/liter). Most of this CRH was attributed to granulosa-lutein cell production, whereas production by resident macrophages of the ovary present in the cultures was also considered. To study the effect of endogenous CRH production on follicular growth, the authors supplemented the culture media with α-helical CRH_{9-41}, a nonselective peptidic CRH-R1/CRH-R2 antagonist. However, the antagonist had no effect when added without CRH, possibly due to the low levels of endogenous production of this neuropeptide. Based on these data, we opted to exclude an antalarmin-only arm from the design of our study.

We found a statistically significant difference in the development of early (two-, four-, and eight-cell) and advanced (morula/blastocyst) embryo stages in the presence of CRH. In fact, in the CRH 10^{-7} mol/liter group, the rates were significantly lower compared with the control group, suggesting that CRH contributes to arrest of mouse embryo development. The addition of the specific CRH-R1 antagonist antalarmin in *in vitro* cultures with CRH yielded higher survival rates in all embryo stages, suggesting that antalarmin reverses the inhibitory effect of CRH.

With regard to steroidogenesis, we found that exposure of preantral mouse follicles to CRH 10^{-7} mol/liter caused a remarkable reduction in estradiol concentrations in the culture medium from d 5–11. In addition, although CRH 10^{-7} mol/liter did not affect progesterone concentrations in the culture medium from d 1–9, it caused a prominent reduction in its release by early luteinized cumulus cells on d 11 after triggering of ovulation. Interestingly, the reduction of both hormone concentrations was reversed by the addition of antalarmin indicating a CRH-R-mediated effect.

The inhibition of FSH-stimulated estrogen production from rat and human granulosa cells cultured in the presence of CRH has been shown to be dose dependent, an effect mediated by the CRH-R, because a selective antagonist was able to overcome it (17). Compatible results from another study in human granulosa-lutein cell cultures showed that increasing concentrations of CRH caused a moderate but consistent reduction in both estradiol and progesterone concentrations. This action appeared to be mediated by CRH-R and IL-1 receptor (12). A receptor-mediated effect of CRH was also shown in rat granulosa cells, where the inhibitory effect of CRH on IGF-I and estrogen release was reversed by a selective CRH-R antagonist (13). The suppressive effect of CRH on the autocrine/paracrine action of IGF-I could possibly explain the decrease in estradiol production in our *in vitro* culture systems. Interestingly, this effect was successfully antagonized by antalarmin, which restored estradiol levels. As expected, progesterone production was prominent in the control group on d 11 after hCG-induced luteinization of granulosa cells. However, no increase in progesterone levels was noted in the CRH 10^{-7} mol/liter group, which could be probably attributed to the suppressive effect of CRH on IGF-I release, because it is known that IGF-I favors progesterone biosynthesis (19).

With regard to β-hCG levels in the CRH 10^{-7} mol/liter group, we were surprised by the fact that although 1.5 IU/ml of this hormone was added on d 10, β-hCG levels were at unexpectedly low levels on d 11. The relatively low levels found in the control and CRH plus antalarmin groups could be hypothetically attributed to hCG binding to LH receptors on granulosa cells, leaving little free hCG in the culture medium. However, in the CRH group, an initial IGF-I-mediated effect could stand as a possible explanation for the diminished hCG levels. In fact, IGF-I induces LH receptor sites in both theca (20) and granulosa cells (19). Furthermore, LH activity is known to up-regulate LH receptors at least in males, because exposure of testicular Leydig cells, the male homolog of ovarian theca cells, to LH or hCG results in a rise in LH receptor (21). Combining these data, we could speculate that CRH-mediated inhibition of IGF-I release causes an initial reduction of LH/hCG-binding capacity, which is followed by a rise in LH receptor sites (up-regulation) induced by the addition of hCG on d 10 of culture. Increasing hCG binding to abundant LH receptors leads thus to a progressive depletion of β-hCG in the culture medium (Fig. 5). Although this hypothetical pathway sounds reasonable, it is awaiting verification in the laboratory setting. To this end, future studies would investigate cumulus-lutein cell LH receptor expression along with IGF-I and hCG concentrations in the culture medium at various time points after triggering ovulation to unravel the underlying mechanism and prove this hypothesis *in vitro*.

Unlike the fact that folliculogenesis can progress in hypoestrogenic conditions, optimal oocyte cytoplasm and oolemma maturation require the presence of estrogens (22). Data derived from Intra Cytoplasmic Sperm Injection (ICSI) on human oocytes reveal that estrogen-rich follicles tend to yield better-quality oocytes with an increased chance to undergo fertilization and result in a successful pregnancy. These data reinforce the aspect that estradiol plays a fundamental role in the process of oocyte maturation, leading to successful embryogenesis. Oocyte

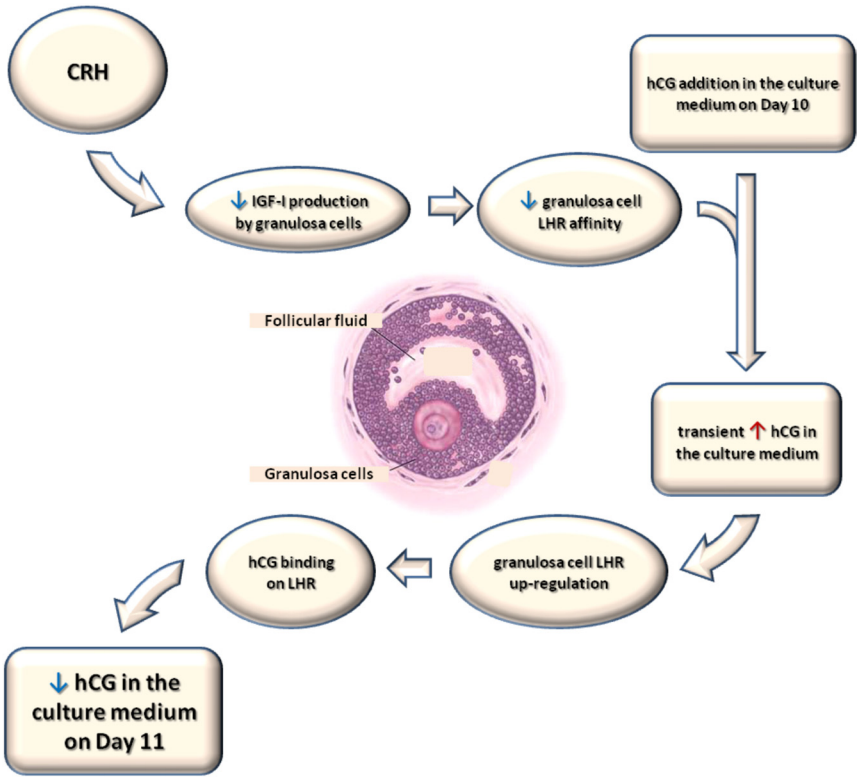

FIG. 5. Increasing hCG to abundant LH/hCG receptors (LHR) leads to a progressive depletion of β-hCG in the culture medium of the preantral mouse follicles in CRH 10^{-7} mol/liter group.

maturation is characterized by changes in its distinct compartments, the nucleus and the cytoplasm. *In vitro* studies have shown that nuclear maturation is cAMP dependent, whereas cytoplasmic maturation is driven by a specific intrafollicular milieu mainly determined by steroids. In particular, they have been shown to affect cytoplasmic maturation either indirectly via granulosa and cumulus cells or directly through changes in the reactivity of the oocyte's Ca^{2+} release mechanism (23). Yoshimura and colleagues (24) used cyanoketone to block ovarian steroid synthesis in rabbit ovaries, concluding that ovarian steroidogenesis is not essential for the resumption of meiosis but participates in cytoplasmic maturation and thus may be essential for the fertilizability of the oocyte.

In our previous study, we demonstrated CRH-associated inhibition in the resumption of meiosis in mouse oocytes through activation of CRH-Rs, which lead to adenylate cyclase activation and subsequent cAMP elevation (16). Herein, although we did not directly assess fertilization rate via two-pronuclei-stage tracing, we found a significant difference in the percentage of two-cell-stage embryos between CRH and control group, suggesting a negative effect of CRH on oocyte cytoplasmic maturation reflected by early embryo development. Basically, CRH was found to block estradiol synthesis, implying that estradiol production is not a prerequisite for the resumption of meiosis but participates in cytoplasmic maturation of the oocyte. Our findings demonstrate a new mechanism involved in oocyte maturation during *in vitro* follicle growth (IVG) conditions driven by CRH, which interferes with nuclear maturation, as previously shown, but also seems to affect cytoplasmic maturation. In particular, the specific antisteroid (antiestrogen) action of CRH alters oocyte's microenvironment with regard to the inducive signals needed to complete cytoplasmic maturation, a mechanism that is reversible by antalarmin. This novel evidence of action in cytoplasmic maturation could offer the basis for further research regarding the physiology of oocyte maturation process, especially in IVG cultures.

In our earlier study, we also reported that *CRH-R1* mRNA is expressed in mouse preantral follicles throughout follicular culture in the presence of either CRH or CRH and antalarmin. In this study, we reappraised the expression of *CRH-R1* mRNA in mouse preantral follicles using real-time RT-PCR and, furthermore, investigated the expression of *CRH-R2* mRNA. We demonstrated that *CRH-R2* mRNA is also expressed in mouse preantral and antral follicles in cultures devoid of CRH and in the presence of either CRH or CRH plus antalarmin. According to the quantitative analysis, we found that mouse preantral and antral follicles express both *CRH-R1* and *CRH-R2*, although *CRH-R2* is expressed to a lesser extent. CRH-R1 has been described in mouse and human ovaries in theca cells, in stroma cells, and in cumulus oophorus (9, 25). In rats under both control and stressful conditions during the gonadal life cycle, expression of the gene encoding the type 1 but not the type 2 CRH-R was found in selective ovarian compartments. The temporal and anatomic selectivity of the ovarian periovulatory *CRH-R1* gene expression indicates that a critical biological action for CRH may be identified during the ovulatory process and that the intraovarian environment may influence the stress-induced transcription of that particular CRH-R subtype in rat ovaries (26). Considering this fact along with the lower expression of *CRH-R2* in preantral and antral follicles, we could speculate that CRH-R2 may not substantially participate in follicular growth and oocyte maturation. Moreover, the anti-CRH role of the selective CRH-R1 antagonist antalarmin has been investigated as a therapeutic

tool in disorders associated with CRH hypersecretion with promising results (27). Two reports in mice lacking CRH-R1 concluded that CRH participates in the behavioral response to stress through CRH-R1 (25, 28). Hence, a possible therapeutic role of antalarmin in the treatment of stress-related anovulatory states could not be ruled out.

We also found that mouse preimplantation embryos of the morula/blastocyst stage express *CRH-R1* but not *CRH-R2* mRNA. In fact, we partially reappraised the findings of a single previous study regarding the expression of *CRH-R1* in mouse embryos (29). Combining these findings with the fact that *CRH-R2* had low expression in mouse preantral and antral follicles, we could enrich the aforementioned aspect that, contrary to CRH-R1, CRH-R2 may be involved to a lesser extent in follicular growth by the suggestion that this receptor may be involved to a lesser extent in early embryo development in an autocrine and/or paracrine manner, if at all. Furthermore, taking into account that a synchronized interaction between the blastocyst and the adhesive endometrium characterizes the complex implantation process and that the endometrial stroma cells produce CRH, with highest expression at the implantation sites (30), the secretion of CRH by preimplantation embryos along with the expression of *CRH-R1* in the hatching blastocyst may determine implantation potential.

In conclusion, our data suggest a new role for CRH in follicular development, which is reversed by its inhibitor antalarmin. This role is characterized by a reduction in steroidogenesis, resulting from a direct and/or indirect, through the IL-1 (12) and/or IGF-I pathways (13), effect of CRH on the granulosa-lutein cells. This evidence could help improve IVG conditions, enhancing fertilization and cleavage rates of the developing embryos. Furthermore, given that stress is defined as the response of the body to any threatening demand (31), a possible clinical application of this knowledge is suggested. As peripheral central nervous system effectors, postganglionic sympathetic nerve fibers may secrete CRH in the ovary and the reproductive tract (32). Thus, increased stress levels may induce regional CRH secretion in the ovary and the fallopian tube milieu, as these organs have sympathetic innervation. This suggests that it might be possible to optimize oocyte quality and embryo cleavage rates during ovulation induction and *in vitro* fertilization techniques by modifying patient stress levels.

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