Life Sciences, Vol. 34, pp. 273-280
Printed in the U.S.A.

Pergamon Press

INHIBITION OF IN VITRO HUMAN CHORIONIC GONADOTROPIN-STIMULATED TESTOSTERONE PRODUCTION IN TESTIS AND OF OVULATION IN THE RAT BY CHARCOAL-TREATED RAT TESTICULAR EXTRACT*

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(Received in final form November 1, 1983)

SUMMARY

Previously, we described the presence of a factor obtained from a 105,000 x g supernatant of rat testis that was found to inhibit human chorionic gonadotropin (hCG) binding to gonadal receptors. In the present study, similarly prepared testicular extract was tested for its effects on in vitro hCG-stimulated testosterone production by isolated testis interstitial cells and for its effect on spontaneous ovulation in the rat. Incubation of interstitial cells with charcoal-treated extract significantly inhibited the steroidogenic response to hCG in a dose-related manner. This inhibition was also apparent after heating the extract for 10 min at 100°C. Preincubation of the cells with charcoal-treated extract resulted in an inhibitory effect that was not readily reversed by subsequent addition of hCG, revealing an element of irreversibility in the mechanism of inhibition. A single i.p. injection of testicular extract given between 1430-1630 h of proestrus inhibited spontaneous ovulation in the rat. This effect was also observed after heating the extract for 10 min at 100°C; in contrast, no significant effect was obtained with the injection of a similar dose of liver extract. Administration of 5 IU hCG after pretreatment with the testicular extract did not reverse the inhibitory effect on ovulation, indicating that this effect was probably not exerted at the hypothalamus-pituitary level. It is concluded that the aqueous testicular extract contains a factor able to antagonize the physiological events mediated by luteinizing hormone (LH)/hCG, and that this factor is consistent with the presence of an LH/hCG-binding inhibitory activity in rat testis.

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*Supported by an Institutional Research Grant (S07-RR05654) from The University of Texas Health Science Center at San Antonio and by the Consejo de Desarrollo Científico y Humanístico de la Universidad de Los Andes. This work was presented in part at the 16th Annual Meeting of the Society for the Study of Reproduction, August 8-10, 1983, Cleveland, Ohio. Address requests for reprints to: Dr. Francisco J. Rojas, Department of Obstetrics and Gynecology, The University of Texas Health Science Center at San Antonio, 7703 Floyd Curl Drive, San Antonio, Texas 78284.

0024-3205/84 $3.00 + .00

Recently, we described the presence of a factor obtained from rat testis having the ability to inhibit luteinizing hormone (LH)/human chorionic gonadotropin (hCG) binding to gonadal receptors (1). This factor was partially heat stable, not steroid in nature and presented a low molecular weight. Binding studies and inhibition kinetics indicated that the inhibitor affects LH/hCG binding mainly by interfering with the formation of the hormone-receptor complex and that it competes with the hormone for the same binding sites. Consequently, it was demonstrated that the inhibitor prevents the interaction of the gonadotropin and its receptors in testicular and ovarian tissues.

In the present report, we extend our previous findings and demonstrate that charcoal-treated testicular extract interferes with hCG-stimulated testosterone production by enzyme-dispersed testicular interstitial cells in vitro and that its administration during proestrus inhibits ovulation in the rat. These results are consistent with the presence of an LH/hCG binding inhibitor in rat testis, suggesting that this factor is not only able to inhibit the binding of the hormone to the receptors, but also to act as an antagonist of the physiological functions mediated by LH/hCG.

Materials and Methods

Preparations of a 105,000 x g supernatant of rat liver and of rat testis containing the LH/hCG binding inhibitor were obtained in either phosphate buffer (0.01 M sodium phosphate buffer, pH 7.5) or in Medium 199 (Gibco Laboratories) as described elsewhere (1). Under these conditions, the resulting aqueous extracts contained 100 mg tissue equivalent per 100 µl of extract. Charcoal treatment involved addition of 200 mg charcoal (Norit A) per 2 ml testicular extract incubated for 30 min at 4°C. Pilot experiments using [^{3}H] testosterone showed that charcoal removed more than 98% of the added steroid. For heating studies, charcoal-treated extracts were boiled in a water bath for 10 min followed by centrifugation at 10,000 x g for 10 min. The resulting clear supernatant was then used for inhibitory activities. In general, heating of the extracts was performed just before their use.

Preparation of dispersed interstitial cells was carried out by collagenase digestion of decapsulated rat testis according to the method described by Dufau et al. (2). Final resuspension of the cells was in Medium 199 at a ratio of 100 mg tissue per 200 µl of cell suspension. The general procedures outlined by Cigorraga et al. (3) were employed for cell incubations. Two hundred microliters of cell suspension were incubated at 34°C under an atmosphere of 95% O_2:5% CO_2 in a final volume of 1 ml of Medium 199. At the end of the incubation period, the mixture was boiled in a water bath for 5 min, centrifuged at 10,000 x g for 10 min and the supernatant stored at -30°C for subsequent determination of total (medium plus tissue) testosterone production by radioimmunoassay. In the presence of an excess of hCG (5 IU; Ayerst Labs, New York), the total testosterone production was linear for at least 3 hours.

The antiserum to testosterone, obtained from Radioassay System Laboratories (Carson, CA), had high specificity for testosterone with relatively low cross-reactivity for dihydrotestosterone (6.6%), Δ^4-androstenedione (0.9%) and 5α-androstane-3β, 17β-diol (2.19%). Radioimmunoassays were performed directly on samples as outlined by Dufau et al. (4). The interassay and intra-assay coefficients of variation were 12.5% and 10.1%, respectively.

Rats of the Sprague-Dawley strain were used. The animals were housed in a controlled environment, 12 L:12 D, with rat chow and tap water available ad libitum, and were sacrificed by decapitation immediately prior to use. For studies on inhibition of ovulation, rats exhibiting at least two consecutive

4-day cycles were selected; the LH surge was observed between 1600 h and 1800 h of proestrus. The influence of charcoal-treated testicular and liver extracts, or charcoal- and heat-treated testicular extracts was assessed in normal proestrous rats receiving a single i.p. injection of 3.5 g tissue equivalent/BW at various times between 1130 h and 1630 h. The rats were sacrificed on the following day (1000 h of estrus) and the number of ova in the oviducts counted under a dissecting microscope. Data were analyzed using Student's t test.

Results

Effects of charcoal-treated testicular extract upon hCG-stimulated steroidogenesis by dispersed testicular interstitial cells in vitro.

Incubation of interstitial cells with 5 IU of hCG in the presence of testicular extracts which had been treated with either charcoal or with charcoal followed by heating for 10 min at 100°C resulted in a significant decrease in the ability of the cells to produce testosterone (Fig. 1). There was a dose-response relationship between the quantity of testicular extract and the degree of inhibition of steroidogenesis, with 400 µl of charcoal-treated extract resulting in a decrease of approximately 85% in the net gonadotropin-stimulated production of testosterone. Inhibition in the presence of 400 µl of the charcoal- and heat-treated extract was about 40%. This ability to retain some inhibitory activity after heating at 100°C agreed with previous data showing that the hCG/LH binding inhibitor is partially heat stable with respect to its ability to interfere with the binding of the hormone (1). In contrast to the testicular extract, addition of 400 µl of charcoal-treated liver extract heated at 100°C for 10 min failed to show detectable inhibitory activity on the hCG-stimulated steroidogenesis of the cells (Fig. 1).

In another set of experiments, interstitial cells were first incubated with charcoal-treated testicular extract for 30 min at 34°C. The inhibitor was then removed and the cells reconstituted with medium and incubated with 5 IU of hCG for 2 h. Results indicated that preincubation with the testicular factor diminished the subsequent stimulation of testosterone production by the gonadotropin added during the second incubation (Table I). This effect may be attributed to the ability of the inhibitor to prevent the formation of the hormone-receptor complex as previously described (1). Furthermore, the observation that inhibition of steroidogenesis in interstitial cells was not reversed after addition of hCG further supports the concept that binding of the inhibitor to LH/hCG receptors may proceed in an irreversible manner (1).

Effects of a single injection of charcoal-treated testicular extract on the spontaneous ovulation in normal cycling rats.

Because the LH/hCG binding inhibitor prevents the interaction of hCG and its receptors in testis and ovary (1), an attempt was made to see whether the testicular factor might have an inhibitory effect on LH/hCG binding in vivo. In preliminary studies, we observed that the administration of testicular extract to immature rats was able to alter the ovulation induced by an s.c. injection of 5 IU hCG, as determined by the number of ova per ovulating rat 72 h after the hormone treatment (data not shown). This effect was most apparent when the testicular extract was injected in single doses of 3 to 5 g tissue equivalent/rat, 54-55 h following hCG administration. Lower doses had no detectable effects. Under the conditions of this experimental model, hCG alone may induce ovulation in about 75% of immature rats and it is thought that the gonadotropin elicits, between 54 and 57 h after the injection, an endogenous surge of LH which is presumably responsible for triggering ovulation (5). Although our initial results were encouraging, they were highly

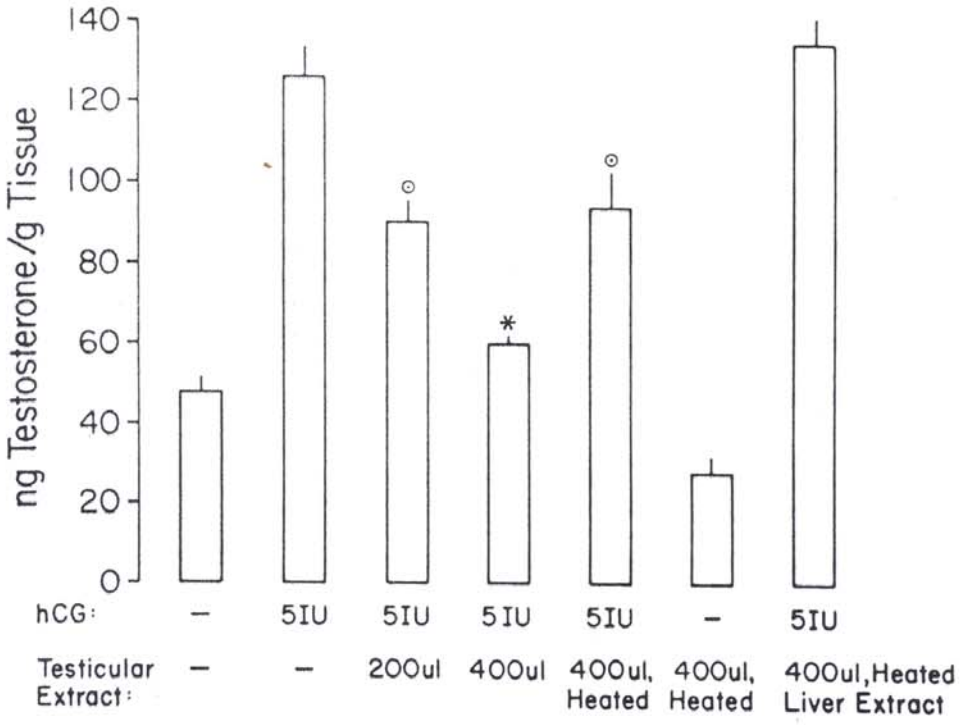

FIG. 1

Effects of the testicular inhibitor on hCG-stimulated testosterone production by dispersed testicular interstitial cells in vitro.

Cell preparations were incubated with 5 IU of hCG in the presence of either charcoal-treated testicular extract, or testicular extract which has been treated with charcoal followed by heating for 10 min at 100°C. The incubations were carried out in Medium 199 at 34°C for 3 h under an atmosphere of 95% O_2:5% CO_2. At the end of the incubation total (medium plus tissue) testosterone production was determined as described under Materials and Methods. Last column at right shows incubation with charcoal-treated liver extract heated for 10 min at 100°C. All tissue extracts were prepared in Medium 199 at a ratio of 100 mg tissue equivalent per 100 µl extract. Each value represents the mean ± SEM of three separate determinations. *, $P < 0.005$; θ, $P < 0.01$ compared to the group having hCG alone.

variable and failed to show a consistent pattern of ovulation-inhibitory response to the extract.

Better and more reproducible results were obtained using normal cycling rats which received an i.p. injection of 3.5 g tissue equivalent/BW of charcoal-treated testicular extract at different times during proestrus. The single dose selected was based on the previous observations using the model of ovulation induction in immature rats described above. Table II shows that injection of testicular extract between 1430-1630 h of proestrus significantly inhibited spontaneous ovulation, as evidenced by the number of animals in which ova were found. In contrast, rats receiving a similar dose of liver extract ovulated normally. As expected, in view of the partial heat stability of LH/hCG binding inhibitor, administration of a single dose of charcoal- and heat-treated extract also resulted in a significant inhibition of ovulation, although to a lesser extent than the nonheated extract. Administration of the testicular extract at 1130 h was not inhibitory, precluding the likelihood that

TABLE I
Effects of Preincubation of Dispersed Testicular Interstitial Cells with Testicular Inhibitor on in vitro hCG Stimulation of Testosterone Production

Assay	First incubation[a]	Second incubation	Testosterone produced (ng/g tissue)	Decrease of hCG-stimulated testosterone production (%)
1	Interstitial cells	hCG	102.0 ± 4.7	control
2	Interstitial cells + 200 µl extract	hCG	74.5 ± 4.2	27[b]
3	Interstitial cells + 400 µl extract	hCG	59.3 ± 7.4	42[b]

[a]Cells were first incubated at 34°C for 30 min with either charcoal-treated testicular extracts or with Medium 199 alone (control). After centrifugation and washing, the cells were resuspended and incubated a second time at 34°C for 2 h in the presence of 5 IU hCG. Total production of testosterone was determined as described under Materials and Methods. Data are the mean (± SEM) of two separate sets of experiments.

[b]$P < 0.005$ vs. control

TABLE II
Inhibitory Activity of Testicular Extract upon Spontaneous Ovulation in Normal Cycling Rats[a]

Extact[b]	Time of Injection[c]	No. ovulating rat	No. ova (± SEM) per ovulating rat	P[d]
Control	—	8/8	11.2 ± 0.9	
Testicular extract				
Charcoal-treated	1130 h	5/5	9.6 ± 2.1	NS
Charcoal-treated	1430 h	6/11	6.6 ± 0.7	<0.01
Charcoal-treated	1530 h	5/13	2.0 ± 0.3	<0.001
Charcoal-treated	1630 h	5/5	3.0 ± 0.7	<0.001
Charcoal-treated and heated	1530 h	4/4	5.3 ± 1.1	<0.01
Liver extract, heated	1530 h	4/4	10.7 ± 0.8	NS

[a]Normal proestrous rats received a single dose of tissue extracts and were sacrificed at 1000 h the next day for counting the number of ova in the oviducts.

[b]When heated, the charcoal-treated extracts were boiled in a water bath for 10 min.

[c]Time of proestrus at which injection was given

[d]P vs. control

the effects described in Table II were attributable to a nonspecific deleterious effect upon the ovary.

In order to investigate further the nature of the inhibition of ovulation, we first injected the rats with the inhibitor at 1530 h of proestrus, followed by an injection of hCG 1 h later. Figure 2 shows that the administration of 5 IU of hCG was unable to reverse the inhibitory effect induced by the single dose of charcoal-treated extract. These results strongly suggest that inhibition may be due to a direct interference at the level of the ovary. Furthermore, the inhibitory effect induced with the charcoal- and heat-treated extract was partially reversed by the subsequent administration of hCG, a result which is in agreement with the partial retention of activity following heating of the inhibitor, and which indicates that the ability to inhibit ovulation was dose-related.

Discussion

The present results demonstrate that the aqueous extract prepared from rat testis contains a factor able to prevent hCG-stimulated testosterone production by testicular interstitial cells in vitro and to inhibit ovulation in the cycling rat. These findings were consistent with the presence of an LH/hCG binding inhibitor in the testicular extract which has been reported to be partially heat stable, non-extractable by charcoal, and able to compete with LH/hCG for the same binding sites in testicular and ovarian tissues (1).

The in vitro studies showed that preincubation with the testicular factor significantly inhibited the subsequent stimulation of steroidogenesis by hCG compared to controls in which the first incubation of the interstitial cells was in medium alone. Thus, the irreversibility in the mechanism of action of the inhibitor observed in binding experiments (1) was also evident in studies concerning steroidogenesis in vitro (Table I). A similar observation has been reported by Kumari et al. (6), using an LH receptor binding inhibitor (LHRBI) obtained from extracts of porcine corpus luteum. These investigators found that the ability of the LHRBI to inhibit progesterone secretion in cultured granulosa cells is not reversed after addition of LH. It is apparent, therefore, that an element of irreversibility may be a common feature in the mode of action of other gonadotropin binding inhibitors.

The inhibitory effects of the testicular factor upon testosterone production by interstitial cells in response to hCG contrasted greatly with those observed with LHRH by Yang and co-workers (7). Studies from these authors show that the LHRBI obtained from the ovaries of pseudopregnant rats inhibits in vitro LH-stimulated progesterone synthesis by ovarian tissue and that, in testicular tissue, the LHRBI preparation not only enhances the in vitro LH stimulation of testosterone, but also, by itself, stimulates the testosterone production (7). These observations might suggest the existence of a dichotomy of behavior between LH receptors from ovaries as compared with the receptors from testis. In contrast, our data demonstrating that the testicular factor is an antagonist of LH/hCG functioning at the gonadal level in both the male and the female do not support such a concept. Furthermore, the evidence that the testicular factor inhibits hCG binding to testicular and ovarian LH/hCG receptors (1) disagrees with the suggestion of Yang et al. (7,8) that ovarian LH receptors are structurally different. The present results, therefore, support the thought that LHRBI obtained from ovaries of pseudopregnant rats and the LH/hCG binding inhibitor obtained from rat testis may be distinct molecular entities with different modes of inhibition.

The precise mechanism of action of the ovulation-inhibiting activity demonstrated in testicular extract is not known, but the fact that suppression of ovulation occurred after administration of hCG suggests that the effect was

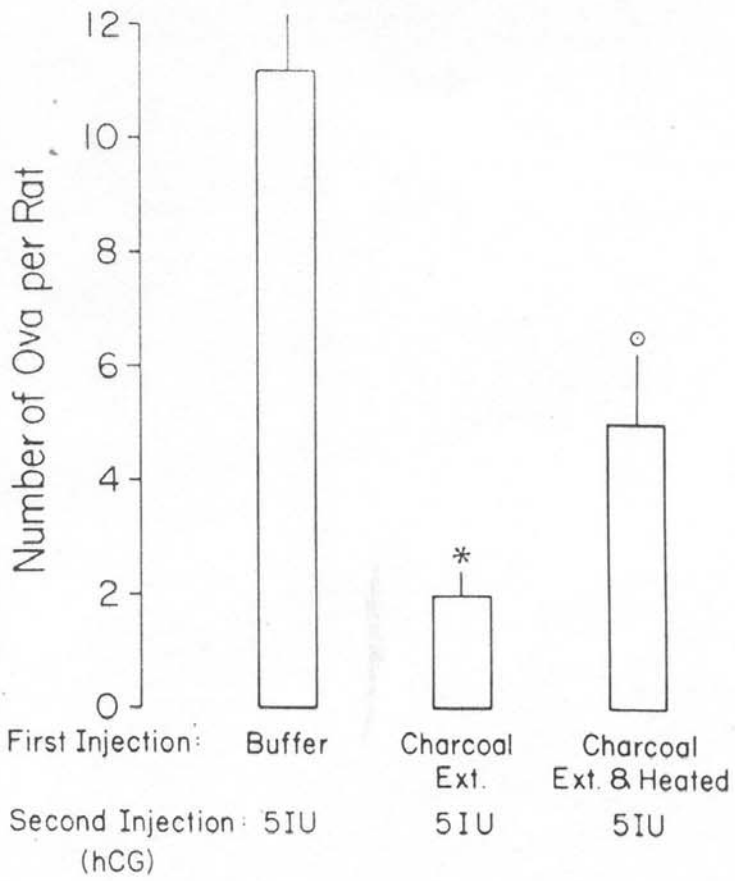

FIG. 2
Administration of hCG to rats pre-treated with testicular inhibitor during proestrus.

Rats were first given a single i.p. injection of either phosphate buffer (0.01 M, pH 7.5), charcoal-treated, or charcoal- and heat-treated testicular extracts at 1530 h of proestrus. Following this pre-treatment, a single i.p. injection of 5 IU hCG was given at 1630 h of proestrus. The animals were then sacrificed at 1000 h next day and the number of ova in the oviduct counted. Testicular extracts were prepared in phosphate buffer and the dose injected was of 3.6 g tissue equivalent/BW. *, $P < 0.001$; θ, $P < 0.01$. Statistical analyses refers to a comparison between the experimental and control (buffer-injected) groups.

probably not at the hypothalamic-pituitary level, but, rather, due to interference in the binding of the gonadotropin to its ovarian receptors. Recently, the inhibitory action of a crude charcoal-treated aqueous extract of porcine corpus luteum upon ovulation in the rabbit was reported (9). This effect was also attributed to a direct inhibitory action on ovarian LH binding, although an effect on hypothalamic-pituitary function was not ruled out (9). Whether the inhibitory substance present in the porcine corpus luteum extract is identical to the LH/hCG-binding factor extracted from rat testis remains to be elucidated.

The existence described here of a testicular factor having ovulation-inhibiting activity suggests its potential use as a contraceptive acting at the ovarian level. Since the preparation of large quantities of the inhibitor is

necessary for more extensive and complete studies, a definitive conclusion awaits its purification and chemical characterization. In the meantime, we feel that efforts to describe the various activities of the gonadal tissue extracts and fluids capable of affecting normal physiological processes are certainly worthwhile.

Acknowledgments

We wish to thank Ms. Gretta Small for her assistance in preparing this manuscript.

References

1. F.J. ROJAS, I. MORETTI-ROJAS, G. DE BELLABARBA and W. BISHOP, Biol. Reprod. 25:519-529 (1981).
2. M.L. DUFAU, C.R. MENDELSON and K.J. CATT, J. Clin. Endocrinol. Metab. 39:610-613 (1974).
3. S.G. CIGORRAGA, M.L. DUFAU and K.J. CATT, J. Biol. Chem. 253:4297-4304 (1978).
4. M.L. DUFAU, K.J. CATT and T. TSURUHARA, Endocrinology 90:1032-1040 (1972).
5. S.K. KOSTYK, E.J. DROPCHO, H. MOLTZ and J.R. SWARTWOUT, Biol. Reprod. 19:1102-1107 (1978).
6. G.L. KUMARI, S. TUCKER and C.P. CHANNING, Biol. Reprod. 21:1043-1050 (1979).
7. K-P. YANG, N.A. SAMAAN and D.N. WARD, Endocrinology 104:552-558 (1979).
8. K-P. YANG, N.A. SAMAAN and D.N. WARD, Proc. Soc. Exp. Biol. Med. 152:606-609 (1976).
9. C.P. CHANNING, S.K. BATTA and I-H. BAE, Proc. Soc. Exp. Biol. Med. 166:479-483 (1981).