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ANDROGEN BINDING PROTEINS IN DIFFERENT TESTIS COMPARTMENTS

E. MARTIN RITZÉN and LARS HAGENÄS
Pediatric Endocrinology Unit, Karolinska Sjukhuset, Stockholm
and
VIDAR HANSSON
Department of Pathology, Rikshospitalet, Oslo
and
FRANK S. FRENCH and SHIHADEH N. NAYFEH
Department of Pediatrics and Laboratories for Reproductive Biology, University of North Carolina, Chapel Hill, N.C., U.S.A.

SUMMARY

Two different high-affinity androgen binding proteins have been demonstrated in the rat testis. The smaller protein (ABP, MW 90,000) is produced in the testis, and transported with the testicular fluid into the epididymis. ABP is found in tubules but not in interstitial tissue or in testicular lymph. Destruction of the spermiogenic cells by physical or chemical agents does not decrease the concentration of ABP in the testis, indicating Sertoli cells to be the site of production. ABP disappears from the testis following hypophysectomy, but reappears following FSH treatment. Other pituitary hormones have no effect. Another androgen binding component, different from ABP, has also been recovered from rat testis tubules. This protein shows characteristics different from ABP but identical to cytoplasmic receptors of epididymis and prostate, indicating the presence of androgen target cells in the testis. Large doses of testosterone or dihydrotestosterone are known to maintain spermatogenesis in the hypophysectomized rat. We postulate that a major action of FSH on spermatogenesis may be mediated by ABP. ABP may facilitate androgen transport into the seminiferous tubules and provide the germ cells with the milieu of androgenic hormones which is required for normal spermatogenesis.

An androgen binding protein (ABP) which was first identified in the 105,000 *g* supernatant of rat epididymis [1, 2] was later found to originate in the testis [3, 4]. ABP was identified in the fluid collected from the testicular efferent ducts, and was found to be present at concentrations high enough to provide binding sites for the large amounts of androgens found in the same fluid. Ligation of the efferent ducts results in a complete disappearance of ABP from the caput epididymis, while ABP accumulated in the testis [4, 5]. ABP was found to have characteristics distinctly different from the cytoplasmic receptors for androgenic hormones in prostate and epididymis [6, 7]. Rat ABP was similar to the testosterone binding globulin (TeBG) which is found in human but not in rat serum. Rabbit ABP was found to be very similar to the TeBG in rabbit serum but could be separated from TeBG by ion exchange chromatography or isoelectric focusing [8].

The present paper will review the origin of ABP within the testis and the regulation of ABP synthesis, as well as the occurrence of androgen *receptors* within the testis. A hypothesis has been formulated for the mode of action of follicle stimulating hormone (FSH) on spermatogenesis [18].

PREPARATION OF SAMPLES

Tissues were homogenized in 3–6 volumes buffer (TEMG = 10 mM Tris, 1,5 mM EDTA, 1 mM 2-mercaptoethanol and glycerol to 10% v/v., or TES = 20 mM Tris, 1·5 mM EDTA and 0·25 M sucrose, both pH 7·4 at 25°C), and centrifuged at 105,000 *g* for 1 h at +1°C. Aliquots of the cytosol were layered on 6·5% polyacrylamide gels and run as described below.

Steady state polyacrylamide electrophoresis for quantitation of steroid binding proteins

Polyacrylamide gel electrophoresis (PAGE) has previously been used for demonstration of proteins in serum[9] and in tissue cytosols[1, 2] that bind radioactive steroids. However, since there is a continuous dissociation of radioactive ligand from its binding sites during electrophoresis, accurate quantitation is difficult unless the rate of dissociation of the binding is relatively slow compared to the time of electrophoresis. For this reason, a method of steady state polyacrylamide gel electrophoresis (SS-PAGE) was developed.

Radioactive steroid is dissolved in the gel solution before polymerization and is stationary in the electrophoretic field until it is bound by protein moving through the gel. Steady state between association and dissociation is reached when the level of radioactivity in front of the binding protein is the same as that behind (Fig. 1). At steady state, the law of mass action can be applied:

$$K_d = \frac{[BP_u] \times [S_u]}{[S_b]}, \quad \text{or} \quad BP_{tot} = S_b\left(\frac{Kd}{[S_u]} + 1\right)$$

where K_d = dissociation constant at the temperature and pH used, $[BP_u]$ = concentration of free binding sites, $[S_u]$ = concentration of unbound steroid, $[S_b]$ = concentration of bound steroid and BP_{tot} = total number of binding sites. Using this technique, binding proteins can be accurately measured (coefficient of variance 10%) at concentrations as low as 10^{-14} mol. For detailed description of the method, see Ritzén *et al.*[10].

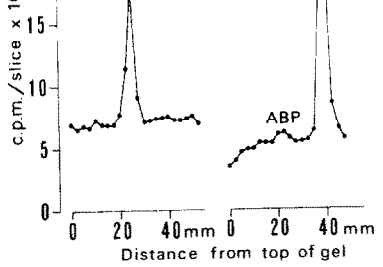

Fig. 1. Steady state polyacrylamide electrophoresis of efferent duct fluid and testicular lymph, collected from the same testis. The efferent ducts were ligated 16 h before collection of the fluids. Testicular lymph was collected under barbiturate anesthesia by introducing a thin polyvinyl catheter with side holes immediately underneath the capsule. Efferent duct fluid was collected by incision of the distended ducts, after the lymph collection was finished. The protein concentration in the lymph was 20·3 mg/ml, in efferent duct fluid 2·4 mg/ml. 50 μl lymph and 24 μl efferent duct fluid was run in polyacrylamide gels containing 1 nm [^{3}H]-dihydrotestosterone. The concentration of ABP in efferent duct fluid (calculated as described in the text) was 15·7 pmol/mg protein, in testicular lymph 0·09 pmol/mg protein. (Hagenäs, Ritzén and Plöen, to be published)

SERTOLI CELL PRODUCTION OF ABP

ABP was previously found to originate in the testis [4, 5]. In looking for the site of production within the testis, two main compartments were first considered; interstitial cells and tubules. If ABP were produced by the interstitial cells and transported into the tubular lumen and into the efferent duct fluid, its concentration in the testicular lymph should be at least as high as in efferent duct fluid. The concentration of ABP in rat testicular lymph was less than 2% of that found in efferent duct fluid from the same animal (Fig. 1). ABP could be recovered from mechanically isolated tubules but not from interstitial cells, as observed also by Vernon *et al.*[12]. These findings were taken as evidence for tubular rather than interstitial production and storage of ABP.

Within the testicular tubules, two major cell types may be considered as the possible origin of ABP; Sertoli cells and germ cells. Since reliable methods for separation of Sertoli cells from germ cells have not yet been developed, indirect methods had to be used to prove whether ABP stems from one or the other of these principally different cell types. Germ cells are very sensitive to physical or chemical injuries. Thirty days after a single dose (600 R) of gamma irradiation of the lower third of adult rats, or following 30 days continuous feeding with nitrofurazone (0·01% mixed with crushed mouse pellets) essentially no germ cells remained. However, the concentration of ABP in the

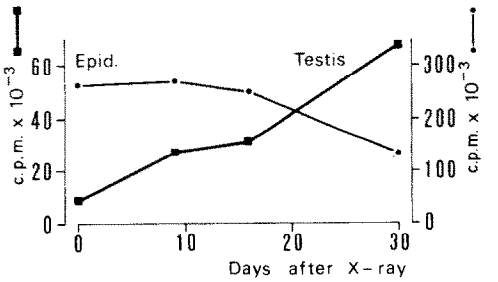

Fig. 2. ABP concentration in testes and caput epidydimis after a single-dose X-ray treatment. Adult rats were irradiated with 600 R over the lower third of the body 9, 16 and 30 days before the experiment. Cytosols were prepared from testes and caput epididymis, and the content of ABP was analyzed by steady state polyacrylamide electrophoresis with [^{3}H]-dihydrotestosterone in the gel. Relative binding is expressed as cpm recovered in the peak of radioactivity bound to ABP. (Hagenäs, Ritzén and Plöen, to be published).

testis *increased* markedly (Fig. 2). This finding indicated that Sertoli cells are the site of production of ABP.

REGULATION OF ABP PRODUCTION

Protein synthesis in testicular tubules has been shown to be stimulated by gonadotrophin [13]. Therefore, adult rats were hypophysectomized, and the concentration of testicular ABP was compared with sham operated littermates. ABP was found to disappear almost completely 19 days following hypophysectomy. (Fig. 3a, b). When hypophysectomized rats were given a combination of LH and FSH for 5 days, starting 19 days after hypophysectomy, ABP was found in *super*normal amounts in the testes (Fig. 3c). This effect was found to be due entirely to FSH. Other pituitary hormones did not stimulate ABP production (Fig. 4). The stimulatory effect of FSH on ABP was dose dependent, and correlated with the biological potency of the FSH preparation [19]. Testosterone alone had no stimulatory effect when given in doses less than 2 mg per rat (Fig. 4). However, when immature hypophysectomized rats were given testosterone and FSH, a remarkable augmentation of the FSH response was seen (Fig. 5). This synergistic effect of FSH and testosterone has not yet been fully explored. However, it may be explained by action on different metabolic events in the Sertoli cells, or by decreased degradation of ABP after synthesis.

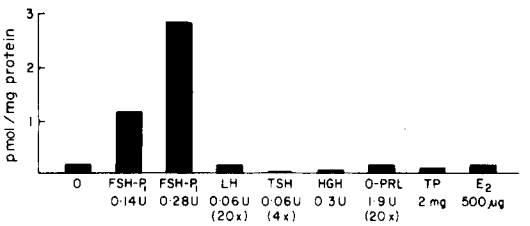

Fig. 4. Effect of various hormones on the level of ABP in caput epidydimis of hypophysectomized immature rats. Rats in groups of 4 were hypophysectomized at 28 days of age and injected with 0·14 or 0·28 NIH-units FSH-P_1 (total dose), starting on day 30. Parallel groups of animals were injected with high doses of LH, thyroid stimulating hormone (TSH), ovine prolactin (O-PRL) human growth hormone (HGH), testosterone propionate (TP) or 17 β-estradiol. (E_2). ABP in the cytosols was measured by steady-state polyacrylamide gel electrophoresis.
(20 ×) = 20 times the maximum contamination of LH or O-PRL in 0·28 units FSH P_1.
(4 ×) = 4 times the maximum contamination of TSH in 0·28 units FSH-P_1. (From Hansson *et al.* [19]).

ANDROGENS AND SPERMATOGENESIS

It has been known for some time that spermatogenesis and fertility can be maintained in hypophysectomized rats by administration of large amounts of testosterone [14] or dihydrotestosterone [15]. The finding that androgens maintain spermatogenesis suggested that certain cells in the germinal epithelium

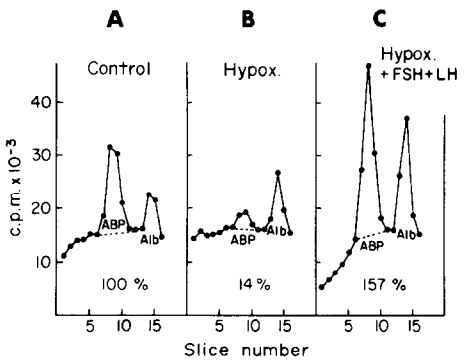

Fig. 3. Effects of hypophysectomy and FSH-LH replacement on ABP levels in testes of adult rats. Rats in groups of 4 were hypophysectomized and kept for 19 days. From day 20 each animal was injected with 75 IU FSH and 25 IU LH (Homogonal®, Leo, Sweden) or saline only. After treatment for 5 days, the testes were examined for ABP as described in the legend to Fig. 1. A: Sham operated. B: Hypophysectomy for 19 days and saline for 5 days. C: Hypophysectomy for 19 days and FSH-LH treatment for 5 days (From Hansson *et al.*[19]).

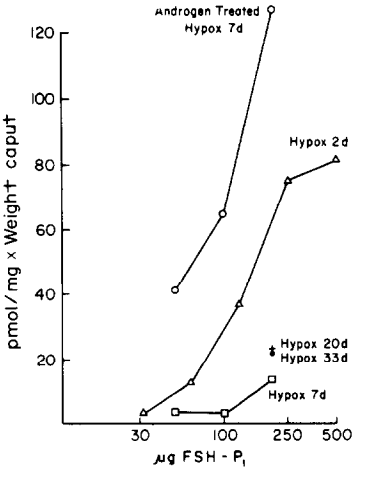

Fig. 5. Effect of increasing doses of NIH-FSH-P_1 on the amounts of ABP in caput epididymis. Animals in groups of 4 were hypophysectomized at 28 days of age and FSH treatment was started 2, 7, 20 or 33 days later. One of the groups was treated with 2 mg testosterone propionate i.m. from the day after hypophysectomy to the day of sacrifice. FSH was administered to all groups twice daily the 3 last days before sacrifice. ABP was measured by steady state polyacrylamide electrophoresis with [^{3}H]-dihydrotestosterone in the gel. (From Hansson *et al.*[19]).

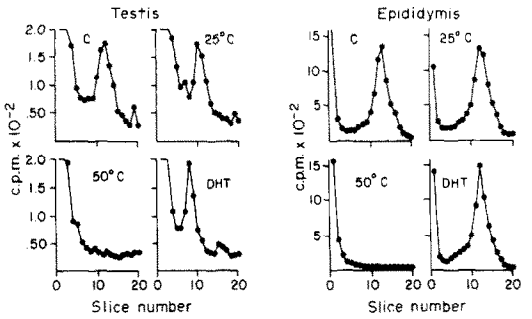

Fig. 6. Effect of temperature and unlabelled dihydrotestosterone (DHT) on binding of radioactive androgens to cytoplasmic receptors in testicular and epidydimal supernatants. Rats were hypophysectomized at 25 days of age. Three days later, the animals were injected with [^{3}H]-testosterone, 105,000g supernatants prepared, and binding analysed by electrophoresis in 3·25% acrylamide gels containing 0·5% agaroce.

C: Control.
25°C: Supernatants heated at 25°C for 30 min.
50°C: Supernatants heated at 50°C for 30 min.
DHT: Excess of unlabelled DHT (10 μg) was added to 0·5 ml supernatants and incubated at 0° for 2 h before electrophoresis.

In these hypophysectomized rats, the level of ABP is very low (seen as a small fast moving peak in the testis control) while the slower moving cytoplasmic receptor is the major peak of radioactivity (From Hansson *et al.* [16]).

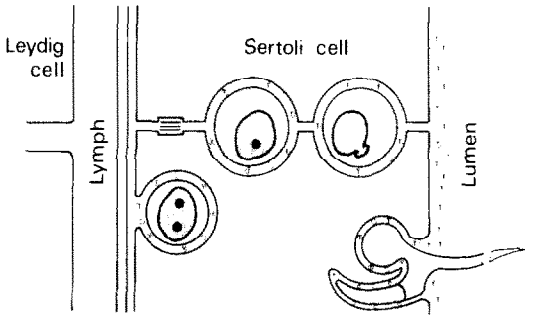

Fig. 7. Schematic representation of the tubular epithelium of the testis with adjacent interstitial tissue. Germ cells are enclosed by Sertoli cells. ABP (open circles), is produced by the Sertoli cells under influence of FSH and is secreted into the seminiferous tubular fluid surrounding the germ cells. Testosterone produced by the Leydig cells in the interstitial compartment, is bound by ABP in the Sertoli cells, and carried to receptors in androgen target cells of the germinal epithelium.

must be targets for androgenic hormones. Most androgen target organs have been shown to contain cytoplasmic androgen "receptors". This has been shown to be the case also for the testis [16]; when [^{3}H]-testosterone was injected i.v. to hypophysectomized and functionally hepatectomized immature rats, radioactive dihydrotestosterone and testosterone was bound to a soluble protein with characteristics identical to cytoplasmic receptors in the epididymis and prostate (Fig. 6). In contrast to ABP which disappeared from testis supernatants within a few days following hypophysectomy, the receptor appeared to remain indefinitely. Cytoplasmic receptors for androgen characteristically release bound steroid slowly ($t_{1/2}$ 0° > 4 days), while androgen complexed with "carrier" proteins like ABP and TeBG dissociate rapidly ($t_{1/2}$ 0° < 60 min). The testicular cytoplasmic androgen receptor has been localized to the tubules, but it is not yet known if it is present in germ cells, Sertoli cells, or both. This intracellular receptor protein is completely different from ABP in several respects: Molecular size, steroid specificity, rate of dissociation of bound steroid, and sensitivity to temperature and to sulfhydryl blocking agents.

The doses of androgens needed for maintenance of spermatogenesis in hypophysectomized rats are notably high. However, the physiological concentration of testosterone in and around seminiferous tubules is normally high. In the ram, testis lymph and rete testis fluid have been found to contain about 10 times the testosterone concentrations found in peripheral serum [17]. In the rat and rabbit, similar ratios between efferent duct fluid and peripheral serum has been found*. Assuming some metabolism and consumption of androgen by the testicular tissues, the concentration of testosterone in and around the tubules should be even higher. Therefore, administration of a "pharmacological" dose of androgen may be needed to maintain a physiological concentration in the seminiferous tubule, especially in the FSH deficient hypophysectomized rat.

In the normal rat, a high concentration of ABP in the seminiferous tubules ($2–4 \times 10^{-8}$ M in efferent duct fluid) may serve to maintain a reservoir of androgen in close proximity to the germ cells (Fig. 7). FSH may exert its major effect on spermatogenesis by stimulating the Sertoli cell production of ABP, which in turn aids in providing the germ cells with an essential supply of androgenic hormones.

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DISCUSSION

Vinson:

I wonder if you have any evidence that before it's bound the testosterone is converted to something else. It seems to me with this sort of capacity for androgen binding that you're demonstrating, it should be possible to show the presence of testosterone in isolated seminiferous tubule preparations which the Rotterdam group seem to fail to do even though they have looked for it quite extensively (cf. Cooke's contribution to the Discussion of an earlier paper).

Ritzén:

Yes, we've been able to mechanically separate the interstitial cells and tubules. We found binding activity in the tubules but not in the interstitial cells.

Jensen:

I wasn't quite clear as to the steroid specificity of the two binding proteins. Is it correct that ABP binds both testosterone and dihydrotestosterone but the receptor protein preferentially binds dihydrotestosterone?

Ritzén:

ABP binds testosterone, but preferentially dihydrotestosterone. The testicular androgen receptor has so far been shown only after *in vivo* injections of [^{3}H]-testosterone. We have isolated and identified both dihydrotestosterone and testosterone bound to the receptor, but we can't say for sure the relative affinities of this in terms of displacement activity.

Korenman:

I would like to congratulate you on this work. I think this really opened up the testis field which had experienced a degree of dormancy. It is very exciting. With regard to the relationship between testosterone and FSH stimulation, it seems as though testosterone seems to condition the Sertoli cell to respond to FSH. Do you have any concepts about how a steroid hormone can condition a cell to its response which is mediated at a cell surface?

Ritzén:

The results could be explained either by stimulated synthesis of ABP by the Sertoli cells or a decreased breakdown of ABP in the tissue. We rather believe that the first situation prevails. Testosterone might influence the number of FSH binding sites or receptors on the Sertoli cell or stimulate a different metabolic pathway that is needed for the full rate protein production, being supplemental to the FSH effect. There are probably even more ways to explain this, but we don't have any proof for either of them.

De Moor:

I wonder if this ABP could be the same substance as the Mullerian duct inhibiting factor. Nathalie Josso cultured Mullerian ducts together with interstitial tubes and found, as expected, that the Mullerian ducts did atrophy. She then repeated this experiment with X-ray irradiated interstitial tubules containing only Sertoli cells and still got diffusion of Mullerian inhibiting factor.

Ritzén:

We are running experiments on fetal rats to try to identify ABP in the fetal gonads and the reproductive system. We don't know whether this may be the Müller inhibiting substance. It's tempting to suggest that ABP is the reason why you get a unilateral effect of fetal castration on the growth of the wolffian ducts.

Munck:

I find your experiments very intriguing and illuminating. The only point on which I wasn't altogether clear was what you meant in your remark in which you suggested that ABP might lead to a high concentration of testosterone. It would seem that, if anything, a binding protein would lower the local concentration. Are you suggesing that the ABP will carry androgen to some place where the androgen by itself wouldn't be able to go?

Ritzén:

ABP leads to high total concentration of testosterone. It's not known how testosterone comes from the Leydig cells to

the tubules. If it is diffusion, which should be dependent on the free concentration of testosterone, then ABP would serve as a large depot, a store of androgenic hormones. We saw yesterday, for instance, how rapidly testosterone production changes in the Leydig cells. ABP would be one way of supplying the germ cells with a continuous high concentration of androgenic hormones, in spite of fluctuations in testosterone synthesis. It's also possible that the Sertoli cells have a directed production of ABP to certain cells or to certain stages of spermatogenesis so that we get the local increase in concentration at that point but not in others.

Sandberg:

This may be a naive question. Is there a special androgen protein in the plasma of the rat?

Ritzén:

No, there is no testosterone binding globulin (TeBG) in plasma of the rat. The rabbit on the other hand, has a high concentration of TeBG in blood as well as a 10 times higher concentration of ABP in the efferent duct fluid. These proteins are very similar, but they can be separated by ion exchange chromatography and by isoelectric focussing.