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AVP receptors of mouse Leydig cells are regulated by LH and E_2 and influenced by experimental cryptorchidism

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Exposure of pubertal mouse Leydig cells for 24 h to increasing concentrations (1–100 ng/ml) of LH elicited a dose-dependent decrease in AVP receptor content. Maximal reduction (50%) was obtained at a dose of 10 ng/ml LH. A similar treatment applied to adult Leydig cells did not influence AVP receptor density. Treatment of adult Leydig cells for 24 h by E_2 (5–500 ng/ml) resulted in a dose-dependent increase in AVP receptor content. About 50% increase was achieved with 500 ng/ml E_2. AVP receptor content in pubertal Leydig cells was not modified irrespective of the concentration of E_2 tested. These changes in AVP receptor number were well correlated with the response of Leydig cells to AVP (10^{-6} M) in terms of testosterone production. 2 weeks bilateral cryptorchidism resulted in reduction of testicular weight, circulating testosterone levels associated with a marked rise in Leydig cell AVP receptor density with no change of affinity. Testosterone production by Leydig cells from cryptorchid testes in response to AVP (10^{-6} M) or hCG (100 ng/ml) stimulation was reduced compared to that of control Leydig cells. This study provides new arguments supporting the concept that AVP could be involved in local regulation of testicular steroidogenesis.

Vasopressin; Receptor; Testosterone; Cryptorchidism; (Leydig cell, Mouse)

1. INTRODUCTION

Previous in vitro studies have demonstrated that the neurohypophysial hormones, AVP and OT, or related peptides can directly affect rat and mouse Leydig cell steroidogenesis. In both species an early stimulatory effect on testosterone production occurred in short-term treatment, but disappeared when the treatment was extended [1,2]. In contrast, a marked reduction in gonadotropin-stimulated androgen production appeared following a prolonged exposure of Leydig cells to the neuropeptides [1–4]. It has been suggested that the stimulatory effect could result from an increase in cholesterol side chain cleavage activity [5] while the inhibitory effect was the consequence of decreased LH/hCG binding capacity [6] and lesion of 17α-hydroxylase/17,20-desmolase activities [2,4]. Altogether these observations allowed to postulate that locally produced AVP-like hormone within the testis [7–9] could act as a paracrine regulatory component of Leydig cell function.

We previously reported [10] that AVP modulates steroidogenesis in purified mouse Leydig cells via specific V_1 subtype. These results are in accordance with those [11,12] showing that AVP activates phosphoinositide breakdown in rat Leydig cells. More recently [13], we have found that at puberty, the AVP receptor density in mouse Leydig cells was markedly reduced as compared to adult, suggesting a possible regulation of this receptor throughout development.

The present report concerns in vitro regulation by LH or E_2 of AVP receptor content in mouse Leydig cells at puberty and adulthood. The findings were correlated with AVP-mediated testosterone stimulation in short-term incubation, used as an index of hormone action [2]. AVP receptor con-

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Abbreviations: AVP, Arg^8-vasopressin; OT, oxytocin; oLH, ovine luteinizing hormone; hCG, human chorionic gonadotropin; E_2, 17β-estradiol

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tent was also determined in Leydig cells derived from bilaterally cryptorchid mouse testes.

2. MATERIALS AND METHODS

2.1. *Chemicals*

Arg^3-Vasopressin (AVP) was provided by Calbiochem (San Diego, CA), [^{3}H]AVP (spec. act. 67 Ci/mmol) by New England Nuclear (Dupont de Nemours, FRG) and human chorionic gonadotropin (hCG, 9000 IU/mg) by Boehringer Mannheim (France). Ovine luteinizing hormone (NIH oLH S1) was a gift from Dr Jutisz (Laboratoire des Hormones Polypeptidiques, Gif-sur-Yvette). Medium 199, L-glutamine, sodium bicarbonate and Hepes were obtained from Eurobio (France). Bovine serum albumin (BSA) and collagenase type I (144 U/mg) were from ICN Pharmaceuticals (Uppsala). Deoxyribonuclease I (DNase I), gentamycin sulphate and 17β-estradiol (E_2) were from Sigma (St. Louis, MO).

2.2. *Experimental cryptorchidism*

Bilateral cryptorchidism was performed on adult Swiss mice by sewing the epididymis on the abdominal wall under ether anesthesia as previously described [14]. Sham-operated animals served as controls. After 2 weeks, animals were killed, and blood was collected for testosterone measurement. Testes were weighed and served either for measurement of E_2 content or for Leydig cell preparation.

2.3. *Preparation and culture of Leydig cells*

Leydig cells were prepared from testes derived from pubertal (35–38 days old), adult (80–90 days old) or cryptorchid mice according to a procedure previously described [15]. The percentage of pure Leydig cell preparations, determined by staining for 3β-hydroxysteroid dehydrogenase activity was 80–90% for all groups. Viability of Leydig cells assessed by trypan blue exclusion was roughly 90%.

Leydig cell cultures were performed as described elsewhere [2]. Culture medium consisted of Medium 199 supplemented with 0.1% BSA, 7 μmol/ml L-glutamine, 350 μg/ml $NaHCO_3$, 1 mmol/ml Hepes, 50 μg/ml gentamycin sulphate and 4.5 mg/ml glucose. Hormones to be tested, LH or E_2, were added at the concentrations required, as indicated in section 3. Cultures were carried out at 37°C under air/CO_2 (95 : 5, v/v) for 24 h. After treatment the cells were washed and recovered for binding assays. When AVP-stimulated testosterone production was studied, Leydig cells were cultured for 24 h in the presence of AVP alone or in combination with LH or E_2. Leydig cells were then washed and reincubated for a subsequent 3 h period in hormone-free medium prior to testosterone measurement. Responsiveness of Leydig cells to hCG was studied in the presence of a maximal dose (100 ng/ml) of hCG as previously reported [2].

2.4. *AVP binding assays*

AVP binding assays were performed as described in [10]. Leydig cells cultured for 24 h in the presence or absence of E_2 or LH, or freshly prepared from cryptorchid and control testes, were used for AVP binding assays. Briefly, about 10^6 Leydig cells/tube were incubated in the presence of [^{3}H]AVP at 4°C for 90 min. Non-specific binding was determined by addition of an excess of unlabeled AVP. Bound and free ligands were separated by cell filtration through Whatman GF/C filters. Radioactivity retained was counted in scintillation fluid (Ready Protein, Beckman).

2.5. *Steroid measurements*

Steroids in the plasma or testicular homogenates were extracted with diethyl ether. Testosterone levels were determined in the extracts or directly in culture media by a specific radioimmunoassay as previously described [16]. E_2 was measured in testicular extracts by ^{125}I-labeled estradiol radioimmunoassay kit (Baxter, FRG).

2.6. *Statistical analysis*

Student's *t*-test or ANOVA and Duncan's new multiple range test were used for statistical analysis of the data.

3. RESULTS

LH treatment of pubertal Leydig cells for 24 h reduced AVP receptor number (fig.1, right). This decrease was dose-dependent and maximal reduction (about 50%) was induced by a dose of 10 ng/ml LH. A similar treatment applied to adult Leydig cells did not modify AVP receptor content whatever the concentration of LH tested. As shown in fig.1 (left), a 24-h E_2 treatment of adult Leydig cells enhanced AVP receptor number in a dose-dependent fashion. Maximal increase (50%) was achieved with 500 ng/ml. E_2 treatment of pubertal Leydig cells had no effect on AVP receptor content at any of the concentrations tested.

As expected, the increase in AVP receptor number in adult Leydig cells after 24 h treatment with 500 ng/ml E_2 is accompanied by a significant

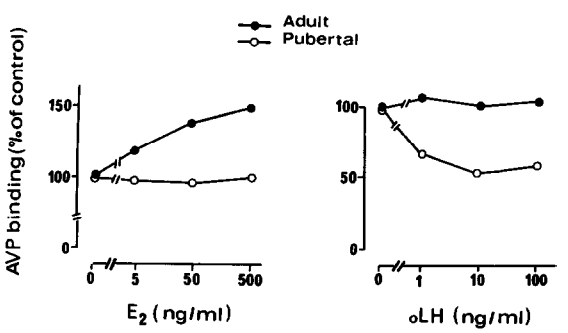

Fig.1. Effects of 24 h treatment of Leydic cells with E_2 or oLH on specific [^{3}H]AVP binding measured in the presence of 1 nM of the ligand. Receptor levels (fmol/10^6 cells) in untreated adult (6.02) and pubertal (2.39) Leydig cells were taken as 100%. Results are means of triplicate determination from a representative of 3 experiments.

($P<0.01$) amplification of the stimulatory effect of AVP (10^{-6} M) on testosterone production (fig.2, left). Since the dose of LH (10 ng/ml) which maximally reduced AVP receptor number in pubertal Leydig cells was also able to increase testosterone production (data not shown), a lower dose of LH which did not influence basal testosterone production was used (fig.2, right). When 0.1 ng/ml LH was concomitantly added with AVP (10^{-6} M) for 24 h, a significant ($P<0.05$) reduction in the stimulatory effect of the neurohypophysial hormone was observed (fig.2, right). Treatment for 24 h of pubertal and adult Leydig cells with E_2 (500 ng/ml) and LH (0.1 ng/ml) respectively did not influence either basal (cells cultured without AVP) or AVP-stimulated testosterone production.

Experimental bilateral cryptorchidism resulted 2 weeks later in a marked reduction (table 1) of testicular weight ($P<0.01$) and plasma testosterone levels ($P<0.05$). E_2 content per testis was not changed between control and cryptorchid, but was 2-3 times greater ($P<0.01$) in the cryptorchid when expressed per g testicular tissue. Binding studies (fig.3) indicate that bilateral cryptorchidism induced a striking rise in the number of AVP receptors in Leydig cells from cryptorchid testes. B_{max} values (fmol/10^6 Leydig cells) rose from 15.6 in control to 49.7 in cryptorchid testes. No difference in the affinity (K_d) was observed between control (2.02 nM) and cryptorchid (2.19 nM) groups. This change in AVP receptor content was associated with a lower degree of testosterone stimulation by hCG (6- vs 46-fold stimulation, table 1). The

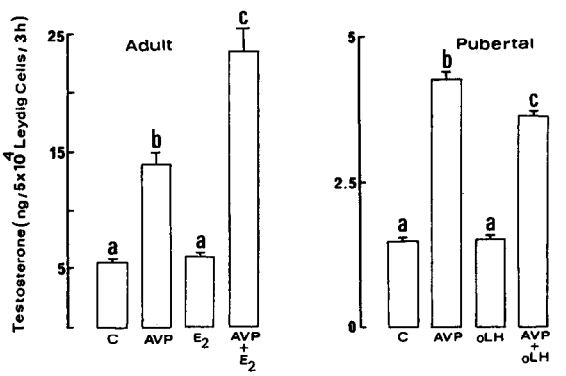

Fig.2. Effects of 24 h treatment of Leydig cells with E_2 (500 ng/ml) or oLH (0.1 ng/ml) on AVP (10^{-6} M) stimulated testosterone production. C: Leydig cells cultured in hormone-free medium. Results are means ± SE of 3 different cultures. Columns with different letters are significantly different from each other ($P<0.05$, Duncan's new multiple range test).

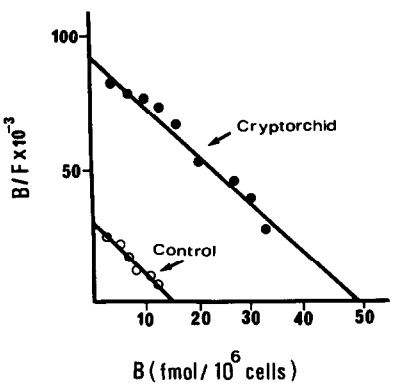

Fig.3. Scatchard plots of [^{3}H]AVP binding in Leydig cells from control or cryptorchid adult mice. Cells were incubated in the presence of increasing concentrations of [^{3}H]AVP (0.2-6 nM). Results are the means of duplicate determinations from a representative of 2 experiments of each group.

Table 1

Effects of cryptorchidism on testicular weight, testicular E_2 content, plasma testosterone and Leydig cell response to hCG

	Testicular weight (mg)	Plasma testosterone (ng/ml)	Testicular E_2		Testosterone production (ng/5 × 10^4 Leydig cells per 3 h)		
			pg/testis	pg/g tissue	− hCG	+ hCG	fold-stimulation
Sham-operated (controls)	135 ± 9.5 (12)	4.18 ± 1.2 (8)	7.75 ± 0.5 (8)	55.06 ± 5.77 (8)	3.07 ± 0.42 (3)	142.9 ± 11.33[c] (3)	46
Cryptorchids	50 ± 4[a] (12)	0.74 ± 0.07[b] (8)	6.81 ± 0.6 (8)	133.25 ± 15.48[a] (8)	7.75 ± 0.30 (3)	47.14 ± 2.77[c] (3)	6

Results are the means ± SE of n determinations. [a] $P<0.01$ vs controls; [b] $P<0.05$ vs controls; [c] $P<0.001$ vs respective unstimulated Leydig cells. Number of animals given in parentheses

respone of Leydig cells from cryptorchid testes to AVP was also reduced. Testosterone production (means ± SE) was 3.07 ± 0.42 and 7.78 ± 0.45 ng/5×10^4 Leydig cells per 3 h in the absence, and 7.75 ± 0.30 and 11.82 ± 0.74 ng/5×10^4 Leydig cells per 3 h in the presence of AVP in Leydig cells from control and cryptorchid testes, respectively.

4. DISCUSSION

The present data demonstrate for the first time that AVP receptors in Leydig cells are regulated by LH or E_2 treatment. They indicate in addition, that these regulations differ markedly according to the maturational stages of the animals. The present results show that LH does not affect AVP receptor number in adult Leydig cells, whereas gonadotropin is able to reduce these receptors at puberty. This latter result is strongly supported by the observation that LH is also able to reduce the stimulatory effect of AVP on testosterone production by pubertal Leydig cells. Another argument in favor of negative control of AVP receptor by LH comes from recent data (Tahri-Joutei et al,, unpublished) indicating that phospholipid breakdown elicited by AVP [11,12] is reduced by LH in pubertal but not in adult Leydig cells. The possibility that such a heterologous down-regulation of AVP receptor by gonadotropin occurs in vivo during puberty may be considered, since the decreased number of AVP receptors at this stage [13] was coincident with the peaking of serum LH [17,18]. Whether this negative control of AVP receptor at puberty has physiological relevance remains to be clarified.

The current study also shows that E_2 is able to increase AVP receptor number associated with amplification of steroidogenic responsiveness of adult Leydig cells to AVP. A similar E_2-mediated up-regulation of AVP receptors has been reported in myometrium [19]. The presence of estrogen receptor has been demonstrated in both mouse and rat Leydig cells [20]. In rat a lower E_2 receptor content in Leydig cells was found during sexual maturation as compared to adulthood [21]. If a similar pattern of E_2 receptor change occurs in the mouse, this could explain the lack of E_2 effect on AVP receptor in pubertal Leydig cells. However, the exact mechanism by which E_2 induces increae of AVP binding in adult Leydig cell is presently unknown. E_2 is known to inhibit in vitro steroidogenesis in adult rat Leydig cells [22], but has no effect in mouse Leydig cells [23]. The insensitivity of mouse Leydig cells to E_2 may be explained by the rapid decrease in vitro of estrogen receptor levels [20]. Nonetheless, the present data clearly demonstrate a stimulatory effect of E_2 on AVP receptor content in cultured mouse Leydig cells. In the rat the inhibitory effect of E_2 on adult Leydig cell function is associated with synthesis of a specific protein of 27 kDa the nature and role of which are still unknown [22]. It is therefore attractive to hypothesize that the protein identified by these authors could be related to AVP receptor.

Cryptorchid animals have been used to investigate the influence of seminiferous tubules on Leydig cell function. Our study shows that disruption of spermatogenesis induced by cryptorchidism resulted in a reduction of testosterone biosynthesis accompanied by a striking increase in AVP receptor number in Leydig cells. Although, in the present study we have used the stimulatory effect of AVP on testosterone production as an index of hormone action [1,2], an inhibitory effect of neurohypophysial hormones on gonadotropin-stimulated androgen production has also been demonstrated in mouse and rat Leydig cells [1–4,6]. On the basis of our observations on the cryptorchid testes, it is tempting to speculate that the main physiological effect of neurohypophysial hormones in vivo is inhibitory. First the in vitro responsiveness of Leydig cells to hCG is markedly reduced in cryptorchid testes. Second, in spite of a 3-fold increase of AVP receptor content in Leydig cells from cryptorchid testes, the AVP-stimulated testosterone production is not positively coupled to the AVP receptor increase. From our data it is presently difficult to ascertain whether the up-regulation of AVP receptors by E_2 obsreved in vitro, occurs in the cryptorchid testis. The fact that E_2 concentration increases when expressed per g testicular tissue in cryptorchid as compared to control (present study, [24]) associated with the previous findings [25] that cryptorchid rat testes produced more E_2 in vitro than controls under hCG stimulation, might support E_2-mediated up-regulation of AVP receptor in cryptorchid testes. Accordingly, the possibility that E_2 and AVP act in a synergistic manner to inhibit Leydig cell steroidogenesis cannot be excluded. Another explanation

for increased AVP receptor number could be that the seminiferous tubule produces a factor which controls AVP receptors in Leydig cells. Alteration of its production by damaged tubules could result in an increase in AVP receptors. We cannot exclude the possibility that up-regulation of AVP receptor results from a homologous down-regulation suppression, since it was demonstrated [26] that immunoreactive AVP content decreased in cryptorchid rat testes. Such a homologous down-regulation of AVP receptors has been reported for WRK1 cells [27].

In conclusion, this study has demonstrated hormonal regulation of Leydig cell AVP receptor density accompanied by modification of the steroidogenic response to the neuropeptide. The changes in AVP receptor content during normal development [13] or after disruption of testicular function by experimental cryptorchidism, provide new insight into the consideration that vasopressin could be one of the major paracrine factors regulating gonadal steroidogenesis.

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