HORMONAL CONTROL OF SPERMATOGENESIS: EXPRESSION OF FSH RECEPTOR AND ANDROGEN RECEPTOR GENES

(HORMONALE CONTROLE VAN SPERMATOGENESE: EXPRESSIE VAN FSH RECEPTOR EN ANDROGEEN RECEPTOR GENEN)

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CONTENTS

ABBREVIATIONS

ABP	:androgen binding protein
AR	:androgen receptor
cAMP	:adenosine cyclic-3':5'-monophosphate
cDNA	:complementary deoxyribonucleic acid
CG	:chorionic gonadotropin
dbcAMP	:dibutyryl adenosine cyclic-3':5'-monophosphate
DHT	:dihydrotestosterone
EDS	:ethane dimethane sulphonate
EGF	:epidermal growth factor
EGTA	:ethyleneglycol-2-(2-aminoethyl)-tetracetic acid
FSH	:follicle-stimulating hormone or follitropin
GnRH	:gonadotropin releasing hormone
IGF-I	:insulin-like growth factor I
IU	:international unit
kb	:kilo base
KD	:kilo Dalton
Kd	:binding affinity
LH	:luteinizing hormone or lutropin
LHRH	:luteinizing hormone releasing hormone
LNCaP	:lymph node carcinoma of the prostate (human)
mRNA	:messenger ribonucleic acid
PmodS	:a Peritubular myoid cell factor which modulates Sertoli cell function
R1881	:17ß-hydroxy-17α-methylestra-4,9,11-trien-3-one
SBP	:sex hormone binding protein
SHBG	:sex hormone binding globulin
T	:testosterone
Tfm	:testicular feminization
TGFα	:transforming growth factor α
TGFß	:transforming growth factor ß
TSH	:thyroid-stimulating hormone
vit-D	:1,25-dihydroxy-vitamin D3

Cover :microscopic image of isolated rat spermatocytes

CHAPTER 1

INTRODUCTION

1.1 The testis

The mammalian testis consists of two compartments: the interstitium and the seminiferous tubules. The composition of the interstitium differs between species, but in general it contains: Leydig cells, macrophages, lymph space and blood vessels, and endothelial cells (Fawcett, 1973). The seminiferous tubules are avascular and contain different types of germ cells embedded in Sertoli cells (de Kretser and Kerr, 1988). In between the two compartments and surrounding the seminiferous tubules, peritubular myoid cells and a basal lamina are located (Fig. 1).

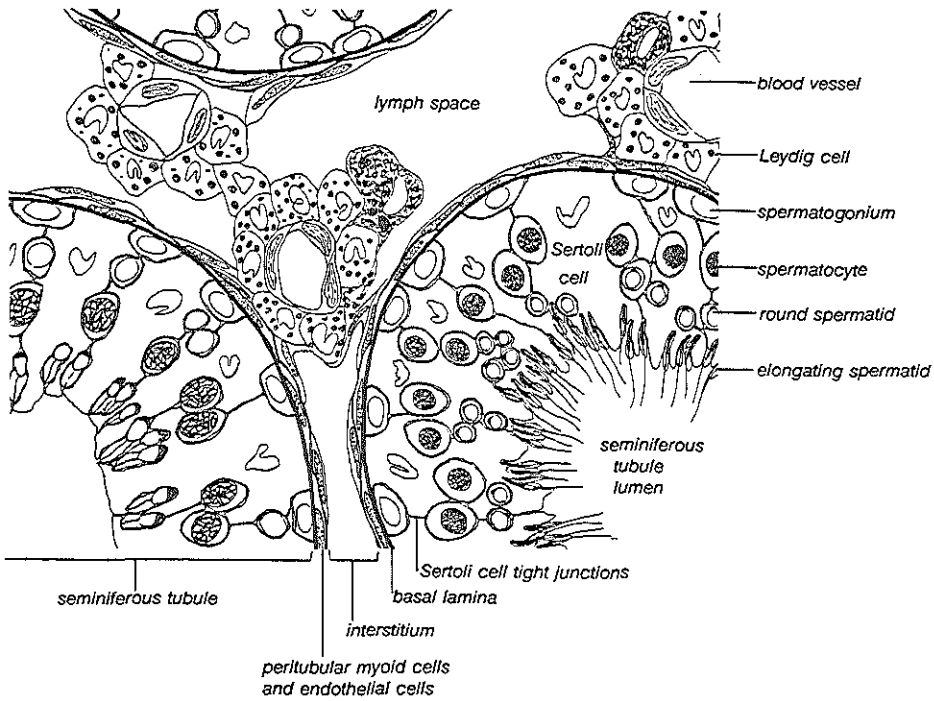

Fig. 1. Schematic representation of a cross-section through part of the rat testis.

1.1.1 Leydig cells

The function of the interstitial Leydig cells is the production and secretion of androgens. Testosterone is produced in large amounts to supply the whole body with androgens. In most androgen target tissues testosterone is converted to dihydrotestosterone, by the enzyme 5α-reductase. Dihydrotestosterone binds to the androgen receptor with a higher affinity than testosterone, and is considered to be the more potent androgen. It is, however, accepted that

testosterone in high concentrations interacts in the same way with the androgen receptor as dihydrotestosterone (Grino et al., 1990). During embryonic development, dihydrotestosterone is necessary for the formation of the male external genitalia and the differentiation of the urogenital sinus into prostate, bulbourethral glands, urethra, periurethral glands and part of the urinary bladder. In the Wolffian ducts, due to the absence of 5α-reductase activity (until late in male differentiation), testosterone is not converted into dihydrotestosterone (Siiteri and Wilson, 1974). However, testosterone is an effective androgen in the differentiation of the Wolffian duct into epididymis, ductus deferens, seminal vesicle and ejaculatory ducts (Wilson and Lasnitzki, 1971). Testosterone and dihydrotestosterone are also involved in the manifestation of male secondary sexual characteristics, and have an important role to play in regulation of spermatogenesis (Hall, 1988).

Leydig cell function is regulated by luteinizing hormone (LH). The secretion of this gonadotropin by the pituitary gland is stimulated by the hypothalamic luteinizing hormone releasing hormone or gonadotropin releasing hormone (LHRH, GnRH) (Hodgson et al., 1983, Fink, 1988), and down-regulated by testosterone (Fig. 3). For example, when circulating testosterone levels are increased by testosterone injection, LH production and secretion by the pituitary gland can be completely blocked, which in turn will result in an almost complete inhibition of testosterone production by Leydig cells (Cunningham et al., 1979).

LH is the main regulator of Leydig cell function, but the cells also take part in a complicated interplay between different cell types in the testis (Fig. 3). Factors secreted by Sertoli cells have been reported to affect Leydig cell mitosis, the number of LH and LHRH receptors, and LH-stimulated testosterone secretion in vivo (Kerr and Sharpe, 1985). Several Sertoli cell factors that might play a role in this paracrine interaction between Sertoli and Leydig cells have been identified. Effects of transforming growth factor α (TGFα) and insulin-like growth factor I (IGF-I) on mitotic activity of cultured Leydig cells have been described recently (Teerds et al., 1992). It should be noted, however, that these putative paracrine factors are also produced by peritubular myoid cells, and that regulatory effects of these growth factors may originate from this source (Skinner et al., 1989; Skinner, 1991) (Fig. 3).

1.1.2 Peritubular myoid cells

Peritubular myoid cells form a layer of flattened cells between the interstitium and the seminiferous tubules. They are in close contact with the androgen producing Leydig cells in the interstitium (Skinner et al., 1991), and together with the Sertoli cells the peritubular myoid cells produce an extracellular matrix and provide structural support for the spermatogenic epithelium (Skinner et al., 1985). Furthermore, these cells seem to be involved in contraction of the tubules which may play a role in the release and transport of the testicular spermatozoa (Clermont, 1958; Fritz and Tung, 1986).

Androgens, but also follicle-stimulating hormone (FSH), stimulate the development of peritubular cells towards a more mature appearance (Bressler and Ross, 1972). The gonadotropic hormone FSH, like LH secreted by the pituitary gland, acts on testicular Sertoli cells (Paragraph 1.1.3). Effects of FSH on peritubular myoid cell differentiation, therefore, indicate communication between Sertoli cells and peritubular myoid cells (Fig. 3).

Peritubular myoid cells produce a potential regulator of Sertoli cell function, termed PmodS (a Peritubular myoid cell factor which modulates Sertoli cell function; Skinner and Fritz, 1985; 1986). In cell cultures, this paracrine factor has a marked impact on a number of Sertoli cell functions: it increases the production of androgen binding protein (ABP, a well known

Sertoli cell product; Tung and Fritz, 1980; Hudson and Stocco, 1981), and transferrin (Holmes et al., 1984; Skinner and Fritz, 1985), and it affects vectorial secretion of proteins (Janecki and Steinberger, 1987; Ailenberg et al., 1988) and alters the histochemical staining of several metabolic enzymes in Sertoli cells (Cameron and Snydle, 1985). The current hypothesis is that PmodS may play an important role in Sertoli cell function and differentiation (Skinner, 1989) (Fig. 3).

Other, so far identified, peritubular myoid cell products that may influence testicular function are: TGFα, TGFß and IGF-I.

1.1.3 Sertoli cells and germ cells

Sertoli cells are the only somatic cell type of the spermatogenic epithelium. Together with endothelial cells (lining blood vessels and lymph space), peritubular myoid cells and the basal lamina, they form blood-testis barriers (Setchell et al., 1969; Dym and Fawcett, 1970; Plöen and Setchell, 1992). The contribution of Sertoli cells to these barriers is important. Tight junctions are located in between neighbouring Sertoli cells and divide the seminiferous tubule into a basal and an adluminal compartment. These junctional contacts between adjacent Sertoli cells restrict the passage of many growth factors, nutrients and hormones from the basal to the adluminal compartment of the seminiferous tubule. In fact, the Sertoli cell tight junctions establish the functional blood-testis barrier (Sharpe, 1983; Saez et al., 1987; Bardin et al., 1988). In rats, the blood-testis barrier is constructed soon after birth but it takes several weeks before this barrier is fully effective (Setchell et al., 1981; 1988). The construction of the blood-testis barrier is correlated with the onset of spermatogenesis and seminiferous tubule fluid production. The tubule fluid may serve as a vehicle for sperm transport and possibly plays a role in further maturation of the testicular spermatozoa (Russell et al., 1989).

Germ cell development takes place in the protected environment of the seminiferous tubules, as a defined series of developmental events, according to a tight schedule in time and place (Clermont and Harvey, 1967). The germ cells are arranged in specific cell associations, called the stages of the cycle of the seminiferous epithelium. For the rat, 14 different stages have been defined, which make up one cycle of approximately 13 days duration. For its complete development, the germ cell traverses the different stages of the cycle 4 times, which takes all together 52 days (Clermont, 1972). Each time the germ cell passes through the same stage of the cycle, it is more advanced in its development towards a full grown spermatozoa (Fig. 2; Dym and Clermont, 1970).

At the beginning of the first wave of spermatogenesis, it is thought that the seminiferous epithelium shows considerable synchronisation. Later, Sertoli cells at adjacent parts of the seminiferous epithelium support very different stages of the cycle.

Spermatogenesis does not seem to rely on direct control of the germ cells by the endocrine system, but rather is dependent on hormone action on Sertoli cells, and cell-cell interactions between germ cells and Sertoli cells (Jegou et al., 1984; Grootegoed et al., 1985; Griswold et al., 1988; Skinner, 1991). In this respect, the Sertoli cell is also referred to as the nursing and supporting cell of the germinal epithelium. For example, Sertoli cells provide an energy-yielding substrate for the developing germ cells in the form of lactate (Jutte et al., 1983), play an important role in detoxification of antispermatogenic compounds (Den Boer et al., 1989), and are involved in spermatid differentiation (Russell, 1980).

Testosterone and FSH are considered to be the most important hormonal regulators of spermatogenesis (Clermont and Harvey, 1967; Steinberger, 1971; Hansson et al., 1975;

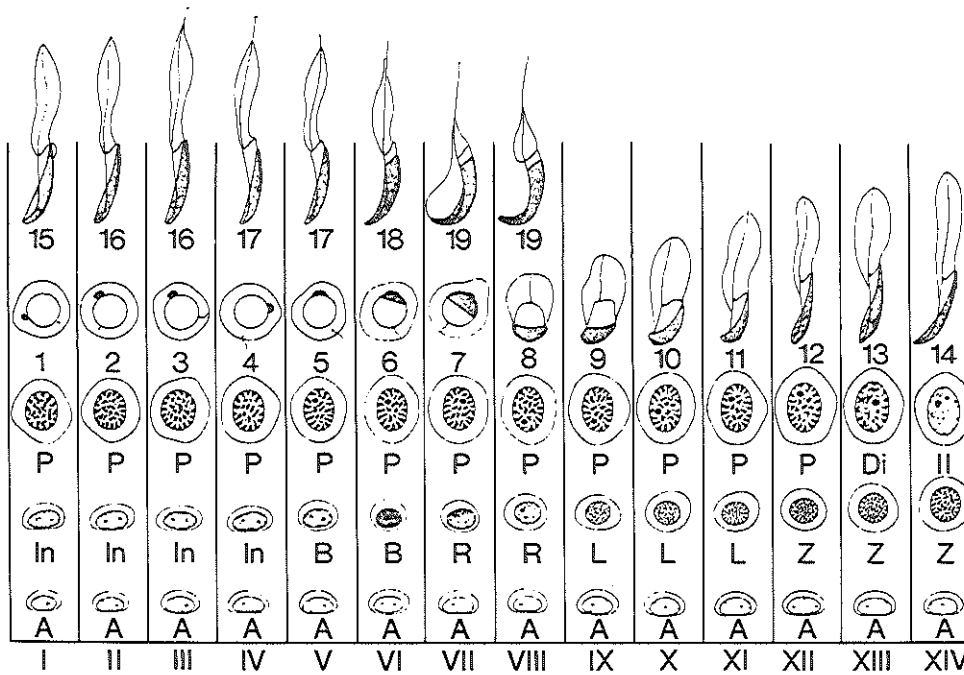

Fig. 2. Diagram illustrating the cell associations in the 14 different stages of the cycle of the seminiferous epithelium in the rat. The 14 stages are designated by Roman numerals. Abbreviations are: A = type A spermatogonia; In = intermediate type spermatogonia; B = type B spermatogonia; R = preleptotene spermatocytes; L = leptotene spermatocytes; Z = zygotene spermatocytes; P = pachytene spermatocytes; Di = primary spermatocytes at the diplotene stage; II = secondary spermatocytes; 1-19 = spermatids at the successive steps of spermiogenesis (From Dym and Clermont, 1970).

Means et al., 1976; Fritz, 1978; Tindall et al., 1984; Russell et al., 1987). Both hormones can exert actions on spermatogenesis by regulating activities of Sertoli cells (Fig. 3) (Means et al., 1980; Sanborn et al., 1983; Cheng et al., 1986; Roberts and Griswold, 1989). The action of FSH on spermatogenesis is certainly direct on Sertoli cells, because this is the only cell type in the male body that expresses the FSH receptor (Dorrington et al., 1975; Dorrington and Armstrong, 1979; Bortolussi et al., 1990; Heckert and Griswold, 1991). Androgen action may not only involve effects on the Sertoli cells, but also on the androgen receptor containing peritubular myoid cells (Buzek and Sanborn, 1988; 1990; Sar et al., 1990, Ruizeveld de Winter et al., 1991). Direct action of androgens on germ cells is not very likely, because several authors have reported that these cells do not contain androgen receptors (Grootegoed et al., 1977; Anthony et al., 1989).

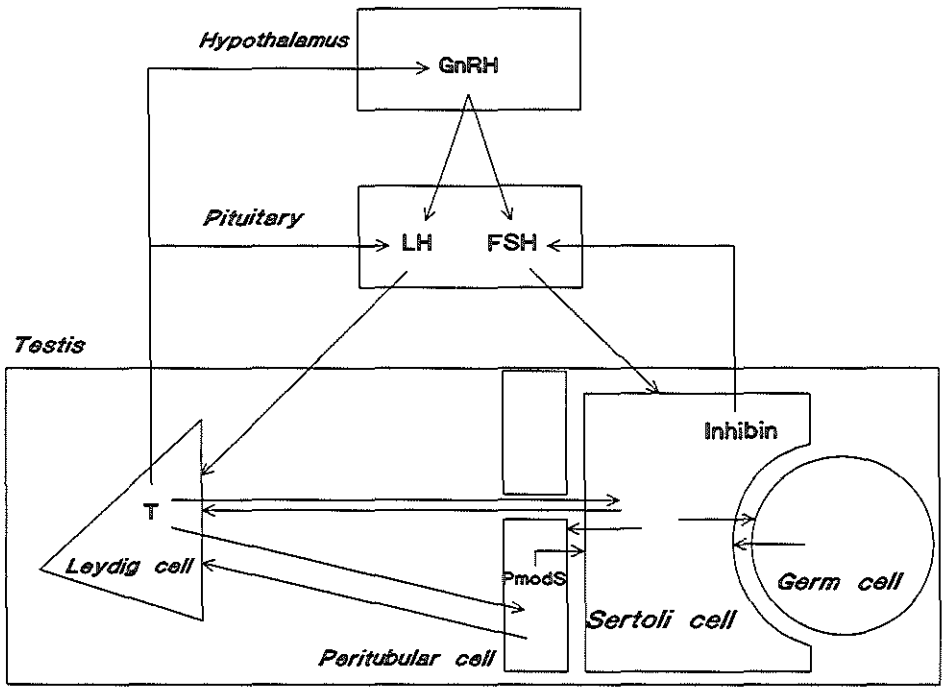

Fig. 3. Interactions involved in the regulation of spermatogenesis by FSH and testosterone.

1.2 Aim and scope of this thesis

FSH and testosterone are the main hormonal regulators of spermatogenesis. The actions of androgens and FSH are mediated by their respective receptors. Receptor gene expression (mRNA and protein), is an important determinant of hormone action. Biochemical aspects of the regulation of androgen and FSH receptor gene expression in the testis were chosen as the subject of the studies described in this thesis. Regulation of the expression of the receptor genes was studied at the level of gene transcription, and at the level of mRNA and protein expression.

In Chapters 2-4, a detailed characterization is given of the effects of FSH on androgen and FSH receptor mRNA and protein expression in cultured immature Sertoli cells. For the androgen receptor, these findings were extended by measurements of androgen receptor gene transcription initiation rate in cultured immature Sertoli cells and LNCaP (lymph node carcinoma of the prostate) cells (Chapter 5).

Preliminary results concerning a putative paracrine factor, produced by Sertoli cells and affecting androgen receptor mRNA expression in peritubular myoid cells, are presented in

Chapter 6.

The effects of testosterone deprivation in vivo on androgen receptor mRNA and protein expression in the adult rat testis were examined as described in Chapter 7. In vitro effects of testosterone on androgen receptor gene expression in cultured testicular cells and LNCaP cells are described in the Chapters 2, 3 and 5.

In the General Discussion (Chapter 8) we have considered some aspects of regulation of spermatogenesis by FSH and testosterone and have discussed them in relation to our experimental data as well as in a broader perspective. This way, we hope that the results which we have presented, and discussions which we have tried to initiate, may contribute to research concerning hormonal control of spermatogenesis, now and in the future.

1.3 Hormonal regulation of spermatogenesis

The emphasis of this thesis will be on the actions of testosterone and FSH on spermatogenesis. There are, however, other factors that can also affect spermatogenesis. Before going into detail about the role of androgens and FSH, some of these other factors are briefly discussed.

Inhibin is a glycoprotein hormone, which inhibits pituitary gonadotropin production and/or secretion, preferentially that of FSH (Burger and Igarashi, 1988). Sertoli cell production and secretion of inhibin is considered to be part of the hypophysis/gonad feedback mechanism that controls FSH (Fig. 3) (Weinbauer et al., 1989). Van Dissel-Emiliani et al. (1989) reported that injections of an excess of inhibin in one of the testes of mice or hamsters, resulted in a small but significant reduction in spermatogonial numbers in the treated testes, but not in the untreated contralateral testes. Furthermore, Woodruff et al. (1992) reported binding of inhibin to isolated rat germ cells. For the inhibin related protein activin, binding to germ cells, and induction of spermatogonial proliferation has also been reported (Mather et al., 1990; Woodruff et al., 1992). Moreover, activin receptor mRNA has been reported to be present in pachytene spermatocytes and round spermatids; the presence of the mRNA in the polysomal fraction indicated translation of this mRNA into activin receptor protein (de Winter et al., 1992). These reports suggest a local role for inhibin and activin in the regulation of spermatogenesis. Furthermore, inhibins, activins, or related proteins, are also synthesized in a number of other tissues, and may play a role in various other processes (Grootenhuis, 1990).

Epidermal growth factor (EGF), produced in high amounts by the mouse submandibular gland, may also play some role in spermatogenesis. It was reported that upon removal of the submandibular gland (sialoadenectomy), spermatogenesis in mice became impaired (Tsutsumi et al., 1986). When EGF was substituted in physiological concentrations to the sialoadenectomized mice, spermatogenesis returned to normal. These data, however, could only partly be reproduced (Russell et al., 1990). In Chapter 6, the possible role of EGF in the rat model is discussed in more detail.

Vitamin A (retinol), and its derivatives retinal and retinoic acid, have been reported to be of essential importance for reproduction, vision, cell differentiation and normal growth (Dicken, 1984). When rats or mice are made vitamin A-deficient, there are two effects in the testis: the developing germ cells degenerate, and spermatogenesis is blocked at the level of the transition of undifferentiated to differentiated A spermatogonia (van Pelt and de Rooij, 1990a). The loss of germ cells in vitamin A-deficient rats, is suggested to be caused mainly by the disruption of the Sertoli cell barrier (Huang et al., 1988), which is part of a more general

effect of vitamin A-deficiency on epithelial cells. The blockade of spermatogonial cell division, however, may be caused by the absence of direct effects of retinoic acid on spermatogonial cells. Kim and Griswold (1990) and van Pelt (1992) reported localization of retinoic acid receptor mRNA (α and) in spermatogonial cells. These findings suggest that retinoic acid may exert a direct effect on spermatogonial cells. On the other hand the Sertoli cell has also been denoted as target for retinoic acid (Karl and Griswold, 1980).

Upon refeeding of vitamin A deficient animals with retinol (Morales and Griswold, 1987; van Pelt and de Rooij, 1990a and 1990b) or a high dose of retinoic acid (van Pelt and de Rooij, 1991), spermatogonial cells start to divide again, and spermatogenesis returns to normal. It should be noted, that in this case a synchronization in the development of different stages of the seminiferous tubule is maintained for some time.

1.3.1 FSH receptor

The molecular mechanism of FSH action on spermatogenesis starts with the binding of FSH to its receptor in the Sertoli cell plasma membrane. FSH is a member of a family of 4 glycoprotein hormones (thyroid-stimulating hormone [TSH], chorionic gonadotropin [CG], LH and FSH). These hormones are composed of two dissimilar glycoprotein subunits, α and ß. Within a given species, the α subunits are identical and the ß subunits are similar but not identical. Because of the similarities between the hormones, it was speculated that their receptors also would be related (Salesse et al., 1991). Cloning of one member of the glycoprotein hormone receptor family (LH/CG (CG is closely related to LH, and binds to the LH receptor) receptor; McFarland et al., 1989; Loosfelt et al., 1989) indeed facilitated the cloning of other members of the family: the TSH receptor (Parmentier et al., 1989; Libert et al., 1989; Nagayama et al., 1989; Misrahi et al., 1990; Akamizu et al., 1990) and the FSH receptor (Parmentier et al., 1989; Sprengel et al., 1990; Minegish et al., 1991).

These glycoprotein hormone receptors have in common a characteristic domain that traverses the membrane 7 times, and a N-terminal domain that is involved in specific hormone binding and are members of a large super-family of G protein-coupled membrane receptors. Through the interaction of the FSH receptor with the GTP-binding protein Gs, FSH binding results in activation of adenylyl cyclase, and thereby in increased levels of intracellular cAMP. Elevated cAMP levels in turn can activate protein kinase A, and the ensuing protein phosphorylation can modify Sertoli cell activities. Recent observations indicated that other signal transduction pathways may also be involved in FSH action on Sertoli cells (Grasso et al., 1989; 1990; 1991, Gorczynska and Handelsman, 1991).

Cloning of FSH receptor cDNA (Parmentier et al., 1989; Sprengel et al., 1990; Minegish et al., 1991) was rapidly followed by studies on regulation of FSH receptor mRNA expression in the ovary and testis (Chapters 4 and 8). As is the case with the LH/CG receptor gene (Wang et al., 1991; Frazier et al., 1990) and the TSH receptor gene (Frazier et al., 1990), the FSH receptor gene also encodes several mRNAs. Heckert and Griswold (1991) showed a predominant 2.6 kb FSH receptor mRNA and a much lower expressed 4.5 kb FSH receptor mRNA. The expression of both FSH receptor mRNAs is limited to Sertoli cells and the ovary; other tissues and cells (epididymis, liver, brain, kidney, spleen, uterus, germ cells) were negative. The two mRNA species probably represent different splice or poly-adenylation variants from the same gene. It is not yet known whether both FSH receptor mRNAs encode full-length receptors; one of them may encode a truncated receptor form. For the LH/CG receptor, using monoclonal antibodies, 3 different proteins were recognized in testicular

membrane extracts (Vuhai-Luuthi et al., 1990). One of the proteins lacked the hormone binding domain, the second represented the full-length LH receptor and the third protein only contained the extracellular domain and is thought to be an extracellular LH-binding protein (Wang et al., 1991).

The expression of FSH receptor mRNAs appears to be very low in the stages VII to X of the cycle of the seminiferous epithelium, as measured in isolated seminiferous tubules from stage-synchronized rats (Heckert and Griswold, 1991). These findings were in agreement with studies from Kangasniemi et al. (1990a; 1990b), who reported a decreased binding of FSH, resulting in a decreased cAMP response to FSH, at these stages of the spermatogenic cycle. Kliesch et al. (1992), however, found almost opposite results using in situ hybridization; a high FSH receptor mRNA expression in the stages IX and X of the spermatogenic cycle of the seminiferous tubule. Hence, either the in situ hybridization gave erroneous results, or there is a marked difference between stage-synchronized Sertoli cells and normal cyclic Sertoli cells.

In the literature, several reports demonstrate a decreased responsiveness of Sertoli cells upon continuous stimulation with FSH (O'Shaughnessy and Brown, 1978; Namiki et al., 1987; Yoon et al., 1990). This decrease is caused by a desensitization process that involves receptor internalisation (O'Shaughnessy, 1980), loss of adenylyl cyclase activity (Verhoeven 1980) and increased phosphodiesterase activity (Conti et al., 1981; 1983). Regulation of FSH receptor mRNA and protein expression may also play a part in the desensitization process. The regulation of FSH receptor gene expression in cultured immature Sertoli cells by FSH was studied in more detail, as described in this thesis (Chapter 4).

1.3.2 FSH and spermatogenesis

There is general agreement that FSH is important for the initiation of spermatogenesis in mammals during puberty (Means et al., 1976; Dym et al., 1979; Russell et al., 1987), or for the reinitiation of spermatogenesis in hypophysectomized rats or in hibernating species. Its role in the maintenance of spermatogenesis in adult monkeys has also been established (Weinbauer and Nieschlag, 1991). In adult rats, however, its role is subject to debate. Experiments showing a role for FSH in adult animals are difficult to design; ideally, an in vivo study, in which FSH and testosterone levels can be regulated completely independent of each other, is needed.

Using hypophysectomized adult rats that were substituted with a highly purified preparation of human FSH and/or silastic implants containing testosterone, Bartlett et al. (1989) found indications that FSH is needed in addition to testosterone for maintenance of full spermatogenic capacity in adult rats. When adult rats were substituted with either FSH or testosterone for two weeks following hypophysectomy, testicular weights were decreased by 45%. Substitution of FSH together with testosterone, however, resulted in almost normal testicular weights. Germ cell counts confirmed that loss in testicular weight was due to impaired spermatogenesis. This study showed a co-operative action of FSH and testosterone in the regulation of spermatogenesis in adult rats. In fact, similar observations concerning maintenance of spermatogenesis have been reported 3-4 decades ago (for a review see Steinberger et al., 1971)

Kerr et al. (1992) treated hypophysectomized rats with ethane dimethane sulphonate (EDS), an alkylating agent which destroys Leydig cells (Morris et al., 1986). FSH and LH were eliminated by hypophysectomy, and testicular testosterone production was completely abolished by destruction of the Leydig cells. At the start of this experiment, the animals were

substituted with no hormone, testosterone, FSH or both hormones. Using this model, Kerr et al. (1992) could show that testosterone and FSH had independent, synergistic and stage-dependent effects on spermatogenesis. A problem in this study, however, was that the normal spermatogenic function of the testis could not be maintained.

At the biochemical level, co-operation between FSH and testosterone has been observed for androgen receptor expression. Verhoeven and Cailleau (1988) reported that FSH increased the concentration of androgen receptors in cultured Sertoli cells. In the present thesis, additional biochemical and molecular data are presented that suggest a role for FSH in regulation of androgen receptor gene expression in Sertoli cells in the immature rat testis (Chapters 2, 3 and 5).

1.3.3 Androgen receptor

With the cloning of androgen receptor cDNA (Chang et al., 1988; Lubahn et al., 1988a; Trapman et al., 1988), a new impulse was given to studies on the mechanism of action of androgens. The availability of androgen receptor cDNA provided tools to study androgen receptor mRNA expression, androgen receptor protein structure and function, and to produce androgen receptor antibodies. The new techniques and probes, together with the already widely used specific ligand binding assays, resulted in new insights in androgen receptor regulation.

Lubahn et al. (1988b) and Tan et al. (1988) were the first to report that androgen receptor mRNA expression in rat ventral prostate was increased upon castration of the animals (24h). This effect was reversed upon injection of testosterone, 24 h after castration. It is clear, therefore, that in the prostate androgens cause down-regulation of androgen receptor mRNA expression. Quarmby et al. (1990) showed that down-regulation of androgen receptor mRNA expression was not restricted to the ventral prostate. In kidney, brain, epididymis and coagulating gland, expression of androgen receptor mRNA was also increased upon castration and subsequently decreased after androgen injection. Using the LNCaP cell line, androgen dependent down-regulation of androgen receptor mRNA expression could also be shown (Quarmby et al., 1990; Tilley et al., 1990; Trapman et al., 1990; Krongrad et al., 1991).

Up-regulation of androgen receptor mRNA expression does not necessarily imply that androgen receptor protein levels are up-regulated also. In fact, the opposite seems to occur. Fiorelli et al (1989) showed that the androgen receptor content of the ventral prostate in adult rats is decreased upon androgen withdrawal. Data on rat penis tissue (Takane et al., 1991), and cultured fat pad adipose precursor cells (de Pergola et al., 1990), gave rise to the same conclusion. Using LNCaP cells, Krongrad et al. (1991) observed an increased level of androgen receptor mRNA that coincided with a decreased level of androgen receptor protein. These findings may partly be explained by observations of Syms et al (1985), who reported an increased stability of the androgen receptor upon binding to its ligand. Due to this ligand-induced stability, the androgen receptor protein level may become increased, while androgen receptor mRNA expression is decreased.

Although down-regulation of androgen receptor mRNA and up-regulation of androgen receptor protein in the presence of testosterone seems to be a rather general phenomenon, there are reports that show a different regulation. In prostatic epithelium from neonatally castrated animals, the level of expression of androgen receptor protein was not different in the presence or absence of dihydrotestosterone, measured on day 10 (Husmann et al., 1991).

Furthermore, Prins (1989) reported differential regulation of androgen receptor protein in the separate lobes of the rat prostate. In ventral prostate, androgen receptors were lost upon castration, whereas in lateral prostate, after an initial decrease, the amount of androgen receptors was restored to precastration levels.

Besides studies that provide additional information about differential regulation of androgen receptor mRNA and protein expression, there are also some conflicting findings. Using monoclonal antibodies, Shan et al. (1990) found that the ventral prostate levels of androgen receptor mRNA and protein were elevated in castrated rats. Takeda et al. (1991), using other techniques (immunohistochemical and in situ hybridization analysis), different antibodies, and a different cDNA probe, reached similar conclusions. It is difficult to interpret the information from these two research groups in relation to other data in the literature. It is clear, however, that more research is needed to obtain conclusive data.

In Chapters 5 and 7 of this thesis, data are presented indicating that regulation of androgen receptor expression (mRNA and protein) in the rat testis is markedly different from the regulation of androgen receptor expression in other rat tissues.

1.3.4 Androgens and spermatogenesis

Leydig cells produce large amounts of androgens. Through their actions on Sertoli and peritubular myoid cells, androgens drive spermatogenesis (Sharpe, 1987). In the literature, several indications can be found that the level of testicular testosterone (measured either in the testicular vein, or in whole testis [Maddocks and Sharpe, 1988]) is considerably higher than needed for ongoing spermatogenesis (Sun et al., 1989; Awoniyi et al., 1989; Huang and Boccabella, 1988). Sharpe (1987) indicated that the techniques used to measure the actual in vivo testicular testosterone level are far from reliable. Research has continued, using different techniques and different approaches, but always resulting in the same outcome: spermatogenesis does not seem to require an exceedingly high local level of testicular testosterone (this topic is discussed in Chapter 8).

In the experiments described in Chapter 7, EDS was used to decrease the testicular testosterone level in adult rats. Subsequently, these rats were treated with testosterone, FSH or both hormones. Upon specific ligand binding, the androgen receptor becomes transformed to a DNA binding form that regulates the expression of androgen dependent genes (Mainwaring, 1977; Schräder et al., 1981; Brinkmann et al., 1983). The amount of androgen receptor present in the testis, and the degree of transformation of these androgen receptors, was measured to determine what amount of testosterone is necessary to occupy and transform all androgen receptors. Furthermore, androgen receptor gene expression was studied in the testis of animals treated according to different protocols.

1.4 Methodology

1.4.1 In vivo versus in vitro

Part of the experiments described in this thesis were performed in vivo (animal experiments), to try to obtain information on the regulation of the androgen receptor in the testis of immature and mature rats. These studies, however, do not yield details on regulation of androgen receptor expression in the different cell types in the testis. This information is of

value because, for example, regulation of androgen receptor number in Sertoli cells might be of more relevance for spermatogenesis than regulation of the number of androgen receptors in peritubular myoid cells. Because Sertoli cells are most directly involved in the regulation of spermatogenesis, this cell type was used in most of the in vitro experiments (cell culture experiments). However, peritubular myoid cells have also been included in some of the experiments. FSH receptor gene expression was studied in vivo in testis from immature rats, and in vitro using isolated Sertoli cells.

In Chapters 2 and 4, isolation methods are described for the isolation of a highly pure Sertoli cell population from immature rats, which can be used in a cell culture system. Immature rats were used to isolate Sertoli cells, for a number of reasons. First, Sertoli cells can be obtained from immature animals at higher purity and better viability, than from more mature animals (Russell and Steinberger, 1989). Second, in these immature animals FSH plays an active role in the initiation of spermatogenesis, and Sertoli cells isolated from immature rats are much more sensitive to FSH stimulation than Sertoli cells from older animals (Steinberger et al., 1975; 1978; Heindel, 1988). Third, in the immature animals the first spermatogenic wave has just started (Russell et al., 1989), and for that reason the Sertoli cells have not yet reached complete a-synchrony with respect to spermatogenic cycle-dependent activities (Parvinen, 1982). Therefore, Sertoli cells isolated from immature animals are probably a much more homogeneous population than Sertoli cells from adult animals.

An important consideration with respect to the interpretation of experimental data, is the extent to which the cultured cells represent the cells in vivo. Rich et al. (1983) showed, using ABP as a functional parameter, that Sertoli cells isolated from 10-day-old rats can develop in culture to a somewhat more mature Sertoli cell that produces more ABP. This increment stops after 10 days in culture; thereafter it reverses. Sanborn et al. (1986) examined differences in secreted proteins between cultured Sertoli cells isolated from rats of different ages, and found, after 3 days of culture, marked differences in Sertoli cell function in relation to the age of the animals that were used to isolate the Sertoli cells from. These data suggest that Sertoli cells do not readily revert to a common basal state within a few days of culture.

In the present experiments, isolated Sertoli cells were seeded at a density of 1-2 x 10^6 cells per cm^2 in plastic tissue culture flasks, to obtain a monolayer of flattened or slightly columnar cells. These cells retain some of the morphological characteristics of Sertoli cells located in the seminiferous tubule. When Sertoli cells are cultured on an extracellular matrix, however, a more optimal morphology of the Sertoli cells seems to be obtained (Tung and Fritz, 1986; Hadley et al., 1985; 1987). Anthony and Skinner (1989) have investigated the effect of the extracellular matrix on hormonal regulation of the production of transferrin and ABP by cultured immature Sertoli cells. Although the cytoarchitecture of the cultured Sertoli cells was significantly improved, they could not show marked effects of the extracellular matrix on hormonal regulation of the expression of biochemical markers for Sertoli cell function. For comparison, it is of interest to briefly discuss another epithelial cell type that has been cultured under various conditions.

Mammary epithelial cells cultured on floating collagen gel express their differentiated properties (production of transferrin and casein) at a much higher level, and for longer periods of time, than cells cultured on plastic. Furthermore, the effect of prolactin on α2-casein and ß-casein mRNA expression and protein synthesis, was less pronounced for the epithelial cells cultured on plastic, than for the cells cultured on floating collagen gel (Lee et al., 1985). These results indicate that the molecular mechanism responsible for hormonal regulation of gene expression was intact but less active in the cells cultured on plastic as compared to the cells cultured on floating collagen.

In this thesis, Sertoli cells were maintained in culture for up to 7 days, without the help of collagen or any other extracellular matrix material. In this manner, some of the morphological and functional properties of the Sertoli cells may have been lost, but biochemical mechanisms can also be studied under these conditions. The results from in vitro studies were compared to results obtained from in vivo studies.

1.4.2 The study of receptor regulation

In view of the main regulatory roles of androgens and FSH in spermatogenesis, the expression of the androgen receptor and FSH receptor genes was chosen as the subject of the studies described in this thesis. The regulation of receptor gene expression was studied at different levels: gene transcription, mRNA stability and protein expression.

Transcription of the androgen and FSH receptor genes was studied using two different methods.

1. Transfection: Part of the androgen receptor gene transcription unit (Baarends et al., 1990) together with flanking sequences, was cloned in front of a reporter gene, and transfected into cultured Sertoli cells or LNCaP cells. The transfected cells were tested for the transcriptional response of the constructs to testosterone and FSH.
2. Nuclear run-on: Transcription of the androgen and FSH receptor genes was allowed to continue in vitro in isolated cell nuclei, in the presence of radioactively labelled mRNA precursors. The obtained radioactive androgen and FSH receptor mRNAs were hybridized to their respective unlabeled cDNAs that were immobilized on blotting paper, and visualized using autoradiography.

Androgen and FSH receptor mRNAs were studied in vivo and in vitro. Coding regions of the genes (cDNAs) were labelled and used as probes for androgen and FSH receptor mRNAs on Northern or dot blots. The hybridization signals were quantified by densitometer scanning and interpreted as direct measurements of the amount of receptor mRNA present in the cultured cells or tissues.

The androgen and FSH receptor proteins were studied, using specific ligand binding assays. Radioactively labelled ligands used were: ^{3}H-methyltrienolone (^{3}H-R1881), which is a synthetic androgen that binds with high specificity to the androgen receptor (Veldscholten et al., 1990a; 1990b), and ^{125}I-FSH, which binds to the FSH receptor. Furthermore, the androgen receptor protein expression was also studied using specific mono- and polyclonal antibodies.

1.4.3 The EDS model

Ethane dimethane sulfonate (EDS) is an alkylating agent that, through an unknown mechanism, destroys all Leydig cells in adult rat testes within 36 h after subcutaneous injection (Bartlett et al., 1986; Jackson AE et al., 1986; Jackson NC et al., 1986; Kerr et al., 1986; Molenaar et al., 1986; Morris et al., 1986). Treatment of adult rats with EDS does not irreversibly damage the testis (Kerr et al., 1985; Bartlett et al., 1986), and does not result in marked testicular defects other than the temporary elimination of Leydig cells (Morris, 1985; Verhoeven et al., 1989). Differentiation of a new population of Leydig cells starts 2 weeks after the EDS injection. The cells are restored to their normal numbers by 42 days after EDS treatment (Kerr et al., 1987), which results in a completely normalized sperm output 3 months

after EDS treatment (Jackson and Morris, 1977).

In Chapter 7, it is described that we have used EDS to destroy Leydig cells, thereby curtailing the circulating and testicular androgen concentration in adult rats (Bartlett et al., 1986). Nuclei from ventral prostate and testis were isolated, to estimate specific R1881 binding sites (Scatchard analysis). The procedure for isolation of the nuclear fraction included tissue fractionation under liquid nitrogen (Barberis et al., 1989) and the use of buffers containing a high concentration of sucrose (Gorski et al., 1986), in order to reduce loss of nuclear androgen receptors. It was observed, however, that unoccupied androgen receptors were not present in the nuclear fraction (Brinkmann et al., 1983). These results seem to suggest that the unoccupied androgen receptor is not located in the nucleus (Simental et al., 1991). However, Husmann et al. (1990) showed, using several different monoclonal antibodies, that in the rat ventral prostate in the presence but also in the absence of androgens, the androgen receptor is predominantly located in the nucleus. In the present experiments, we have taken the position that untransformed (unoccupied) androgen receptor molecules, are not in the tight nuclear binding form, and therefore are lost from the nuclei upon cell fractionation. To enable us to measure the total amount of androgen receptors, the unoccupied androgen receptors were transformed to the occupied, tight nuclear binding form. This was attained by treatment of the rats with a very high dose of testosterone (10mg, i.p.) 2 h prior to killing (T-pulse). Using this approach, the number of occupied androgen receptors (without T-pulse) and the total amount of androgen receptors (with T-pulse) could be measured in the nuclear fraction. This is illustrated in Fig. 4.

From now on we will use the term occupied androgen receptor for the receptor that is transformed to the tight nuclear binding form.

The experiment was chosen to last for 5 days, because it had been described that during the first 7 days after EDS treatment, in addition to the destruction of the Leydig cells, no marked damage to spermatogenesis could be observed (Bartlett et al., 1986). Ventral prostate and epididymis were also included in this study, because these organs have been reported to react to changing testosterone levels.

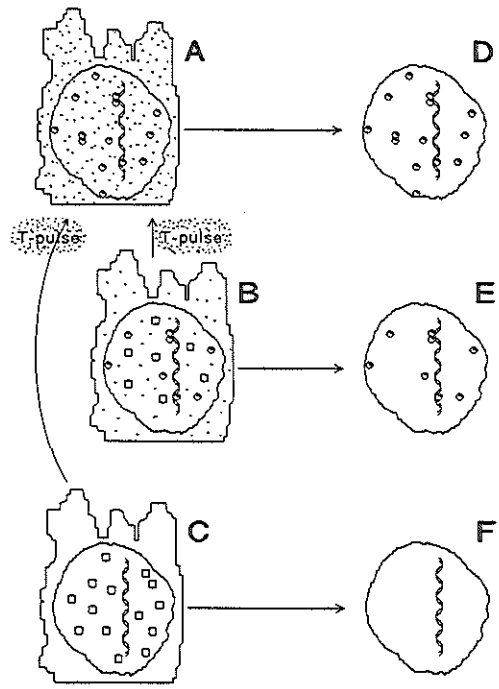

Fig. 4. The EDS model. A, B and C represent whole cells; A is the situation in the intact control rat testis, B is the situation in the ventral prostate from intact control rats, and C is the situation 5 days after EDS treatment. D, E and F represent isolated nuclei from A, B and C, respectively. The unoccupied androgen receptors (□) from B and C can be transformed to the occupied form (◔) by T-pulse treatment. The unoccupied androgen receptors in B and C are lost upon isolation of the nuclei (E and F). T-pulse = testosterone pulse treatment, (▒) represents testosterone concentration.

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CHAPTER 2

Follicle-stimulating hormone regulates androgen receptor mRNA in Sertoli cells

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Rapid Communication

Follicle-stimulating hormone regulates androgen receptor mRNA in Sertoli cells

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Summary

Follicle-stimulating hormone (FSH) and testosterone stimulate the production of a variety of proteins by immature Sertoli cells. A highly purified Sertoli cell preparation was incubated for 3 days with FSH and testosterone. Both androgen receptor protein and mRNA concentrations were markedly increased by FSH. Testosterone also increased the androgen receptor protein concentration, but did not increase the expression of the androgen receptor mRNA. It is concluded that FSH plays a role in the responsiveness of Sertoli cells to testosterone.

Introduction

Spermatogenesis is supported by Sertoli cells. The two principal hormonal regulators of spermatogenesis, follicle-stimulating hormone (FSH) and testosterone show a synergistic effect on spermatogenesis in immature rats (Russell et al., 1987). These hormones are important for Sertoli cell maturation (Fritz, 1978) and stimulate the production and secretion of a large number of different proteins by Sertoli cells (Cheng et al., 1986). Although the production of many of these proteins is stimulated by both FSH and testosterone, different mechanisms of hormone action are involved.

The regulatory effects of FSH and testosterone on spermatogenesis involve stimulation of Sertoli cells (Grootegoed et al., 1977; Fritz, 1978). It is not clear, however, to what extent peritubular myoid cells play a role in mediating the hormonal signals. Skinner and Fritz (1985) reported that peritubular cells secrete a protein under the influence of androgens, which modulates Sertoli cell function.

Recently, Verhoeven and Cailleau (1988) have shown that the androgen receptor concentration in Sertoli cells from immature rats was increased 2- to 3-fold after adding testosterone or FSH to the cell cultures. In the present experiments, we have studied the hormonal regulation of the expression of the androgen receptor mRNA and protein in rat Sertoli cells, using a highly purified cell preparation with a minimal number of peritubular cells. The present results extend the findings of Verhoeven and Cailleau (1988) and indicate that

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FSH stimulates the androgen receptor concentration in Sertoli cells through stabilisation of the mRNA and/or stimulation of transcription of the gene encoding the androgen receptor.

Materials and methods

Isolation and culture of Sertoli cells. Testes of 3-week-old Wistar rats were decapsulated and chopped (at a distance of 0.5 mm in two perpendicular directions). Subsequently, 10–12 chopped testes were incubated in phosphate-buffered saline (PBS; Dulbecco and Vogt, 1954) containing 1 mg/ml DNase (DN-25; Sigma, St. Louis, MO, U.S.A.), for 30 min at 37 °C in a 100 ml Erlenmeyer flask, in a shaking waterbath (120 cycles/min). During this treatment, tubule fragments became dissociated from the interstitial cells. Blood vessels were taken out using a glass pipette and the interstitial cells were removed by 3-fold sedimentation at unit gravity for 2 min, in a volume of 50 ml PBS containing 5 μg/ml DNase. The tubule fragments were subsequently incubated in 20 ml PBS containing 1 mg/ml collagenase (CLS-I; Cooper Biomedical, Freehold, NJ, U.S.A.), 1 mg/ml hyaluronidase (I-S; Sigma) and 5 μg/ml DNase, under the same conditions as described above. During this treatment peritubular cells became detached, resulting in partial loss of the cells from the tubular wall. After two sedimentation steps, the volume was corrected to 15 ml and the suspension was dounced 5 times, using a loose-fitting pestle as described by Oonk et al. (1985). The remaining Sertoli cell aggregates were washed twice (sedimentation at $7 \times g$ for 2 min) to remove the dislodged peritubular and germ cells. Sedimentation was followed by treatment of the cell aggregates as described above with 1 mg/ml hyaluronidase and 5 μg/ml DNase for 30 min. Many remaining peritubular cells were detached during this incubation and were removed by washing 3 times. The last wash step was performed using Eagle's minimum essential medium (MEM), supplemented with non-essential amino acids and antibiotics (fungizone, streptomycin and penicillin). The resulting cell preparation was allowed to attach, either in 24-well plates (for estimation of ^{3}H-R1881 binding) or in 25 cm^2 culture flasks (for RNA isolation). The cells were seeded at a density of 1×10^6 cells/cm^2 and incubated for 3 days in MEM containing 1% FCS, non-essential amino acids and antibiotics, under an atmosphere of 5% CO_2 in air. Subsequently, the Sertoli cells were subjected to a hypotonic shock (0.1 × MEM for 2 min) to remove the remaining germ cells (Galdieri et al., 1981; Oonk and Grootegoed, 1987). The cells were then cultured in MEM containing non-essential amino acids, antibiotics and 0.1% (w/v) bovine serum albumin (BSA, fraction V; Sigma) for 24 h, before the start of hormone treatment.

Hormone treatment. The Sertoli cells were incubated for 3 days in the presence of FSH (10 ng/ml NIH-FSH-S16) and/or testosterone (1 μM), in medium containing 0.1% BSA. The medium was replaced daily by fresh MEM containing the hormones. After the hormone treatment, the cells were used either to assay the binding of ^{3}H-R1881 (NEN Products, Stevenage, Hertfordshire, U.K.) or to isolate total RNA. At the end of the incubations, the number of peritubular cells in the Sertoli cell cultures was estimated using cytochemical detection of alkaline phosphatase activity, which is present in peritubular myoid cells but not in Sertoli cells isolated from rat testis (Palombi and Di Carlo, 1988).

Androgen binding assay. After incubation in the presence of hormones, the Sertoli monolayers were washed 4 times, each washing for 15 min at 37 °C with MEM containing 0.1% BSA. The cells were then treated for 1 h with triamcinolone acetonide (4 μM), which occupies the progesterone receptors (Zava et al., 1979). This was followed by an incubation of the cells in the presence of ^{3}H-R1881 (7 nM) with or without an excess of unlabelled R1881 (10 μM), for 2 h at 37 °C. Subsequently, the cells were placed on ice and washed 4 times, for 15 min, with ice-cold MEM, to remove the free ^{3}H-R1881. The cellular proteins were dissolved in 1 M NaOH and stored at −20 °C, until ^{3}H-R1881 binding and the total protein and DNA content were measured.

RNA extraction and hybridisation. Total RNA was extracted using a guanidinium thiocyanate lysis buffer, followed by centrifugation through CsCl, phenol extraction and ethanol precipitation (Chirgwin et al., 1979). RNA was blotted on Gene-screen (NEF-972; NEN Products) and hybridised (stringency: 5 × SSC) with a 0.5 kb

[1] Materials and Methods, line 7 should read:

... 'containing 1 mg/ml trypsin (TRL, Worthington, Freehold, NJ, U.S.A.) and 25 μg/ml DNase (DN-25; Sigma, St. Louis, MO, U.S.A.)' ...

(*Eco*RI–*Eco*RI) human androgen receptor cDNA probe (Trapman et al., 1988), corresponding to a part of the steroid-binding domain and the 3′ untranslated region of the receptor mRNA. The blots were washed in 0.1 × SSC containing 0.1% SDS, for 50 min at 50 °C.

Results

The effects of FSH and testosterone on androgen receptor protein and mRNA were estimated using a highly purified Sertoli cell preparation, containing less than 0.1% peritubular myoid cells. The presence of peritubular cells in the Sertoli cell cultures was evaluated using an alkaline phosphatase staining procedure (Fig. 1).

After culture in the presence or absence of FSH and testosterone for 3 days, the DNA and protein content of the culture wells was not changed (not shown). The binding of ^{3}H-R1881, however, was markedly increased after incubation in the presence of hormones (Fig. 2) ($P < 0.001$, compared with control; *t*-test). Testosterone elevated the number of androgen binding sites to a lesser extent than FSH, but testosterone in combination with FSH resulted in a cumulative effect (Fig. 2) ($P < 0.01$, compared with FSH or testosterone).

Using the same cell preparation method and the same hormone treatment schedule, the expression of the androgen receptor mRNA was estimated. In the cultured Sertoli cells a single androgen receptor mRNA species of 10 kb was observed (Fig. 3). This 10 kb mRNA was also detected using total testis RNA (not shown). After incubation of Sertoli cells in the presence of testosterone, the expression of the androgen receptor mRNA was not increased, but rather seemed to be slightly decreased. FSH treatment of the cells caused a marked increase of the androgen receptor mRNA level.

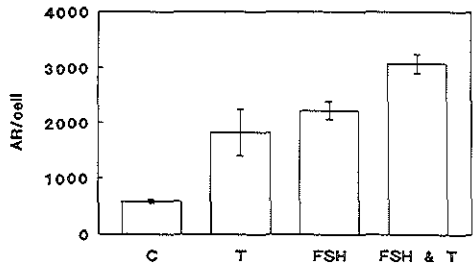

Fig. 2. Effect of testosterone and FSH on the number of androgen receptors in Sertoli cells. Specific binding of ^{3}H-R1881 was estimated after 3 days of culture in the absence (C) or presence of testosterone (T) and/or FSH. The binding was expressed as number of androgen receptors (AR) per cell. The results represent the mean ± SD of four experiments with triplicate incubations.

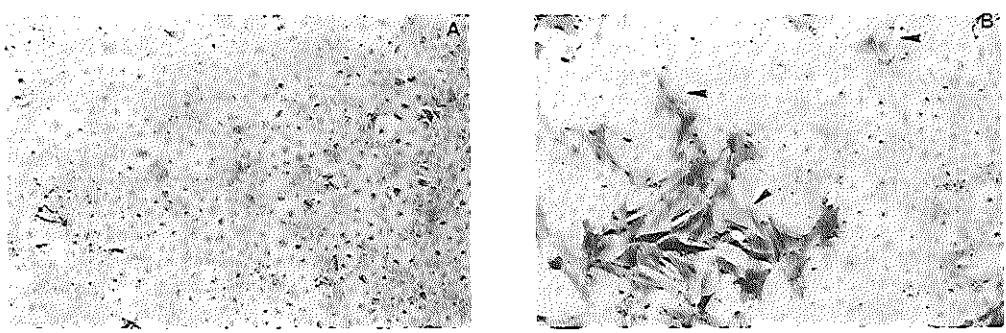

Fig. 1. Alkaline phosphatase staining of peritubular cells present in Sertoli cell cultures. A highly purified Sertoli cell preparation, prepared as described in Materials and Methods (*A*), compared with a less pure Sertoli cell preparation, which was prepared using a different method (*B*). The arrowheads in the less pure preparation point to contaminating peritubular myoid cells, detected with the alkaline phosphatase staining.

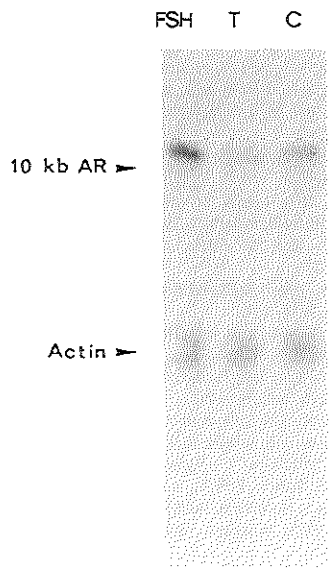

Fig. 3. Northern blot analysis of Sertoli cell RNA using a human androgen receptor cDNA probe. Total RNA was extracted from the Sertoli cells after 3 days of culture in the absence (C) or presence of testosterone (T) of FSH. An amount of 50 μg total RNA was applied per lane, and the blot was hybridised with a human androgen receptor (AR) cDNA probe. Hybridisation with a rat actin cDNA probe was used to confirm that approximately equal amounts of RNA were applied to the different lanes of the gel.

Discussion

In the present experiments, ^{3}H-R1881 was used to estimate the effects of FSH and testosterone on the number of androgen binding sites, whereas Verhoeven and Cailleau (1988) have used ^{3}H-mibolerone. On the basis of K_d (0.7 nM), detailed saturation curves and binding competition studies, Verhoeven and Cailleau (1988) concluded that ^{3}H-mibolerone binding was indicative for the number of androgen receptors per mg protein. A similar K_d value was reported for binding of ^{3}H-R1881 to the androgen receptor in rat Sertoli cells by Buzek and Sanborn (1988). The present findings on hormonal stimulation of the number of androgen receptors in Sertoli cells are in agreement with the results reported by Verhoeven and Cailleau (1988). Furthermore, the concentration of the androgen receptor in non-hormone-treated cells was similar to the data reported by Buzek and Sanborn (1988). However, Verhoeven and Cailleau (1988) did observe the effect of testosterone on receptor numbers, only after the Sertoli cells had been washed and incubated for 15 h in the absence of testosterone. In the present experiments, binding of ^{3}H-R1881 was estimated immediately following the culture period in the presence of testosterone and/or FSH. The binding was decreased rather than increased, after 15 h of incubation in the absence of testosterone (not shown). The reason for this discrepancy is not clear.

Northern blot analysis indicated that FSH, but not testosterone, caused an increased expression of a 10 kb androgen receptor mRNA in the cultured Sertoli cells. Recent data indicate that in other cell types testosterone may suppress androgen receptor mRNA expression. Tan et al. (1988) reported that a 10 kb androgen receptor mRNA in rat prostate was up-regulated after castration, and subsequently down-regulated if testosterone was administered to the castrated rats. The observation that testosterone does not increase the expression of androgen receptor mRNA in cultured Sertoli cells is seemingly in contrast with the results on androgen binding, which showed that testosterone enhances the androgen receptor level in the Sertoli cells 2- to 3-fold. A possible explanation is that testosterone exerts an effect on the translation of the androgen receptor mRNA or on the stability of the androgen receptor. In this context, it is of interest that Syms et al. (1985) have reported that stimulation of a hamster smooth muscle tumour cell line by testosterone resulted in a doubling of the half-life of the androgen receptor.

The Sertoli cell preparation used for the present experiments contained only a very low number of peritubular myoid cells. It is very likely, therefore, that the effect of testosterone on the androgen receptor concentration represents a direct effect of testosterone on Sertoli cells, which is not mediated by the peritubular cells.

The stimulatory effect of FSH on the number of androgen receptors per Sertoli cell was correlated with an increased expression of the androgen receptor mRNA. However, it is not known whether FSH can directly stimulate transcription of the androgen receptor gene. Possibly, this FSH effect on androgen receptor mRNA is an indirect effect which involves regulation of the transcription of other genes by FSH.

From the present results, it is concluded that the molecular mechanism of FSH action on immature Sertoli cells includes a stimulatory effect on androgen receptor mRNA expression, which in combination with post-transcriptional effects of testosterone may lead to an increased responsiveness of the cells to testosterone. This integrated hormone action may play a key role in the hormonal regulation of the initiation and maintenance of spermatogenesis.

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CHAPTER 3

Transient down-regulation of androgen receptor mRNA expression in Sertoli cells by follicle-stimulating hormone is followed by up-regulation of androgen receptor mRNA and protein

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ABSTRACT

Cooperative actions of follicle-stimulating hormone (FSH) and androgens on initiation, maintenance and restoration of spermatogenesis have been described. In the present experiments, regulatory effects of FSH on androgen receptor (AR) gene expression in Sertoli cells were studied.

In immature rats, injection of FSH (i.p., 1 μg/g body weight), resulted in a rapid down-regulation of testicular AR mRNA expression (4h), followed by recovery to control level (10 h). Using cultured immature Sertoli cells, a similar transient effect on AR mRNA expression was observed after addition of FSH (500 ng/ml) or dibutyryl cAMP (0.5 mM). Cycloheximide treatment of the cells did not prevent the rapid FSH-induced down-regulation of AR mRNA expression, indicating that de novo protein synthesis is not required for this effect. Furthermore, using a transcriptional run-on assay, no marked decrease in the rate of AR gene transcription was found upon treatment of the cultured Sertoli cells with FSH for 2 or 4 h. This demonstrates that the short-term effect of FSH on AR mRNA expression reflects a change in mRNA stability. The AR protein level was not markedly affected by the transient decrease in AR mRNA expression.

When immature Sertoli cells were incubated with FSH for longer time periods (24 - 72 h), both AR mRNA and protein expression were increased. In Sertoli cells isolated from 15-day-old rats, this increase was higher (mRNA 2- to 3-fold and protein 2-fold) than in Sertoli cells isolated from 25-day-old animals.

The results indicate that FSH plays a complex role in the regulation of androgen receptor expression in immature rat Sertoli cells.

INTRODUCTION

Follicle-stimulating hormone (FSH), an important hormonal regulator of spermatogenesis, exerts its effects on germ cell development through regulation of Sertoli cell activities (1-3). The addition of FSH to cultured Sertoli cells from immature rats results in various changes in synthesis and secretion of a number of proteins (4). In FSH action, the cAMP signal transduction pathway plays a central role. The ability of FSH to induce changes in intracellular calcium levels, however, has also been documented (5-8). The available information indicates that the involvement of the phosphatidyl inositol pathway in FSH action is not very pronounced (9-10).

Testosterone, the other major hormonal regulator of spermatogenesis, is produced by Leydig cells and exerts actions on peritubular myoid cells and Sertoli cells (11). Germ cells do not express the androgen receptor (12-13). It is likely, therefore, that effects of testosterone on spermatogenesis are mediated by peritubular myoid cells and Sertoli cells.

The androgen receptor is an intracellular protein, which, upon androgen binding, is transformed to a DNA-binding form (14). This transformed receptor can regulate the expression of androgen-dependent genes (15-16).

Substitution of either FSH or testosterone to hypophysectomized rats can maintain spermatogenesis qualitatively, at approximately 70% of the normal level. Only when both hormones are administered together, quantitatively normal spermatogenesis can be maintained (17). From these, and many other experiments (1, 18-20), it has become clear that the actions of FSH and testosterone on spermatogenesis show a cooperative effect.

In long-term stimulation experiments of cultured Sertoli cells from immature rats in the

presence of FSH (3 days), the amounts of androgen receptor mRNA and protein were elevated (21-23). In the present study, the effects of FSH on expression of the androgen receptor gene were examined in more detail, with an emphasis on short-term effects. Experiments were conducted using intact immature rats and cultured Sertoli cells isolated from these animals. Northern blotting, nuclear run-on transcription experiments and specific ligand binding assays were performed to analyze androgen receptor gene expression.

MATERIALS AND METHODS

Isolation of Sertoli cells

Sertoli cells from 15-, 21- or 25-day-old rats were isolated using a method described by Themmen et al. (24). The resulting cell population was allowed to attach, at a density of 10^6 cells/cm^2, either in 24-well plates (for estimation of ^{3}H-R1881 binding) or in 75 cm^2 culture flasks (for RNA extraction), for 2 days. The culture conditions were: Eagle's Minimum Essential Medium, supplemented with antibiotics and non-essential amino acids (MEM), at 37 ^{0}C, in air containing 5% CO_2, in the presence of 1% FCS. Subsequently, the cells were subjected to a hypotonic shock (0.1 x MEM for 2 min) to remove remaining germ cells (25-26). The cells were allowed to recover for 24 h in MEM containing 0.1% (w/v) BSA. The cells were then cultured in the presence or absence of hormones, in MEM containing 0.1% BSA. The experiments were repeated 4 - 5 times, using different cell preparations. Within one experiment, all incubations were terminated at the same time (control and hormone-treated). The hormone treatment protocols are described in the Results section.

Alkaline phosphatase staining was used to evaluate the purity of the cell preparations (27). When 15-day-old rats were used, 0.5% contamination with peritubular myoid cells was observed; when 21- or 25-day-old rats were used, the contamination was below 0.1% (during and at the end of the incubations).

The ligand binding assay was carried out as described by Blok et al. (22) and the RNA extraction and hybridization was carried out as described by Blok et al. (28).

Anova and Tukey's test were used to determine significant differences ($p < 0.01$). Data are expressed as mean $\pm$ S.D.

Nuclear mRNA elongation (run-on)

Nuclei were isolated from immature Sertoli cells that had been cultured in the presence of FSH (500 ng/ml) for 0, 2, or 4 h. The whole procedure was carried out at 4^{0}C. The incubation was terminated by placing the culture flasks on ice. The cells were washed twice with phosphate-buffered saline (PBS) and once with Tris buffer (10 mM Tris, 10 mM NaCl and 3 mM $MgCl_2$, pH 7.4). Subsequently, the cells were incubated in lysis buffer [0.5% v/v Nonidet P40 (BDH Chemicals Ltd, Poole, England), 10 mM Tris, 10 mM NaCl and 3 mM $MgCl_2$, pH 7.4] for 20 min. The cells were detached from the plastic by dynamic shaking. The nuclei and remaining whole cells were centrifuged (500 x g, 5 min) and the pellet was washed in lysis buffer. The pellet was resuspended in 5 ml sucrose buffer A [0.32 M sucrose (BDH), 3 mM $CaCl_2$, 2 mM Mg-acetate, 0.1 mM EDTA, 10 mM Tris, 5 mM dithiothreitol (Sigma), pH 8.0] containing 0.1% Triton X-100 (BDH), to lyse the remaining cell membranes. Different protease inhibitors [0.6 mM bacitracin (Janssen Chimica, Beerse, Belgium) and 0.6 mM PMSF] were added. The

suspension was kept on ice for 5 min, and homogenised by 30 strokes with a dounce homogeniser (pestle type B, Wheaton Scientific, Millville, N.J., USA). The resulting homogenate was mixed with 11 ml of sucrose buffer B [2.05 M sucrose (BDH), 5.0 mM Mg-acetate, 0.1 mM EDTA, 10 mM Tris, pH 8.0]. The mixture, with a final sucrose concentration of approximately 1.4 M, was applied to a 4 ml cushion of sucrose buffer B and centrifuged using a Beckman SW 40 Ti rotor (Beckman, Geneva, Switzerland)(48.000 x g , 45 min, 0°C). This method, using non-ionic detergents to lyse the cells and sucrose gradient centrifugation to recover the nuclei, resulted in a very clean nuclear pellet which could be used in the run-on assay. The nuclei were resuspended in 100μl storage buffer (25% glycerol, 5 mM Mg-acetate, 0.1 mM EDTA, 50 mM Tris, 5 mM dithiothreitol, pH 8.0), frozen in liquid nitrogen and stored at -70°C.

Nuclear elongation was performed essentially as described by Marzluff and Huang (29) and Ausubel et al. (30). In short: 25 x 10^6 nuclei were incubated with ATP, CTP, UTP and [α-^{32}P]-GTP (3000 Ci/mmol; Amersham, Buckinghamshire, U.K.). Sephadex G-50 columns were used to separate labelled pre-mRNA from not incorporated NTPs. Labelled RNA (3 x 10^6 cpm) was added to 2 ml of hybridization mixture. Plasmid DNA was bound to Biotrans nylon blotting filter (ICN Biomedicals, Inc, Irvine, CA, U.S.A.) using a dot blot apparatus (Schleicher and Schuell, Dassel, Germany)(10 μg plasmid DNA was applied per dot). Plasmids used were: pTZ as a control for background hybridization; pTZ-AR-910 (31) and pTZ-0.3A (32) human androgen receptor cDNA; pRK-FSHR-NH2 and pRK-FSHR-COOH rat FSH receptor cDNA (33); α7/pUC18 rat inhibin cDNA (28); pUC9-Act hamster actin cDNA. The hybridization was allowed to carry on for 65 h at 55°C. The blots were washed at 55°C using 1 x SSC for 15 min in case of pTZ, androgen receptor and FSH receptor run-on assays. Inhibin and actin run-on hybridizations were washed with 0.2 x SSC containing 0.25% (w/v) SDS for 45 min at 55°C. Blots were sealed in plastic and autoradiographed. The run-on experiments were repeated 3 times, using 3 different Sertoli cell isolations.

Experimental animals

Wistar rats (substrain R-1 Amsterdam) were housed, 6 per cage, in a controlled environment with a light cycle of 12L : 12D, and received rat diet ad libitum. Ten animals were injected intraperitoneally with FSH (1 μg ovine FSH (FSH-S16)/g body weight), dissolved in 0.9% (w/v) NaCl containing 0.1% (w/v) bovine serum albumin (BSA, fraction V; Sigma, St. Louis, MO, USA). Four control animals received only 0.9% (w/v) NaCl containing 0.1% BSA. The animals were sacrificed 2 - 10 h later for testicular RNA isolation. Other untreated animals were killed for Sertoli cell isolation.

All animal experimentation described in this manuscript were conducted in accord with the highest standards of human animal care, as outlined in "Guidelines for Care and Use of Experimental Animals".

RESULTS

Short-term effects of FSH

Intact immature rats (21-day-old) were injected with FSH (i.p., 1 μg ovine FSH-S16/g body weight) and sacrificed 0, 2, 4, 6, 8, and 10 hours after injection, to analyze the amount of

androgen receptor mRNA present in the testis (Fig. 1). Within 4 h after injection of FSH, the amount of androgen receptor mRNA in the testis was decreased to a very low level (10 ± 6% of control; mean ± SD of 3 animals). At later time points after FSH injection, the amount of androgen receptor mRNA returned approximately to control level (Fig. 1). All Northern blots were hybridized to a hamster actin cDNA probe, to verify the amount of total mRNA that was applied to the lanes on the gel.

Androgen receptors in the testis are located in different somatic cell types, including Sertoli cells. To investigate the effect of FSH on purified Sertoli cells, these cells were isolated from 21-day-old rats and cultured in the presence of 500 ng/ml ovine FSH-S16, which is known to be a maximally stimulating dose (34). It was observed that during the first 5 h after FSH administration, the level of androgen receptor mRNA decreased to a hardly detectable value (15 ± 4% of control; mean ± SD of 5 experiments)(Fig. 2). This low level was rapidly restored to control level, between 5 and 8 h of culture in the continuous presence of FSH (Fig. 2). Similar results were obtained using Sertoli cells isolated from 15- and 25-day-old rats (not shown).

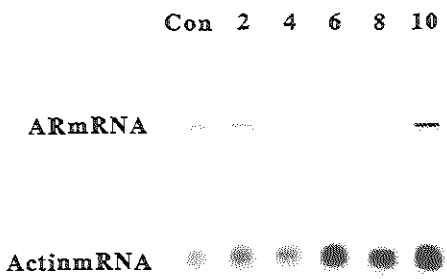

Fig. 1. Effect of FSH on testicular androgen receptor mRNA expression. The 21-day-old rats were injected (i.p.) with 1μg ovine FSH-S16/g body weight and sacrificed 2, 4, 6, 8 or 10 h later. Con = control; saline injected and sacrificed 4 h later. For Northern analysis, 20 μg of total RNA, isolated from total testis, was applied per lane and analyzed using a human androgen receptor cDNA probe and a hamster actin cDNA probe. Actin was used to verify the amounts of mRNA that were applied per lane on the gel.

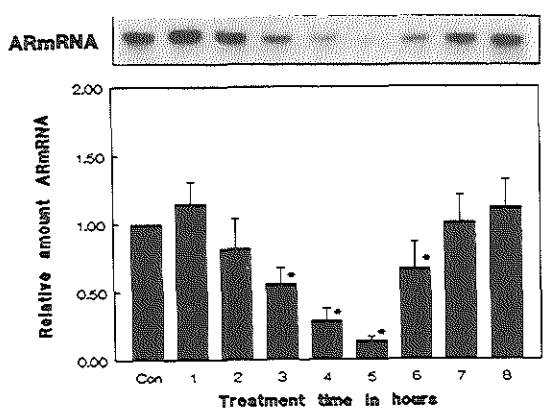

*Fig. 2. Effect of FSH on androgen receptor mRNA expression in cultured Sertoli cells. Sertoli cells from 21-day-old rats were cultured for 1, 2, 3, 4, 5, 6, 7 and 8 h in the presence of ovine FSH-S16 (500 ng/ml). Con = control. For Northern analysis, 20 μg of total RNA was applied per lane and analyzed using a human androgen receptor cDNA probe. The different lanes were scanned and the amount of androgen receptor mRNA was determined relative to the control lane (1.00). The experiment was repeated 5 times (the bars represent the mean ± SD, whereas the autoradiograph is a representative sample from a single experiment). * = significantly different from control ($p < 0.01$).*

A dose-response curve was produced for FSH-induced androgen receptor mRNA down-regulation (Fig. 3). The ED_{50} was approximately 10 ng/ml. For comparison; the ED_{50} for several other, non related, effects of FSH on cultured Sertoli cells, including stimulation of cAMP production, c-fos expression, glycolysis and inhibin α-subunit expression (34-36), was found to be approximately the same.

The cAMP transduction pathway plays an important role in FSH action. Therefore, immature Sertoli cells were also cultured in the presence of dibutyryl cAMP (dbcAMP) or forskolin. It was found that both dbcAMP (Fig. 4) and forskolin (not shown) mimicked the effect of FSH.

Incorporation of L-[1-^{14}C]-leucine in cultured Sertoli cells treated with cycloheximide (50 μg/ml) was reduced by 98% within 30 min. As shown in Figure 5, this cycloheximide treatment stimulated the level of androgen receptor mRNA 1.7 $\pm$ 0.4-fold (mean $\pm$ SD of 4 experiments). When FSH was added to the cell cultures for 4 h, starting 30 min after cycloheximide administration, the amount of androgen receptor mRNA was decreased. The

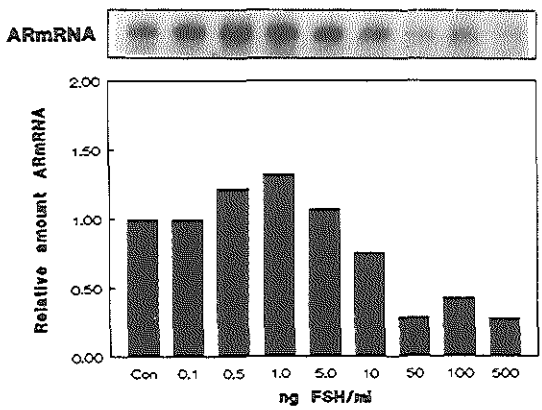

Fig. 3. FSH dose-response curve for FSH-regulated androgen receptor mRNA expression. Sertoli cells from 21-day-old rats were cultured for 4 h in the presence of 0.05, 0.1, 0.5, 1, 5, 10, 50, 100 and 500 ng/ml ovine FSH-S16. Con = control. For Northern analysis, 20 µg of total RNA was applied per lane and analyzed using a human androgen receptor cDNA probe. The different lanes were scanned and the amount of androgen receptor mRNA was determined relative to the control lane (1.00).

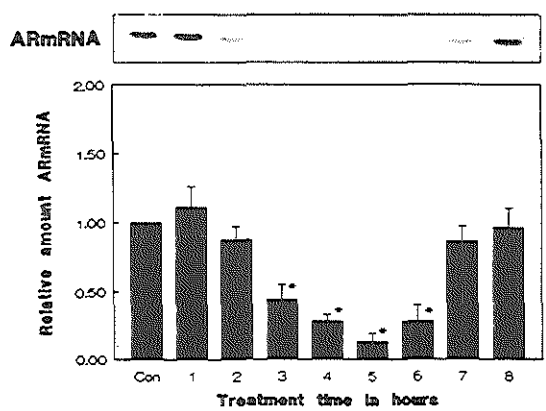

Fig. 4. Effect of dibutyryl cAMP on androgen receptor mRNA expression in cultured Sertoli cells. Sertoli cells from 21-day-old rats were cultured for 1, 2, 3, 4, 5, 6, 7 and 8 h in the presence of dbcAMP (0.5 mM). Con = control. For Northern analysis, 20 µg of total RNA was applied per lane and analyzed using a human androgen receptor cDNA probe. The different lanes were scanned and the amount of androgen receptor mRNA was determined relative to the control lane (1.00).
*The experiment was repeated 4 times (the bars represent the mean $\pm$ SD, whereas the autoradiograph is a representative sample from a single experiment). * = significantly different from control ($p < 0.01$)*

magnitude of this decrease was comparable to the decrease found after FSH treatment without cycloheximide (0.7 units on the Y-axis) (Fig. 5). It can be concluded that de novo protein synthesis is not needed for FSH-induced down-regulation of androgen receptor mRNA.

Down-regulation of mRNA expression in general can result from a lowered transcription rate and/or decreased mRNA stability. To study androgen receptor gene transcription, nuclear RNA elongation experiments (run-on analysis) were performed. Double-stranded probes, used to capture the labelled mRNA, can also detect antisense mRNAs. The presence of antisense transcripts, however, is not very likely, because only one distinct 10 kb androgen receptor mRNA transcript was detected in cultured Sertoli cells and testicular tissue. Recently, Faber et al. (32) have given a detailed characterisation of possible androgen receptor gene transcripts. Using several probes that were located at different parts of the cDNA, they found 10kb and 7kb transcripts. The 7 kb transcript represents an alternative splice product, that is not found in testicular cells, and was detected using a 0.5 kb EcoR1/EcoR1 human androgen receptor cDNA probe. In the present experiments, the same probe was used. A 4 kb androgen receptor gene transcript has been described, but this appeared to be a breakdown product from the 10 kb androgen receptor mRNA (28).

Using a nuclear run-on assay, no marked change in transcription rate of the androgen receptor gene was observed, after incubation of Sertoli cells for 2 to 4 h in the presence of FSH (Fig. 6). Klaij et al. (37) have shown that stimulation of expression of inhibin α-subunit mRNA by FSH in Sertoli cells takes place within 2 h. This effect is thought to involve a direct effect of FSH on gene transcription (37); the promoter region contains a cyclic AMP response element (CRE)(38). In the present run-on experiments, inhibin α-subunit gene transcription indeed was elevated by FSH within 2 h (Fig. 6). This represents a positive control. No induction or suppression of actin gene transcription was observed (Fig. 6).

The amount of androgen receptor present in Sertoli cells was measured by specific ligand (^{3}H-R1881) binding assay. It was observed that the pronounced down-regulation of androgen receptor mRNA did not result in a marked decrease in androgen receptor protein (Fig. 7).

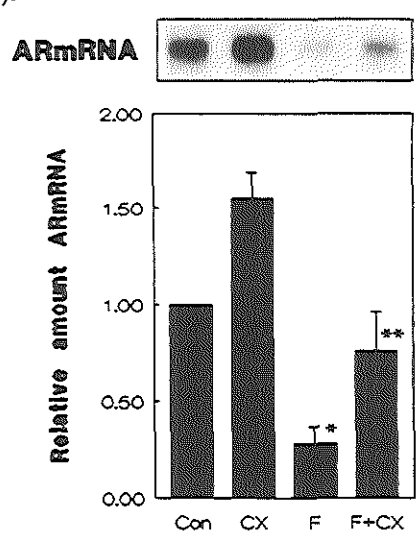

*Fig. 5. FSH regulation of androgen receptor mRNA expression in the presence of cyclo-heximide. Sertoli cells from 21-day-old rats were cultured for 4 h in the presence of cycloheximide (50 µg/ml) and or ovine FSH-S16 (500 ng/ml). Con = control; CX = treated for 4.5 h with cycloheximide; FSH = treated for 4 h with FSH; F + CX = 30 min after cyclo-heximide addition, FSH was added to the cells for 4 h. For Northern analysis, 20 µg of total RNA was applied per lane and analyzed using a human androgen receptor cDNA probe. The different lanes were scanned and the amount of androgen receptor mRNA was determined relative to the control lane (1.00). The experiment was repeated 4 times (the bars represent the mean ± SD, whereas the auto-radiograph is a representative sample from a single experiment). * = significantly different from control ($p < 0.01$). ** = significantly different from cycloheximide treated group ($p < 0.01$).*

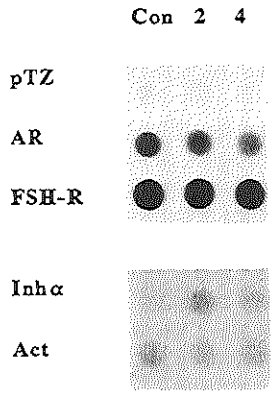

Fig. 6. Quantitation of the initiation of androgen receptor gene transcription in cultured Sertoli cells. Sertoli cells, isolated from 21-day-old rats, were cultured in the presence of ovine FSH-S16 (500 ng/ml), for 2 or 4 h. Nuclei were isolated and transcriptional run-on experiments were performed as described in Materials and Methods. Con = control; AR = androgen receptor; FSH-R = FSH receptor; Inhα = inhibin α-subunit; Act = actin.
The experiment was repeated 3 times with essentially the same results. The autoradiograph shows the results from one experiment.

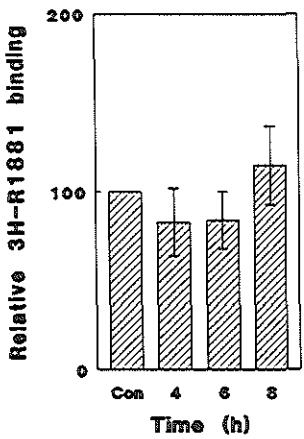

Fig. 7. Short-term effect of FSH on ^{3}H-R1881 binding to Sertoli cells. The cells were isolated from 21-day-old rats and cultured in the presence of ovine FSH-S16 (500 ng/ml) for 4, 6 and 8 h. Con = control. The experiment was repeated 4 times (the bars represent the mean ± SD).

Long-term effects of FSH

The short-term effects described herein are seemingly in contradiction to a stimulatory effect of FSH on androgen receptor expression in cultured Sertoli cells, described previously (21-23). These published observations, however, concern long-term experiments. In the present series of experiments, we have incubated Sertoli cells with FSH also for longer time periods (24 - 72 h). At the end of these incubations, a net increase in androgen receptor mRNA level was observed (Fig. 8). This long-term effect of FSH on androgen receptor mRNA expression in Sertoli cells was more pronounced in Sertoli cells from 15-day-old rats as compared to cells isolated from 25-day-old rats.

Measurement of the total binding of ^{3}H-R1881 to Sertoli cells from 15-day-old rats incubated for 24 - 72 h with FSH, revealed a FSH-induced up-regulation of ligand binding (1.8 ± 0.2-fold; mean ± SD of 4 experiments), which was much less pronounced using Sertoli cells from 25-day-old rats (Fig. 9).

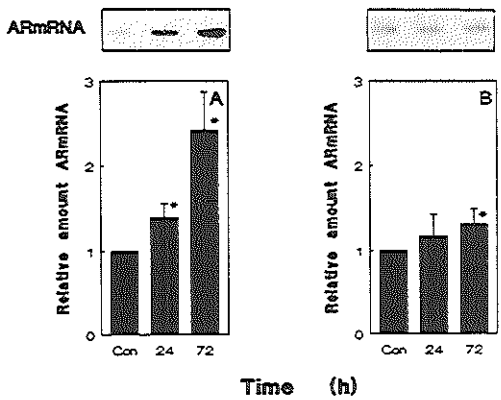

*Fig. 8. Long-term effect of FSH on androgen receptor mRNA expression. Sertoli cells from 15-day-old (A) and 25-day-old (B) rats were cultured for 24 and 72 h in the presence of ovine FSH-S16 (500ng/ml). Con = control. For Northern analysis, 20 µg of total RNA was applied per lane and analyzed using a human androgen receptor cDNA probe. The different lanes were scanned and the amount of androgen receptor mRNA was determined relative to the control lane (1.00). The experiment was repeated 4 times (the bars represent the mean ± SD, and the autoradiograph shows the results from one experiment). * = significantly different from control (p < 0.01).*

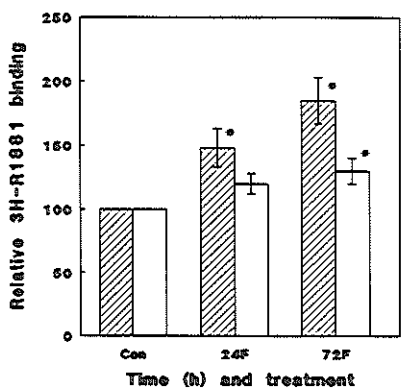

Fig. 9. Long-term effect of FSH on ^{3}H-R1881 binding to Sertoli cells. The cells were isolated from 15-day-old (hatched bars) and 25-day-old (open bars) rats and cultured in the presence of ovine FSH-S16 (500 ng/ml) for 24 and 72 h. Con = control. The experiment was repeated 4 times (the bars represent the mean ± SD).
** = significantly different from control (p < 0.01).*

Addition of testosterone alone, or on top of FSH addition, to the cultures did not significantly change the level of androgen receptor mRNA expression (not shown). However, the binding of ^{3}H-R1881 to cells incubated with testosterone was increased 3.2 ± 0.6-fold (mean ± SD of 3 experiments, not shown). Furthermore, when FSH was added together with testosterone to the Sertoli cell cultures, the ^{3}H-R1881 binding was increased to 4.1 ± 0.7-fold (mean ± SD of 3 experiments, not shown).

DISCUSSION

Short-term effects of FSH

FSH administration (i.p.) to immature rats resulted in a rapid, but transient, decrease of androgen receptor mRNA expression in the testis. Apparently, the circulating FSH level is not

a maximally stimulating dose (39). When highly purified immature Sertoli cells were used, a similar effect of FSH on androgen receptor mRNA expression was observed. In both situations, in vivo and in vitro, the amount of androgen receptor mRNA was decreased to a very low level within 4 - 6 h, and then returned to the control level within several hours. The circulating FSH levels in vivo were not measured, but it can be expected that these levels are very high shortly after FSH injection, while they will return to the normal physiological range 10 h after FSH injection. To further investigate androgen receptor mRNA expression in the continuous presence of a defined dose of FSH, highly purified cultured Sertoli cells from immature rats were used.

The effect of FSH on cultured immature Sertoli cells involves rapid stimulation of the cellular cAMP level, which is followed by a decrease within a few hours. This decrease is caused by a desensitization process that involves receptor internalisation (24,40), loss of adenylate cyclase activity (41) and increased phosphodiesterase activity (42-43). In concordance with this, the effects of dbcAMP on Sertoli cells are usually much more persistent then the effects of FSH. This was described also for FSH-induced down regulation of FSH receptor mRNA levels in cultured immature Sertoli cells (24), showing a transient effect of FSH, but a prolonged effect of dbcAMP. In the present experiments, however, the short-term effect of dbcAMP on androgen receptor mRNA expression also was transient and virtually equivalent to the effect produced by FSH. This transient effect of dbcAMP is difficult to explain. Possibly, it involves activation of a second messenger system other than cAMP (e.g. a calcium mediated response; 44).

Addition of cycloheximide, to block protein synthesis, resulted in an increased androgen receptor mRNA expression in cultured Sertoli cells. Stabilization of distinct mRNAs is not an uncommon event after cycloheximide treatment, and several explanations have been suggested (45 - 47). FSH-induced down-regulation of androgen receptor mRNA expression did occur also in the presence of cycloheximide. This indicates that de novo protein synthesis is not required for this FSH effect. FSH action may result in activation or de-activation of pre-existing proteins that are involved in regulation of androgen receptor mRNA expression.

The results of the run-on experiments indicate that FSH-induced down-regulation of androgen receptor mRNA expression in Sertoli cells cannot be accounted for by a lowered rate of gene transcription. It is possible that the stability of the androgen receptor mRNA is decreased upon FSH stimulation. Secondary structures in the untranslated regions of mRNAs may serve as binding sites for proteins that are involved in mRNA stability regulation (48). In future experiments we will study whether the large 5' and 3' untranslated regions of androgen receptor mRNA may play a role in regulation of its stability.

The amount of androgen receptor protein in cultured Sertoli cells, measured using a ^{3}H-R1881 binding assay, did not follow the short-term FSH-induced diminution of androgen receptor mRNA expression. This can be explained when the half-life of the mRNA is much shorter than the half-life of the protein. Under FSH-induced down-regulation conditions, the androgen receptor mRNA showed a calculated half-life of approximately 1 h, while the reported half-life for an unoccupied androgen receptor, in the ductus deferens smooth muscle tumor cell line DDT$_1$MF-2 is 3.1 h (49).

Long term effects of FSH

In Sertoli cells from 15-day-old rats, incubated for 3 days with FSH, androgen receptor mRNA expression was 2- to 3-fold increased. Using Sertoli cells from 25-day-old rats, only a small

increase (less than 1.5-fold) in androgen receptor mRNA expression was observed.

The effect of age can be explained. Steinberger et al. (50) found that the amount of cAMP that is produced by cultured Sertoli cells upon FSH stimulation, decreased with age of the animals used for cell isolation. In their 4-day cultures of Sertoli cells isolated from 18-day-old rats, the FSH-induced cAMP production was 8-fold higher than that in 4-day cultures of Sertoli cells from 24-day-old rats.

The androgen receptor protein concentration (^{3}H-R1881 binding) closely followed the FSH-induced long-term changes in androgen receptor mRNA expression. This indicates that the long-term action of FSH is mainly on androgen receptor mRNA expression, rather than on mRNA translation and/or receptor stability. The sensitivity of the nuclear run-on assay is not sufficient to detect the long-term, relatively small changes in transcription rate. Therefore, androgen receptor promoter constructs (51), cloned in front of a reporter gene, are presently used by us to study whether FSH regulates androgen receptor gene transcription.

Incubation of the Sertoli cells in the presence of testosterone pointed to a marked post-transcriptional effect of testosterone on androgen receptor protein expression (specific ligand binding was increased 3-fold by testosterone, without an effect on androgen receptor mRNA expression). This observation is in agreement with our previous results using Sertoli cells (22), and with results from Syms et al. (49), who reported increased stability of the androgen receptor, when occupied with ligand in the ductus deferens smooth muscle tumor cell line DDT_1MF-2.

In conclusion, marked effects of FSH on the regulation of androgen receptor expression in Sertoli cells were observed. Short-term transient FSH-induced down-regulation of androgen receptor mRNA expression takes place in vivo as well as in vitro. However, this down-regulation in vitro, in cultured Sertoli cells, did not result in a short-term decrease of androgen binding to the cells. Long-term stimulation of cultured Sertoli cells with FSH resulted in elevated levels of both androgen receptor mRNA and protein. It is suggested that FSH plays an important role in regulating the androgen receptor concentration in Sertoli cells in the testis of immature rats.

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CHAPTER 4

Follitropin receptor down-regulation involves a cAMP-dependent post-transcriptional decrease of receptor mRNA expression

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Rapid Paper

Follitropin receptor down-regulation involves a cAMP-dependent post-transcriptional decrease of receptor mRNA expression

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Summary

The regulation by FSH (follitropin; follicle-stimulating hormone) of FSH receptor mRNA and protein (FSH binding) was studied using cultured Sertoli cells isolated from 21-day-old rats. FSH induced a dose-dependent and almost complete down-regulation of receptor mRNA at 4 h after addition of the hormone. At subsequent time points (16 h and later) the FSH receptor mRNA levels had returned close to control values. The effect of FSH was mimicked by dibutyryl cyclic AMP (dbcAMP) and forskolin, and the phosphodiesterase inhibitor methyl-isobutylxanthine (MIX) prolonged the FSH action. These findings indicate that the effect of FSH on its receptor mRNA was mediated by cAMP. A down-regulatory effect of FSH and dbcAMP on FSH receptor mRNA was also observed in the presence of the protein synthesis inhibitor cycloheximide, suggesting a direct effect of FSH/dbcAMP on the expression of the FSH receptor gene. Transcriptional run-on experiments revealed that FSH did not inhibit initiation of the FSH receptor gene; hence a post-transcriptional mechanism is involved. Binding of ^{125}I-FSH to the cultured Sertoli cells was rapidly (4 h) decreased when the cells were incubated with FSH or FSH in combination with MIX. This effect can be explained by ligand-induced receptor sequestration. In contrast, incubation of Sertoli cells with dbcAMP had no effect on binding of ^{125}I-FSH after 4 h, but resulted in a 60% loss of FSH binding sites after 24 h, probably caused by decreased mRNA expression.

In conclusion, FSH receptor down-regulation in Sertoli cells is effected not only by the well-documented ligand-induced loss of receptors from the plasma membrane, but also involves a cAMP-mediated decrease of FSH receptor mRNA through a post-transcriptional mechanism.

Introduction

Follicle-stimulating hormone (follitropin; FSH), a glycoprotein hormone produced by the pituitary gland, is essential for normal reproductive function. FSH acts on testicular Sertoli cells and ovarian granulosa cells, the only cells in the body that express a detectable amount of receptors for FSH. It is generally assumed that FSH acts through the stimulating GTP-binding protein G_s on adenylyl cyclase, resulting in increased levels of cAMP, although more than one second messenger pathway may be involved in the transduction of the FSH signal (Grasso et al., 1991).

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Several mechanisms exist to control the level of stimulation of Sertoli cells by FSH. In the hypothalamus-pituitary-testis axis, testosterone acts as a major negative feedback regulator of FSH secretion (Drouin and Labrie, 1981), and the Sertoli cell product inhibin also may play a role in this respect (de Jong, 1988). In addition, FSH sensitivity of Sertoli cells is regulated at the cellular level. Upon binding of FSH to its receptor and stimulation of the adenylyl cyclase enzyme, the hormone-receptor complex is thought to be internalized and transported to an intracellular compartment, where the bound FSH is degraded (Fletcher and Reichert, 1984; Shimizu and Kawashima, 1989); there are indications that the unoccupied receptor is recycled to the plasma membrane (Saez and Jaillard, 1986). Yet another mechanism of cellular control of sensitivity is the increase of phosphodiesterase activity observed in Sertoli cells after stimulation with FSH (Conti et al., 1981, 1983).

In the present study, we investigated the involvement of regulation of FSH receptor mRNA expression in the cellular regulation of FSH sensitivity, using cultured Sertoli cells. It was found that the loss of FSH receptors at the plasma membrane is the initial site of regulation, while a subsequent contribution results from a decrease of FSH receptor mRNA expression.

Materials and methods

Isolation and culture of Sertoli cells. All glassware used for the Sertoli cell isolation procedure was sylanized (Oonk et al., 1985). Sertoli cells were isolated from testes of 21-day-old Wistar rats (substrain R-1 Amsterdam). Rats were sacrificed using cervical dislocation, and the testes were removed and decapsulated. 10–12 testes were shaken (120 cycles/min) at 37°C in 20 ml phosphate-buffered saline (PBS) (Dulbecco and Vogt, 1954), supplemented with 5 μg/ml DNase I (type DN25; Sigma, St. Louis, MO, U.S.A.) (PBS/DNase), 1 mg/ml collagenase (type CLS; Worthington, Freehold, NJ, U.S.A.), 1 mg/ml hyaluronidase (type I-S; Sigma) and 1 mg/ml trypsin (type TRL; Worthington) in a 100 ml Erlenmeyer flask for 20 min. The tubule fragments obtained by this enzyme treatment were washed 4 times with 40 ml PBS/DNase by sedimentation for 2 min at unit gravity. The volume was readjusted to 20 ml PBS/DNase and made 1 mg/ml collagenase and 1 mg/ml hyaluronidase, and incubated for 20 min as described above. The tubule fragments then were washed 4 times (see above), and fragmented further using a dounce homogenizer (five strokes) as described by Oonk et al. (1985). The small fragments were washed 4 times with PBS/DNase, and once with Eagle's minimal essential medium (MEM; Gibco, Grand Island, NY, U.S.A.) by sedimentation at $100 \times g$ for 2 min in a calibrated glass tube to measure the cell pellet volume. MEM was supplemented with non-essential amino acids, glutamine, fungizone, streptomycin and penicillin (Oonk et al., 1985). The cell pellet was resuspended in 9 volumes of MEM with 1% fetal calf serum (FCS; Gibco), and plated at a density of 1 ml cell suspension per 75 cm^2 culture flask area. The cells in the culture flasks were incubated in MEM with 1% FCS at 37°C, under 5% CO_2 in air for 48 h. To eliminate contaminating germinal cells, the Sertoli cell cultures were exposed to an osmotic shock, using 10-fold diluted MEM in water for 2 min (Galdieri et al., 1981; as modified by Toebosch et al., 1989). Cells were then incubated for a further 24 h period in MEM with 0.1% (w/v) BSA (bovine serum albumin; fraction V; Sigma), followed by the incubations and the experiments described in the Results and Discussion section. The experiments were repeated 2–3 times, using different cell preparations.

The Sertoli cell isolation method described above yielded a highly purified preparation of Sertoli cells, as evidenced by staining of the cultures for alkaline phosphatase activity as described by Blok et al. (1990). The number of alkaline phosphatase-positive cells, representing peritubular myoid cells (Palombi and Di Carlo, 1988), was less than 0.1% up to the end of the culture period.

RNA analysis. All general molecular biology techniques were carried out as described in Sambrook et al. (1989) and Davis et al. (1986). Recently, the cDNA encoding the rat FSH receptor was cloned (Sprengel et al., 1990). In a study using a PCR approach to obtain the cDNA of the TSH receptor, a human partial cDNA clone was isolated that was expressed only in ovary and

testis (Parmentier et al., 1989). This sequence is homologous with the rat FSH receptor sequence. We have used this human probe (pHGMP09) to study the regulation of expression of FSH receptor mRNA in cultured rat Sertoli cells.

Total RNA was isolated from flash-frozen cells (−80°C) using the LiCl/urea method (Aufray and Rougeon, 1980). RNA was separated on 1% agarose/formaldehyde gels, and blotted using Hybond N$^+$ nylon membrane filters (Amersham, 's-Hertogenbosch, The Netherlands) or Biotrans nylon membrane filters (ICN, Irvine, CA, U.S.A.). The blots were hybridized with the ^{32}P-labelled pHGMP09 probe for 48 h. After hybridization, the blots were washed to a final stringency of 1 × SSC/0.5% (w/v) SDS (1 × SSC = 0.15 M NaCl, 0.015 M sodium citrate) at 42°C, and autoradiographed using Hyperfilm-MP film (Amersham). The hybridization signals of the 3.5 kb FSH receptor mRNA band were quantified using a BioRad-1D gel scanner (BioRad, Utrecht, The Netherlands). Hybridization of the blots with an actin probe showed that in all lanes similar amounts of RNA were applied.

^{125}I-FSH binding. ^{125}I-FSH binding was measured using a whole cell binding assay, essentially as described for porcine Sertoli cells by Saez and Jaillard (1986). In brief, the attached cells were washed twice with 10 mM Tris-HCl pH 7.4, 5 mM $MgCl_2$, 0.1% (w/v) BSA (TB 7.4) at 37°C, and once with 10 mM Tris-HCl pH 3.9, 5 mM $MgCl_2$, 0.1% (w/v) BSA (TB 3.9) at 4°C to remove bound FSH from the cell surface, and subsequently twice with TB 7.4 at 37 °C. These washed cells were incubated for 1 h in 1 ml of TB 7.4 containing 50,000 cpm ^{125}I-FSH (specific activity: 135 μCi/μg; NEN-Du Pont de Nemours, 's-Hertogenbosch, The Netherlands) per well (Costar 12-well plate), at 37°C under 5% CO_2/95% air. To remove unbound ^{125}I-FSH, the wells were rinsed twice with TB 7.4 at 4°C. Bound ^{125}I-FSH was recovered by dissociation from the membrane in TB 3.9 for 10 min at 4°C. The supernatant was collected and the radioactivity was determined. Routinely, the remaining cells were dissolved in 1 N NaOH to determine the remaining amount of cell-associated ^{125}I-FSH. Under all incubation conditions less than 1% of the added ^{125}I-FSH was found in the NaOH lysate. Binding assays were performed in triplicate, with non-specific binding controls in triplicate containing 500-fold excess of unlabelled ovine FSH (NIH S16; NIH, Bethesda, MD, U.S.A.). Maximal specific binding was 3–5% of total added ^{125}I-FSH.

Transcriptional run-on. Transcriptional run-on experiments were performed essentially as described by Marzluff and Huang (1984) and Ausubel et al. (1989). Typically, 25×10^6 nuclei were recovered from 100×10^6 Sertoli cells. Elongation of the in vivo initiated mRNAs was performed in the presence of [^{32}P]α-GTP, and labelled pre-mRNAs were separated from unincorporated nucleotides. Hybrid selection was performed as follows. Plasmid DNA (5 μg of pRK-FSHR-NH2 and 5 μg of pRK-FSHR-COOH, rat FSH receptor cDNA (Sprengel et al., 1990) for determination of FSH receptor pre-mRNAs and 10 μg pTZ as a control for background hybridization) was bound to nylon membrane filter using a dot blot apparatus (Schleicher and Schuell, 's-Hertogenbosch, The Netherlands). The filters were hybridized to 3–10^6 cpm of labeled RNA for 65 h at 55 °C, washed at 55 °C using 1 × SSC for 15 min, and autoradiographed.

Results and discussion

A time course study using the cultured rat Sertoli cells from 21-day-old rats showed a rapid, but transient, down-regulatory effect of FSH on FSH receptor mRNA expression (Fig. 1). This effect was maximal after 4 h incubation with FSH, while the amount of FSH receptor mRNA had returned to 80% of control at the 16 h time point. The ED_{50} of FSH in down-regulating FSH receptor mRNA expression was between 5 and 50 ng/ml FSH (Fig. 2). This is close to the ED_{50} for other effects of FSH, such as stimulation of cAMP, c-*fos* expression, aromatase activity, glycolysis and α-inhibin expression (Oonk et al., 1985; Hall et al., 1988; Toebosch et al., 1989).

FSH is thought to act mainly through the cAMP pathway (Means et al., 1976). Concurrent with this, the down-regulatory effect of FSH was mimicked by dbcAMP (Fig. 3*A*) and forskolin (not shown). This dbcAMP/forskolin induced down-regulation was effective at 4 h and persisted up to at least 24 h. The effect of dbcAMP

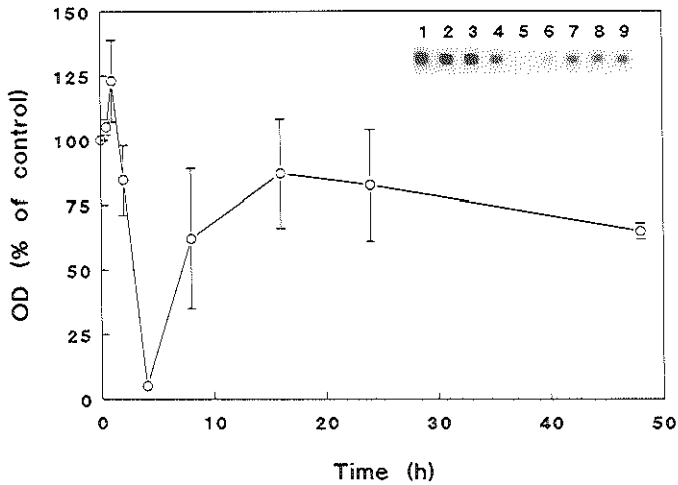

Fig. 1. Time course of the effect of FSH on FSH receptor mRNA expression in Sertoli cells. Cells were incubated for different time periods in the presence of 500 ng/ml FSH. Subsequently, the cells were harvested and total RNA was isolated. The FSH receptor mRNA levels were assessed by Northern analysis, using 20 μg of total RNA per lane. The FSH receptor mRNA band was scanned and the resulting optical density (OD) measurements were expressed relative to the control value (100%). Inset: Autoradiogram of one representative experiment. To lanes 1–9 RNA from cells incubated for 0, 0.5, 1, 2, 4, 8, 16, 24 and 48 h was applied. In the graph the combined results of three experiments are represented. Values given are means ± SD for three experiments.

at 24 h was more pronounced than that of FSH itself, suggesting that FSH down-regulates the activity of the FSH receptor signal transduction system, while dbcAMP does not have such an effect.

Experiments in the presence of the phosphodiesterase inhibitor methyl-isobutylxanthine (MIX) yielded results comparable to those observed using dbcAMP (Fig. 3*A*). This indicates that the transient FSH effect on receptor mRNA expression is related to a transient FSH effect on cAMP production, which is explained by increased levels of phosphodiesterase activity (Conti et al., 1981, 1983).

The intracellular and secreted amounts of cAMP were determined at different time points after addition of FSH or FSH in combination with MIX (Fig. 4). Treatment with FSH increased cAMP rapidly; the maximal intracellular concentration was reached within 15 min, while cAMP had returned to control values at 2 h. Secreted cAMP levels raised slower, reaching a plateau value at 2 h after addition of FSH. Phosphodiesterase activity in Sertoli cells is able to rapidly decrease intracellular cAMP concentrations, as is evidenced by the effects of MIX (Fig. 4). These results confirm earlier observations, that cultured Sertoli cells are rapidly desensitized to FSH with

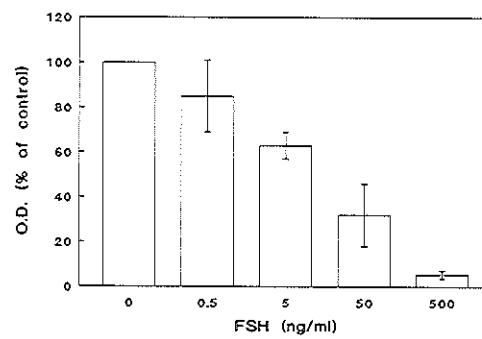

Fig. 2. Effect of FSH dose on the FSH receptor mRNA level in Sertoli cells. Cells were incubated for 4 h in the presence of different concentrations of FSH. Subsequently, the cells were harvested and the FSH receptor mRNA was measured as described in the legend to Fig. 1. Values given are means ± SD for three experiments.

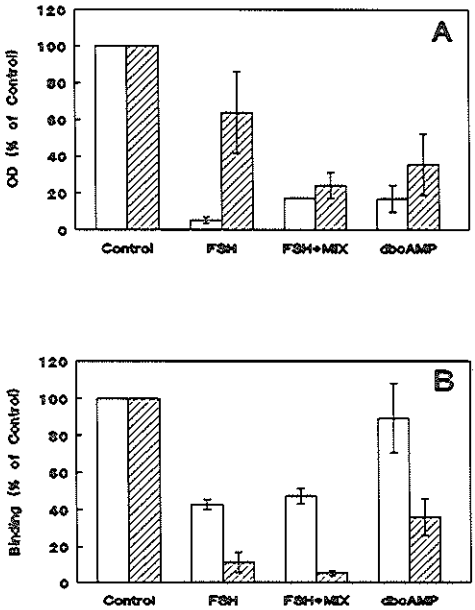

Fig. 3. Involvement of the cAMP pathway in the regulation of FSH receptor mRNA expression and FSH binding in Sertoli cells. *A*: FSH receptor mRNA. Cells were incubated for 4 h (open bars) or 24 h (hatched bars) in the presence of 500 ng/ml FSH, 500 ng/ml FSH in combination with 0.2 mM MIX, or 0.5 mM dbcAMP. Subsequently, the cells were harvested and the FSH receptor mRNA was measured as described in the legend to Fig. 1. Values given are means ± SD for three experiments. *B*: FSH binding. Cells were incubated for 4 h (open bars) or 24 h (hatched bars) in the presence of 500 ng/ml FSH, 500 ng/ml FSH in combination with 0.2 mM MIX, or 0.5 mM dbcAMP. Subsequently, ^{125}I-FSH binding was measured as described in Materials and Methods. Values given are means ± SD for three experiments.

respect to cAMP formation (Verhoeven et al., 1980, 1981; Oonk et al., 1985).

In cells treated with the protein synthesis inhibitor cycloheximide, FSH receptor mRNA still was down-regulated by FSH or dbcAMP, although CX treatment resulted in an overall increase of the receptor mRNA levels (Table 1). The latter effect of CX, that has been observed in many other cell types as well (Almendral et al., 1988), is probably caused by a general effect on transcription and/or mRNA stability (Wilson and Treisman, 1988). The present results indicate that

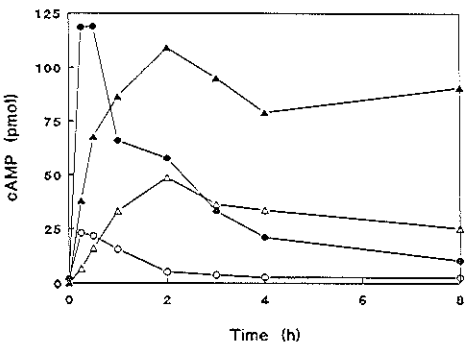

Fig. 4. Effect of FSH on cAMP production by Sertoli cells. Cells were incubated in the presence of 500 ng/ml FSH (open symbols) or 500 ng/ml FSH in combination with 0.2 mM MIX (closed symbols). At different time points, cAMP was determined in the cells (cellular; circles) and in the medium (secreted; triangles) as described previously (Themmen et al., 1986). Values given are means of duplicate determinations of total production of cAMP per well. Differences between duplicates were not larger than 5% of the mean.

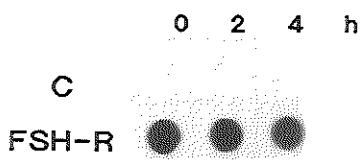

Fig. 5. Initiation of FSH receptor gene transcription. Sertoli cells were incubated for 0, 2 or 4 h with 500 ng/ml FSH. Nuclei were isolated and transcriptional run-on experiments were performed as described in Materials and Methods. C: control plasmid without cDNA insert; FSH-R: two plasmid DNAs containing the complete FSH receptor cDNA.

TABLE 1

EFFECT OF CYCLOHEXIMIDE ON DOWN-REGULATION OF FSH RECEPTOR mRNA

	Addition	
	−CX	+CX
Control	100	328 ± 45
FSH	5 ± 2	169 ± 2
dbcAMP	17 ± 7	215 ± 2

Cells were incubated for 4 h with 50 μg/ml cycloheximide in the presence of 500 ng/ml FSH or 0.5 mM dbcAMP. Subsequently, the cells were harvested and the FSH receptor mRNA was measured as described in the legend to Fig. 1. Values given are means ± SD for three experiments.

the homologous down-regulation of FSH receptor mRNA operates through a direct mechanism which does not require de novo protein synthesis.

The attenuation of FSH receptor mRNA expression in cultured Sertoli cells after incubation with FSH or dbcAMP might be the result of a rapid decrease in transcription. Transcriptional run-on experiments were performed to investigate this mechanism. No decrease in transcription initiation of the FSH receptor gene could be observed, using nuclei isolated from Sertoli cells that had been incubated for 2 or 4 h in the presence of FSH. In the same series of experiments, initiation of α-inhibin gene transcription was consistently induced by FSH (not shown). These results indicate that decrease of initiation of transcription is not the dominant mechanism of FSH receptor mRNA down-regulation.

To study whether the observed down-regulation of FSH receptor mRNA finally resulted in down-regulation at the protein level, we have also measured FSH receptor binding sites on the cultured rat Sertoli cells (Fig. 3*B*). First, the binding characteristics of the FSH receptor was confirmed. A K_d of 45 pM and a B_{max} of 40 fmol per mg protein was generated from a straight Scatchard plot; these data are in agreement with values from the literature (Fletcher and Reichert, 1984). Incubation of the cells with either FSH or FSH in combination with MIX showed a rapid and marked down-regulation of FSH receptor number at 4 h, and a further decrease in binding sites at 24 h. dbcAMP had no effect at 4 h, but had markedly reduced FSH binding after a 24 h incubation period. Thus, the early effect of FSH on the receptor number is probably not mediated by cAMP, but rather reflects a direct effect at the plasma membrane, viz. receptor internalization. Down-regulation of the FSH receptor number by dbcAMP at 24 h can be explained by down-regulation of FSH receptor mRNA expression.

The results presented above indicate that FSH receptor expression is regulated through at least two independent mechanisms: a rapid down-regulation of FSH binding, which involves sequestration of membrane receptors, and a more slow regulation at the post-transcriptional level. The latter effect of FSH is mediated by cAMP.

Exposure of cells to hormones almost invariably leads to hormone-specific desensitization and down-regulation of the hormone receptors. The responsiveness of cells to hormones can be regulated through several mechanisms. Upon activation of a GTP-binding protein, the receptor may assume a low affinity state (Cerione et al., 1984; Zhang et al., 1991), and rapid phosphorylation also has been implicated in receptor desensitization (Benovic et al., 1987; Leeb-Lundberg et al., 1987). These rapid effects are followed by sequestration of the hormone-receptor complex (Lloyd and Ascoli, 1983).

In the case of the FSH receptor, several regulatory mechanisms have been studied. Pulse-chase studies with ^{131}I-FSH in cultured mouse Sertoli cells have shown that after hormone binding FSH receptors are internalized rapidly (within 50 min), and that all ^{131}I-FSH surface binding has disappeared at 100 min (Shimizu and Kawashima, 1989). Similar results have been obtained using ^{125}I-FSH and rat or pig Sertoli cells (Fletcher and Reichert, 1984; Saez and Jaillard, 1986). Furthermore, a decrease of the activity of the second messenger cAMP through activation of phosphodiesterase has been observed (Conti et al., 1981, 1983). In the present paper, we show yet another level of regulation of FSH receptor activity, viz. the expression of FSH receptor mRNA.

FSH acts on FSH receptor mRNA expression through a post-transcriptional mechanism, rather than through regulation of transcription. Both regulatory mechanisms have been shown to occur for other G-protein coupled receptors. The half-life of β_2-adrenergic receptor mRNA in DDT$_1$-MF-2 cells, for example, was decreased from 12 h to approximately 5 h after treatment of the cells with the β_2-adrenergic agonist isoproterenol (Hadcock et al., 1989). Interestingly, it has been found that β_2-adrenergic receptor gene transcription is stimulated acutely by cAMP in response to short-term agonist exposure (Collins et al., 1989). In the present experiments, a rapid induction of FSH receptor mRNA by FSH was not clearly observed, although FSH receptor mRNA expression seemed to be slightly increased (not statistically significantly different from controls) at 1 h after FSH addition (Fig. 1). This will be studied further using a more sensitive and quantitative solution hybridization method.

Similar to our results, it has been shown that LH/CG receptor mRNA in mouse MA-10 Leydig tumour cells also is under homologous down-regulatory control. In these cells, hCG and 8-bromo-cAMP transiently up-regulated LH/CG receptor mRNA, and caused a subsequent decline in mRNA level (Wang et al., 1990, 1991). In preovulatory follicles from hCG-treated immature rats, the situation is more complex: a low concentration of cAMP appeared to induce LH/CG receptor mRNA level, whereas a high dose of the second messenger inhibited the mRNA expression (Segaloff et al., 1990).

In conclusion, we have shown that, quantitatively, the primary cause of FSH receptor down-regulation in Sertoli cells is receptor internalization and/or degradation at the plasma membrane; a later contribution results from the loss of FSH receptor mRNA. This FSH-induced decrease of FSH receptor mRNA expression is mediated by cAMP through a post-transcriptional mechanism.

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CHAPTER 5

Transcriptional regulation of androgen receptor gene expression in Sertoli cells and other cell types.

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SUMMARY

Regulation of androgen receptor (AR) mRNA expression was studied in Sertoli cells and peritubular myoid cells isolated from immature rat testis, and in the lymph node carcinoma cell line derived from a human prostate (LNCaP). Addition of dibutyryl-cyclic AMP (dbcAMP) to Sertoli cell cultures resulted in a rapid transient decrease in AR mRNA expression (5h), which was followed by a gradual increase in AR mRNA expression (24 - 72 h). This effect of dbcAMP mimicked FSH action. In peritubular myoid cells, there was only a moderate but prolonged decrease during incubation in the presence of dbcAMP, and in LNCaP cells no effect of dbcAMP on AR mRNA expression was observed.

When Sertoli cells or peritubular myoid cells were cultured in the presence of androgens, AR mRNA expression in these cell types did not change. This is in contrast to LNCaP cells, that showed a marked reduction of AR mRNA expression during androgen treatment.

In the present experiments, transcriptional regulation of AR gene expression in Sertoli cells and LNCaP cells was also examined. Freshly isolated Sertoli cell clusters were transfected with a series of luciferase reporter gene constructs, driven by the AR promoter. It was found that addition of dbcAMP to the transfected Sertoli cells resulted in a small but consistent increase in reporter gene expression (which was interpreted as resulting from AR promoter activity); a construct that only contained the AR 5' untranslated region of the cDNA sequence, did not show such a regulation. The same constructs, transfected into LNCaP cells, did not show any transcriptional down-regulation when the synthetic androgen R1881 was added to the cell cultures. A nuclear transcription elongation experiment (run-on), however, demonstrated that androgen-induced AR mRNA down-regulation in LNCaP cells resulted from an inhibition of AR gene transcription.

The present results indicate that in Sertoli cells and LNCaP cells, hormonal effects on AR gene transcription play a role in regulation of AR expression. However, AR gene transcription in these cells is differentially regulated.

INTRODUCTION

In the mammalian testis, follicle-stimulating hormone (FSH) and testosterone exert co-operative actions in the initiation and maintenance of spermatogenesis (Clermont and Harvey, 1967; Steinberger, 1971; Hansson et al., 1975; Fritz, 1978; Russell et al., 1987; Marshall and Nieschlag, 1987; Matsumoto and Bremner, 1989; Bartlett et al., 1989). In general, the quality and quantity of spermatogenesis is used as a parameter to evaluate the relative importance of FSH and testosterone. Using cultured Sertoli cells, several specific or common actions of FSH and testosterone, in particular on protein synthesis, have been observed (Cheng et al., 1986). Recently, co-operation between FSH and testosterone has been observed on the level of androgen receptor (AR) regulation. In cultured immature rat Sertoli cells, long-term (72 h) addition of FSH to the culture medium resulted in an increased expression of both AR mRNA and protein (Verhoeven and Cailleau, 1988; Blok et al., 1989; Sanborn et al., 1991: Blok et al., 1992b). Testosterone addition to the cultured cells did not markedly affect AR mRNA expression, but resulted in increased stability of AR protein (Blok et al., 1989, 1992b). The

effects of FSH and testosterone on the expression of AR protein appeared to be additive (Blok et al., 1989; 1992b).

Sertoli cells and peritubular myoid cells are an important source of AR protein in the rat testis (Takeda et al., 1989; Sar et al., 1990). To obtain more information on regulation of AR mRNA expression in these testicular cell types, Sertoli cells and peritubular myoid cells were isolated from immature rats and cultured in the presence or absence of FSH, dbcAMP or testosterone. LNCaP cells (cell line derived from a lymph node metastasis from a human prostate carcinoma; Horoszewicz et al., 1983) were included in this analysis, because androgen-induced down-regulation of AR mRNA expression is a more general phenomina, which also occurs in these cells (Quarmby et al., 1990; Tilley et al., 1990; Trapman et al 1990; Krongrad et al., 1991). Northern blotting was performed to analyze the regulation of AR mRNA expression in the different cell types.

Structural analysis of the rat and human AR promoter region (Baarends et al., 1990; Faber et al., 1991) did not reveal the presence of consensus sequences that could act as cyclic AMP response elements (CRE) through which FSH could regulate AR gene transcription. Several glucocorticoid response element (GRE) half-sites were found in the AR promoter, but it remains to be established whether these GRE half-sites are functional in the regulation of AR gene transcription. To study whether or not regulation of AR mRNA expression by FSH (Sertoli cells) and testosterone (LNCaP cells) is caused by changes in AR gene transcription, rather than through an effect on mRNA stability, reporter constructs were made that contained up to 7 kb 5' upstream sequences of the AR gene start site. These constructs were transfected into cultured immature Sertoli cells and LNCaP cells, to test regulation of their transcription. Regulation of AR gene transcription by FSH or testosterone was also studied using a nuclear transcription elongation (nuclear run-on) assay.

MATERIALS AND METHODS

Cell culture for RNA isolation and nuclear run-on

Sertoli cells were isolated from 15-day-old rats, and cultured as described by Themmen et al. (1991). Peritubular myoid cells were obtained from the same testes as the Sertoli cells, as follows: The testes were decapsulated and incubated in 20 ml phosphate-buffered saline (PBS) containing 5 μg/ml DNase (DN-25; Sigma, St. Louis, MO, U.S.A.), 1 mg/ml collagenase A (Boehringer Mannheim GmbH, Mannheim, Germany), 1 mg/ml hyaluronidase (I-S; Sigma), and 1 mg/ml trypsin (TRL, Cooper Biomedical, Freehold, NJ, U.S.A.), for 25 min at 37°C in a 100 ml Erlenmeyer flask, in a shaking waterbath (120 cycles/min). During this treatment, interstitial and peritubular myoid cells became dissociated from the seminiferous tubule fragments; these fragments were removed by sedimentation at unit gravity for 2 min in 50 ml PBS containing 5 μg/ml DNase. The supernatant was filtered through a 60 μm nylon filter, and centrifuged for 2 min at 7xg. The cells in the supernatant were precipitated by centrifugation at 30xg for 2 min. The interstitial and peritubular myoid cells were seeded at a density of approximately 3 x 10^4 cells per cm^2, in Eagle's Minimum Essential Medium (Gibco BRL, Middlesex, U.K.), supplemented with antibiotics and non-essential amino acids (MEM; Oonk et al., 1985) and containing 10% foetal calf serum (FCS). At the beginning of the culture period, the cell preparation contained approximately 50% peritubular myoid cells, as determined by alkaline phosphatase staining (Palombi et al., 1989). The cultures were grown to near confluency in 3 days. The purity at that time was higher than 95%. All experiments with

peritubular myoid cells were performed in MEM containing 0.1% bovine serum albumin (BSA; fraction V, Sigma). The cells were cultured in the presence or absence of hormones as indicated in the Results section.

LNCaP cells (passages 45 - 50) were cultured in RPMI, containing antibiotics (see above) and 7.5% FCS, to 50% confluency. To study regulation of AR mRNA expression (Northern blotting and nuclear run-on analyses), the cells were cultured in RPMI containing 7.5% Dextran-coated charcoal-treated FCS (DCC-FCS), supplemented with hormones as indicated in the Results section.

Total RNA was isolated from Sertoli-, peritubular myoid- and LNCaP cells as described by Blok et al. (1992a). The nuclear run-on assays were carried out in duplicate as described by Blok et al. (1992b). In this assay, the transcription elongation, was allowed to continue for 30 min in the isolated nuclei, in the presence of labelled mRNA precursors. The mRNAs were hybridized to the complete human AR cDNA (Trapman et al., 1988; Faber et al., 1991; Jenster et al., 1991), and autoradiographed. The films were scanned using a video densitometer (BIO-RAD, Model 620).

Isolation and transfection of Sertoli cell clusters

Tubule fragments obtained from testes from 15-day-old rats in the procedure for isolation of peritubular myoid cells (see above), were the source of Sertoli cell clusters that were used for transfection. The tubule fragments, cell clusters consisting of Sertoli cells with a few adherent germ cells (mainly spermatocytes), were allowed to attach in plastic Petri dishes (60 x 15 mm, Nunclon, Roskilde, Denmark), at a density of approximately 3×10^5 cells/cm^2, for 4h before transfection. The Sertoli cell clusters were cultured in MEM, at 37°C under an atmosphere of 5% CO_2 in air, in the presence of 5% DCC-FCS.

DNA precipitate was produced using the calcium phosphate method (Chen and Okayama, 1987). Per culture dish with 4ml medium, 250 μl precipitate containing 5 μg plasmid DNA construct was added, and kept on the cells for 15 h. Subsequently, the cells were washed with PBS (without Ca^{+2} and Mg^{+2}) before being subjected to a 1.5 min shock-treatment with 15% (v/v) glycerol in PBS (without Ca^{+2} and Mg^{+2}). This was followed by two wash steps and addition of fresh medium (MEM supplemented with 5% DCC-FCS). After a 6 h incubation period, the medium was again replaced by fresh medium supplemented with or without hormones. The incubations of the Sertoli cells, with or without hormones were continued for 24 h because Blok et al. (1992b) have reported a significant increase in AR,RNA and protein expression after 24 h of culture in the presence of FSH. In a number of experiments, the pCH110 ß-galactosidase expression plasmid (Pharmacia LKB Biotechnology, Uppsala, Sweden) was co-transfected as an internal control. Within one experiment, all incubations were terminated at the same time point (control and hormone-treated). The hormone treatment protocols are described in the Results section.

The cells were harvested in 0.5 ml extraction buffer (100 mM Tris pH 7.8, 8 mM $MgCl_2$, 1 mM dithiothreitol, 15% glycerol, 0.2% Triton X-100). For estimation of luciferase activity, a small test tube containing 40 μl of this reaction mixture was placed in a luminometer (Pico-lite 6100; Packard, Downers Grove, IL, U.S.A.), and the reaction was initiated by injection of 10 μl of 0.5 mM luciferin (Sigma), 50 mM ATP, pH 7. Light emission was measured during the first 15 seconds of the reaction (integral mode). The transfection experiments were repeated 4 - 5 times, using different cell isolates and plasmid preparations. The presented luciferase activities are from 4 different transfections in one representative experiment.

Alkaline phosphatase staining was used to evaluate contamination with peritubular myoid cells in the Sertoli cell clusters (Palombi et al., 1989). This contamination was below 3%, and the contamination with spermatocytes was approximately 10% (at the end of the incubations).

LNCaP cell transfection

LNCaP cells were cultured in MEM containing 7.5% DCC-FCS, and transfected as described for Sertoli cells). The transfected cells were stimulated for 24 h with hormones, as described in the Results section, and harvested to measure the luciferase activity. The transfection experiments were repeated 4-5 times. The luciferase activities are from 4 different transfections in one representative experiment.

Plasmids

For construction of most plasmids, the pSLA3 vector was used (van Dijk et al., 1991), that is a derivative of pSuperCAT. Several restriction fragments derived from GrAR2 (Baarends et al., 1990) were subcloned into pSLA3, using standard methods (Sambrook et al., 1989). In a number of constructs, an octamer binding protein promoter (mouse Oct-6; kindly provided by Dr D. Meijer, Rotterdam, The Netherlands) was used as a minimal promoter (Fig. 1).

The **pSLA3-E3K** promoter/reporter construct was generated as follows: A 1.5 kb Kpn1/EcoR1 fragment derived from GrAR2, containing the AR promoter and 5' untranslated region of the cDNA (-435 to +966) (Fig. 1), was inserted in the Kpn1 and EcoR1 sites of pGEM (pGEM-E3K). From this vector, a Cla1/EcoR1 fragment was isolated and ligated into the Cla1/EcoR1 sites of pSLA3. **pSLA3-H2/3-E3K** was constructed by cloning a HindIII/HindIII fragment, derived from GrAR2, into the HindIII site of a Bluescript vector (pBLUE-H2/3). A pBLUE-H2/3 Cla1/Kpn1 fragment (1.3 kb) was placed in front of the E3K fragment in pSLA3-E3K (in Cla1 and Kpn1 sites). **pSLA3-8kb** was constructed by integration of a 6.5 kb BamH1/Kpn1 fragment, derived from GrAR2, into the BamH1 and Kpn1 sites in front of the E3K fragment in pSLA3-E3K. **pSLA3-CRE-tk** was constructed by ligation of a BamH1/PvuII fragment (containing 2 CRE consensus sequences derived from the human fibronectin gene; kindly provided by Dr R. Offringa) into the BamH1/Sma1 sites of pSLA3-tk (van Dijk et al., 1991). To obtain **pSLA3-oct,** a pGEM7 polylinker region was inserted in front of the tk-promoter of the pSLA3-tk vector. A HindIII/Xho1 fragment containing the Oct-6 minimal promoter was then cloned into the HindIII/Xho1 sites of the polylinker region of the pSLA3-pl-tk vector (pSLA3-pl-oct-tk). Finally, the tk-promoter was removed from pSLA3-pl-oct-tk by using BglII/Xho1 (pSLA3-oct). **pSLA3-oct-E3K** was constructed by insertion of the Kpn1/EcoR1 fragment, derived from pSLA3-E3k, into the Sma1 site of the pSLA3-oct. **pSLA3-oct-H3.1** and **pSLA3-oct-H3.2** were constructed by cloning both HindIII fragments [H3.1 (= 411 bp, containing the AR promoter region) and H3.2 (= 610 bp, containing a large portion of the AR 5' untranslated cDNA sequence)] separately into the HindIII site of pSLA3-oct. **pSLA3-oct-GRE** was constructed by cloning a HindIII fragment from pG-29G-tkCAT (Schüle et al., 1988), into the HindIII site of the pSLA3-oct plasmid.

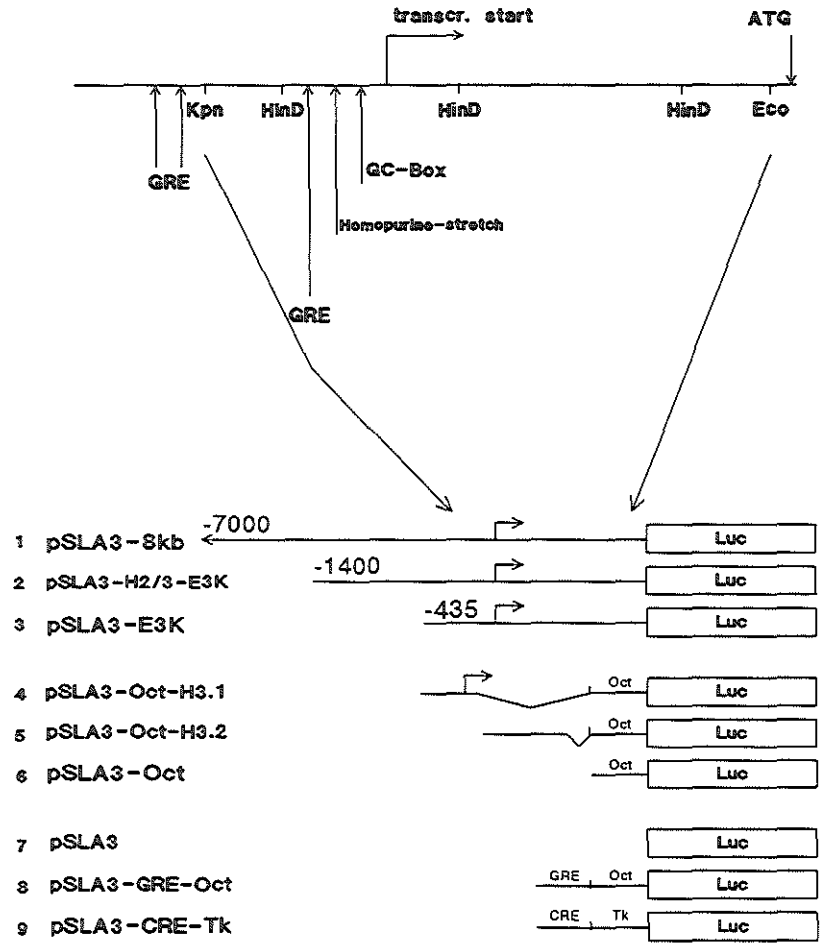

Fig. 1. AR gene promoter/reporter constructs. Constructs 1 - 4 contain regions of the AR gene promoter (Baarends et al., 1990). Construct 5 contains the 5' untranslated region of the cDNA sequence from the AR gene, cloned in front of a minimal promoter (Oct-6 promoter). Constructs 6 and 7 are controls for the basal rate of luciferase gene transcription. Construct 7 is a positive control for AR-stimulated transcription, and construct 8 is a positive control for dbcAMP-stimulated transcription.

RESULTS

AR mRNA expression

When Sertoli cells were cultured in the presence of FSH or dbcAMP for 5 h, a rapid but transient decrease in AR mRNA expression was observed (Fig. 2a). However, when FSH or

dbcAMP was present for longer time periods (72 h), AR mRNA expression became elevated to values above the control level (FSH gave a 2.4 ± 0.2 -fold induction, and dbcAMP a 2.1 ± 0.3 -fold induction; mean ± SD of 4 experiments). Addition of the synthetic androgen R1881 to the cultures did not result in changes in AR mRNA expression. Actin mRNA expression was not significantly affected by the hormone treatments (Fig. 2b).

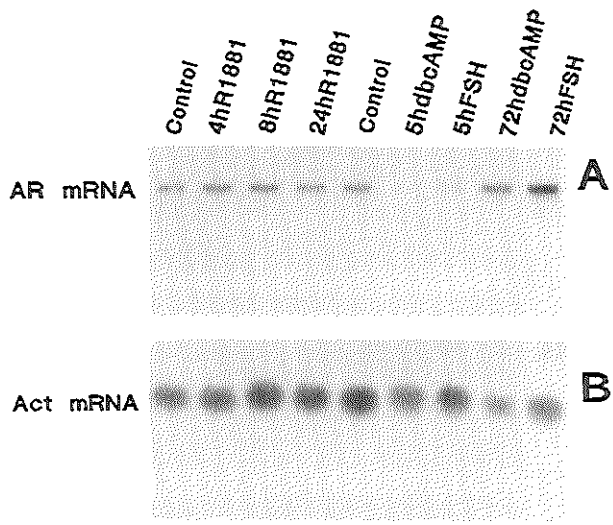

Fig. 2. Effect of FSH, dbcAMP and R1881 on AR mRNA expression in cultured Sertoli cells. Sertoli cells from 15-day-old rats were cultured in the presence of ovine FSH-S16 (500 ng/ml), dbcAMP (0.5 mM) or R1881 (10^{-8} M) for different time periods. Con = control; 4hR1881, 8hR1881, 24hR1881 = cultured for 4 h, 8 h or 24 h in the presence of R1881; 5hdbcAMP, 72hdbcAMP = cultured for 5 h, or 72 h in the presence of dbcAMP; 5hFSH, 72hFSH = cultured for 5 h or 72 h in the presence of FSH. For Northern analysis, 20 µg of total RNA was applied per lane and analyzed using a human AR cDNA probe (A); a hamster actin cDNA probe (B) was used to verify whether equal amounts of mRNA were applied to each lane on the gel.

Peritubular myoid cells showed a small decrease in AR mRNA expression, 5 h and 72 h after addition of dbcAMP (30% decrease; Fig. 3a). Such a decrease was also observed for the actin mRNA level (Fig. 3b), but not for GAPDH mRNA expression (Fig. 3c). Therefore, it was concluded that dbcAMP had a small inhibitory effect on the expression of both actin and AR mRNAs in cultured peritubular myoid cells. This effect, however, was different from the transient decrease (5h) and gradual increase (72 h) of AR mRNA expression in dbcAMP-treated Sertoli cells. Incubation of peritubular myoid cell preparations in the presence of FSH had no effect on AR mRNA expression, confirming that this cell preparation did not contain a substantial number of Sertoli cells. Addition of R1881 to the cultured peritubular myoid cells did not result in changes in the expression of AR mRNA or actin mRNA (Fig. 3a and 3b).

In LNCaP cells, no short- or long-term regulation of AR mRNA expression by dbcAMP

could be observed (Fig. 4a). Addition of R1881 to LNCaP cell cultures, however, did result in a marked reduction of the AR mRNA level (Fig. 4a). This contrasts with the absence of an effect of R1881 on AR mRNA expression in Sertoli and peritubular myoid cell cultures (Figs. 2a and 3a). Actin mRNA expression remained unaltered during the different hormonal treatments of LNCaP cells (Fig. 4b).

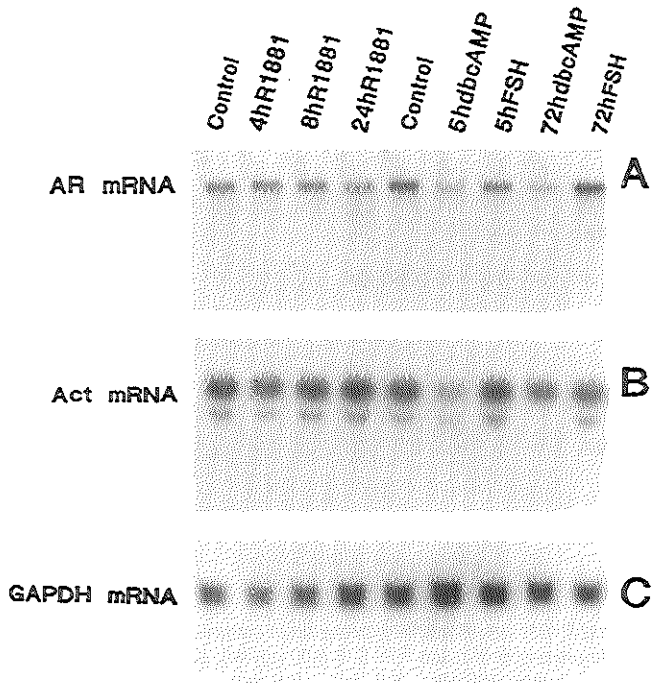

Fig. 3. Effect of FSH, dbcAMP and R1881 on AR mRNA expression in cultured peritubular myoid cells. Peritubular myoid cells from 15-day-old rats were cultured in the presence of ovine FSH-S16 (500 ng/ml), dbcAMP (0.5 mM) or R1881 (10^{-9} M) for different time periods, as described in the Legend to Fig. 2. For Northern analysis, 20 µg of total RNA was applied per lane and analyzed using a human AR cDNA probe (A); a hamster actin cDNA probe (B) and a rat GAPDH cDNA probe (C) were used to verify whether equal amounts of mRNA were applied to each lane on the gel.

Transfection of Sertoli cells

Various methods were examined, to try to transfect primary Sertoli cell cultures. Monolayers of Sertoli cells from immature rats, cultured for several days in the presence or absence of hormones, showed very little uptake and expression of various CAT reporter gene-constructs. Higher construct uptake was observed using freshly isolated Sertoli cell clusters that were transfected shortly after isolation. Furthermore, the sensitivity of the assay was increased by using luciferase as a reporter gene; luciferase-constructs give a 30- to 1000-fold higher signal

than CAT-constructs (de Wet et al., 1985, 1987; Ow et al., 1987). The transfected promoter constructs (Fig. 5; constructs 1 - 5) produced a luciferase activity of approximately 10- to 30-fold over background. Hormone induced changes in luciferase activity, were interpreted as changes in AR promoter activity (de Wet et al., 1987). In some experiments, ß-galactosidase was used as an internal control for transfection efficiency. Different plasmid preparations showed approximately 2-fold variation in transfection efficiency. However, no differences were observed in ß-galactosidase transfection efficiency between the dbcAMP, R1881 or control incubated cells.

Because the Sertoli cell clusters were isolated in the presence of trypsin and used within 4 h after isolation, there was the possibility that FSH receptors were damaged by the protease. Therefore, stimulation of transcription of the transfected promoter constructs was performed using dbcAMP rather than FSH. As a control, the mouse Sertoli cell line TM4 (Mather, 1980) was transfected and incubated with FSH.

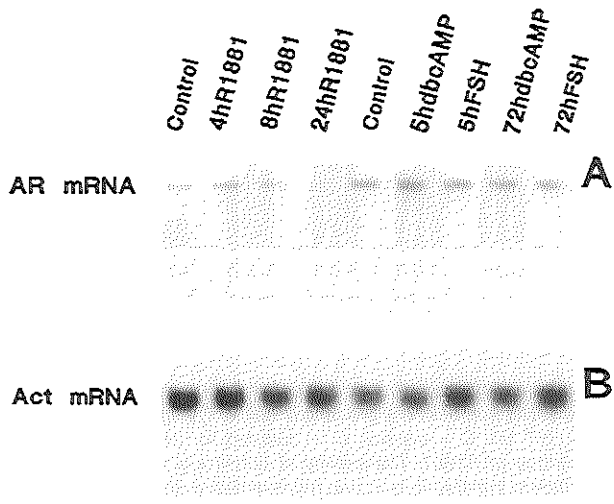

Fig. 4. Effect of FSH, dbcAMP and R1881 on AR mRNA expression in cultured LNCaP cells. LNCaP cells from passages 45 - 50 were cultured in the presence of ovine FSH-S16 (500 ng/ml), dbcAMP (0.5 mM) or R1881 (10^{-9} M) for different time periods, as described in the Legend to Fig. 2. For Northern analysis, 20 µg of total RNA was applied per lane and analyzed using a human AR cDNA probe (A); a hamster actin cDNA probe (B) was used to verify whether equal amounts of mRNA were applied to each lane on the gel.

AR gene transcription

Several constructs (Fig. 1) were used to study AR gene transcription in Sertoli- and LNCaP cells. In transfected Sertoli cells, transcription of the luciferase reporter gene from constructs containing AR promoter sequences was increased approximately 50% - 100% by incubation of the cells in the presence of dbcAMP (Fig. 5; constructs 1 - 4). This increase was not found using construct 5, that contained only 5' untranslated cDNA sequences. A positive control,

containing cAMP response elements (CREs), also showed a 1.5 to 2 -fold stimulated transcription. In primary Sertoli cell cultures, AR promoter activity was not stimulated by FSH (constructs 1 - 4; not shown), most likely due to loss of FSH receptors during the cell isolation procedure. However, when these constructs were transfected into TM4 cells, their transcription was stimulated approximately 1.5-fold by FSH (not shown).

The 1.5- to 2-fold stimulation of construct transcription by dbcAMP/FSH in the transfected Sertoli cells and TM4 cells indicates that FSH may stimulate the transcription of the AR gene in Sertoli cells, through the cAMP pathway.

Addition of R1881 to the Sertoli cell cultures had no effect on the transcription of the transfected constructs (Fig. 5).

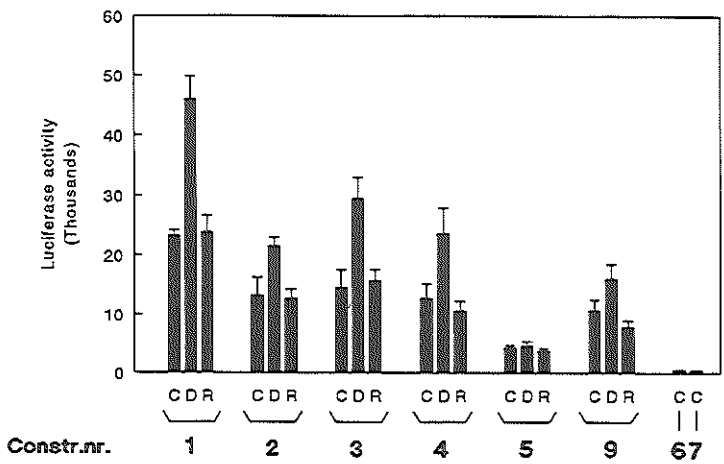

Fig. 5. Luciferase activity in whole cell lysates from transfected Sertoli cells. The Sertoli cells were transfected with different constructs (construct numbers are presented below the figure), and cultured in the presence of dbcAMP (0.5mM) or R1881 (10^{-8} M). C = control; D = cultured for 24 h in the presence of dbcAMP; R = cultured for 24 h in the presence of R1881. The activity of constructs 6 and 7 was very low. The luciferase activity was measured in 4 different transfections in one representative experiment. The bars represent the mean ± SD.

In nuclear run-on experiments, performed on isolated Sertoli cell nuclei, no marked increase in AR gene transcription rate was observed when the nuclei were isolated after 24 or 72 h of culture of the cells in the presence of FSH (Fig. 7a).

Transfection of LNCaP cells

In non-transfected LNCaP cells, cultured in the presence of R1881 for 8 or 24 h, a marked inhibition of AR mRNA expression was observed (Fig. 4a). However, LNCaP cells transfected with AR gene constructs (Fig. 1; constructs 1 - 5) and cultured in the presence of R1881, did not show an inhibition of luciferase transcription (Fig. 6; only the pSLA3-H2/3 construct is shown, but all other constructs were also negative). A positive control construct containing a glucocorticoid response element, showed a dose-dependent increase in transcription, up to 20-fold induction (Fig. 6). From these results, it cannot be concluded that androgen-

induced down-regulation of AR mRNA expression in LNCaP cells results from decreased AR gene transcription. Measurement of transfection efficiency in the different treatment groups revealed no significant differences.

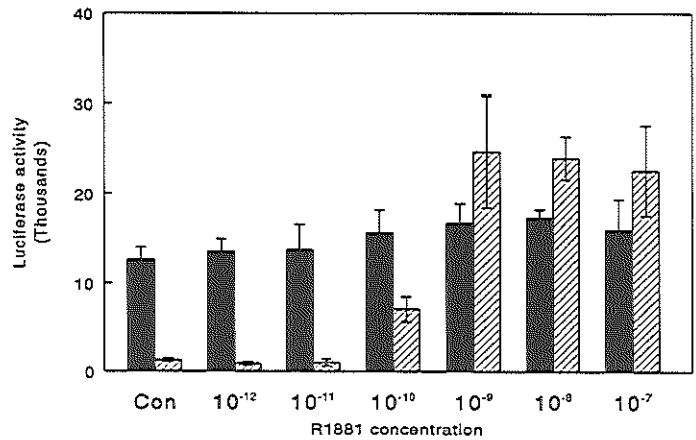

Fig. 6. Luciferase activity in whole cell lysates from transfected LNCaP cells. LNCaP cells were transfected with pSLA3-GRE-oct (a positive control; shaded bars), and with pSLA3-H2/3-E3k (construct 2, an AR promoter containing construct; closed bars). After transfection, the cells were incubated for 24 h in the presence of different concentrations of R1881 (12 = 10^{-12}M to 7 = 10^{-7} M). The luciferase activity was measured in 4 different transfections in one representative experiment. The bars represent the mean ± SD.

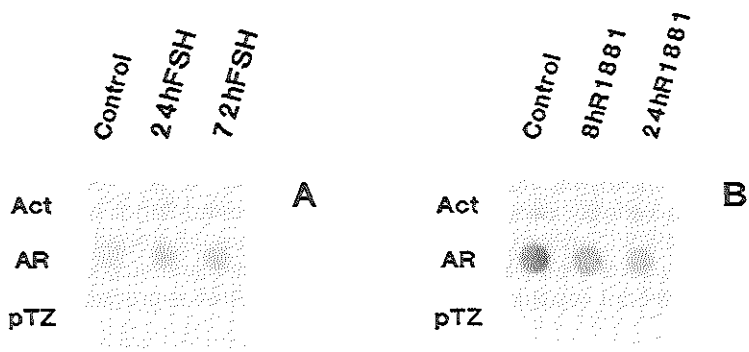

Fig. 7. Measurement of the initiation rate of AR gene transcription in cultured Sertoli cells (A) and LNCaP cells (B). Sertoli cells were cultured in the presence of ovine FSH-S16 (500 ng/ml), for 24 or 72 h. LNCaP cells were cultured in the presence of R1881 (10^{-8} M), for 8 or 24 h. Nuclei were isolated, and transcriptional run-on experiments were performed as described in Materials and Methods. Con = control; 24hFSH, 72hFSH = cultured for 24 h or 72 h in the presence of FSH; 8hR1881, 24hR1881 = cultured for 8 h or 24 h in the presence of R1881; AR = AR; Act = actin; pTZ = control plasmid. The experiment was repeated 2 times with the same results. The autoradiograph shows the results from one experiment.

To study whether or not transcription of the native androgen receptor gene, possibly containing one or several negative androgen response elements (nARE) far upstream or in one of the introns, can be regulated by androgens, a nuclear run-on experiment was performed. The results from the run-on assay indicated that AR gene transcription in LNCaP cells is subject to moderate down-regulation by androgens (Fig. 7b). Inhibition of AR gene transcription rate in LNCaP cells was quantitated by denrsitometric scanning. The measured values were 0.86/0.84 after 8 h, and 0.70/0.76 after 24 h of culture in the presence of R1881.

DISCUSSION

Differential regulation of AR mRNA expression by FSH, dbcAMP and androgens

Regulation of AR mRNA expression by FSH is a cell specific event, because Sertoli cells are the only cells in the male body that express detectable amounts of the FSH receptors (Dorrington et al., 1975). We have shown (Blok et al., 1992b) that addition of FSH to cultured immature Sertoli cells resulted in a short-term transient decrease (5 h) in AR mRNA expression, that was followed by a long-term increase (24-72 h). Dibutyryl-cyclic AMP (dbcAMP) mimicked the FSH effect, indicating that also for this effect cAMP is used as an important second messenger to transduce the FSH signal from the Sertoli cell surface to the interior of the cell. Obviously, the cAMP signal transduction pathway, activated by G protein-coupled receptors, is not Sertoli cell specific. Therefore, we have tested whether the addition of dbcAMP to other AR containing cells might exert a similar effect on AR mRNA expression.

In peritubular myoid cells from the testis, a small decrease in AR mRNA expression was observed when the cells were cultured in the presence of dbcAMP (5 h and 72 h). In LNCaP cells, there was no effect of dbcAMP at all. This contrasts with the marked regulatory effects of dbcAMP (or FSH through cAMP) on Sertoli cells.

With respect to androgen action, AR mRNA expression in LNCaP cells was reduced after the addition of androgens to the culture medium. Other authors have observed a similar down-regulatory androgen effect, in LNCaP cells (Quarmby et al., 1990; Tilley et al., 1990; Trapman et al 1990; Krongrad et al., 1991). Furthermore, androgen administration to castrated rats down-regulated AR mRNA expression in ventral prostate (Lubahn et al., 1988; Tan et al., 1988; Quarmby et al., 1990; Blok et al., 1992a), kidney, brain, coagulating gland (Quarmby et al., 1990) and epididymis (Quarmby et al., 1990; Blok et al., 1992a). Such a down-regulatory androgen effect, however, was not found in cultured Sertoli and peritubular myoid cells (Blok et al., 1989; Sanborn et al., 1991). Furthermore, androgen withdrawal from the adult rat testis, by use of the toxic compound ethane dimethane sulphonate that eliminates Leydig cells, also did not result in altered testicular AR mRNA expression (Blok et al., 1991, 1992a).

The present data underline that regulation of AR mRNA expression in rat testis by FSH, dbcAMP and androgens is different, when compared with AR mRNA expression regulation in LNCaP cells or several other rat tissues and organs.

AR gene transcription

For the short-term (5 h) down-regulation of AR mRNA expression in Sertoli cells by FSH, it has been shown that the rate of initiation of AR gene transcription was not markedly changed

(Blok et al., 1992b). The FSH/dbcAMP-induced decline in AR mRNA expression seems to be the result of a marked decrease in AR mRNA stability (Blok et al., 1992b).

For the long-term (24 - 72 h) FSH-induced stimulation of AR mRNA expression in Sertoli cells, and for the androgen-induced decrease in AR mRNA expression in LNCaP cells, AR gene transcription was studied in the present experiments.

Two different approaches were chosen to examine AR gene transcription. First, several different AR gene promoter regions were inserted in front of a reporter gene. These constructs were transfected into Sertoli and LNCaP cells, which were then incubated in the presence or absence of hormones. After 24 h, reporter activity was measured. Second, using a nuclear transcription elongation assay (run-on), the transcription initiation rate was measured in FSH-stimulated Sertoli cells (0, 24 and 72 h), and in androgen-treated LNCaP cells (0, 8, 24 h).

Stimulation of AR gene transcription by FSH or dbcAMP in Sertoli cells

Using different constructs that contained the AR gene promoter region, a stimulation of construct transcription by dbcAMP was observed in transfected Sertoli cells, that was not observed for the construct that contained only the 5' AR untranslated region of the cDNA sequence. The transcription of a positive control, a CRE-containing construct, was stimulated 50%, which is low when compared to a 5- to 7-fold stimulation of the transcription of this CRE construct when transfected into other cell types (Dean et al., 1989). In LNCaP cells we observed a 5-fold increase in transcription of the CRE construct (not shown).

The results indicate, that there is a small but reproducible increase, induced by dbcAMP, in the transcription of the AR promoter containing constructs transfected into Sertoli cells. Furthermore, the present results suggest that sequences in the promoter region of the AR gene play a role in the stimulatory effect of FSH (dbcAMP) on the transcription of this gene in Sertoli cells. Analysis of the sequence of the AR promoter region (Baarends et al., 1990), however, revealed no known CRE consensus sequences that could be responsible for the observed effects.

In the current investigations, the transcription of AR promoter containing constructs was elevated by only 50 - 100% upon dbcAMP addition to the Sertoli cell cultures. These differences were judged to small to warrant identification of the sequences that are involved in cAMP-induced regulation of AR gene transcription in Sertoli cells by means of mutation analysis.

Inhibition of AR gene transcription by androgens in LNCaP cells

The general concept of androgen action involves that androgens bind to the AR, that subsequently becomes transformed to its DNA binding form and then regulates transcription of androgen responsive genes (Schräder et al., 1981; Beato, 1990; Wahli and Martinez, 1991). LNCaP cells contain large amounts of AR protein, and these ARs become transformed to the tight nuclear binding form in the presence of R1881 (Veldscholte et al., 1990). Furthermore, a clear down-regulatory effect of R1881 addition on AR mRNA expression (8 - 24 h) was found (Krongrad et al., 1991). In the present experiments, it was investigated whether these observations can be explained by a reduced level of AR gene transcription.

To this end, the AR gene promoter constructs were transfected into LNCaP cells, and assayed for their response to androgen treatment. The positive control, a construct containing

a glucocorticoid responsive element which can also be regulated by the androgen receptor (GRE; Schüle et al., 1988) cloned in front of the Oct-6 minimal promoter, showed a marked stimulation of transcription after androgen addition to the LNCaP cells. The transcription of the AR promoter constructs, however, appeared not to be regulated by androgens. Although the constructs, contained large portions of the 5' upstream region of the AR gene promoter, these experiments do not exclude the possibility that androgen response elements are located in other parts of the gene. For example for the C3 gene, encoding a component of prostatic binding protein, an androgen response element has been described in the first intron of the gene (Claessens et al., 1990; Tan et al., 1992). The putative nARE, however, can also be located further upstream, in an area of the AR gene that has not been investigated sofar. Nuclear run-on experiments were performed to investigate whether or not the native gene is subject to transcriptional regulation.

Measurement of the transcription initiation rate of the AR gene in LNCaP cells indicated that androgen treatment resulted in a 30% reduction of transcription. Therefore, androgen-induced inhibition of AR mRNA expression in LNCaP cells may result in part or completely from decreased AR gene transcription.

In conclusion, regulation of the expression of the AR gene in Sertoli, peritubular myoid and LNCaP cells differs markedly. In Sertoli cells, FSH/dbcAMP can stimulate AR gene transcription, whereas androgens have no effect. In LNCaP cells, dbcAMP has no effect on AR mRNA expression, while androgens inhibit AR gene transcription. In peritubular myoid cells neither dbcAMP nor androgens were found to have a marked effect on AR mRNA regulation. Therefore, it appears that the mode of control of AR gene transcription is cell type dependent.

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CHAPTER 6

A putative Sertoli cell factor regulating androgen receptor mRNA expression in peritubular myoid cells

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INTRODUCTION

In mammalian species, testosterone is essential for the maintenance of spermatogenesis, while FSH is more important for the initiation of spermatogenesis. However, FSH has also a significant role to play in the maintenance of spermatogenesis, in particular in monkeys (Clermont and Harvey, 1967; Steinberger, 1971; Hansson et al., 1975; Means et al., 1976; Fritz, 1978; Tindall et al., 1984; Russell et al., 1987).

Bartlett et al. (1989) used hypophysectomized rats that were substituted with no hormone, testosterone, FSH or both hormones. Hypophysectomy alone resulted in a reduction of pachytene spermatocyte and round spermatid numbers to 20% of the normal intact control, and no elongating spermatids were found. When either FSH or testosterone was substituted to the hypophysectomized animals, the number of developing germ cells was increased, but was still markedly reduced compared to the control. Only when FSH and testosterone were administered together, almost normal spermatogenesis was observed.

In order to remove all androgens from the testis, Kerr et al. (1992) treated hypophysectomized rats with ethane dimethane sulphonate to eliminate Leydig cells (Morris, 1985). In these rats, treatment with FSH together with testosterone could not restore complete spermatogenesis. Co-operation between FSH and testosterone in the maintenance of spermatogenesis, however, was still evident.

Co-operation between FSH and testosterone has also been observed on the level of androgen receptor (AR) regulation. In Sertoli cells isolated from immature rats, the rate of transcription of the AR gene was elevated approximately 75% when the cells were cultured in the presence of dbcAMP for 24 h (Blok et al., 1992c). As a result of this, AR mRNA (Blok et al., 1989; Sanborn et al., 1991; Blok et al., 1992b and c) and AR protein (Verhoeven and Cailleau, 1988; Blok et al., 1989; 1992b) levels became elevated. A detailed characterization of AR regulation by FSH (Blok et al., 1992b) revealed that, besides a long-term (24-72h) increase in AR gene transcription, a short-term (5 h) decrease in AR mRNA stability could be observed upon addition of the hormone to cultured immature Sertoli cells. This short-term decrease in AR mRNA expression was also observed when immature rats were injected with FSH (i.p., 1 μg/g bodyweight) and AR mRNA levels were measured in RNA extracted from total testis.

Using monoclonal antibodies, the testicular AR was detected in Sertoli cells, Leydig cells, and peritubular myoid cells (Takeda et al., 1989; Sar et al., 1990; Hardy et al., 1991; Ruizeveld de Winter et al., 1991). In the adult rat testis, Sertoli cells and peritubular myoid cells are the main source of AR protein (Takeda et al., 1989: Sar et al., 1990; Blok et al., 1992a), while in the immature rat testis Leydig cells also contain substantial amounts of ARs (Hardy et al., 1991). AR mRNA has been observed in cultured Sertoli cells (Blok et al., 1989, 1992b and c; Sanborn et al., 1991), peritubular myoid cells (Sanborn et al., 1991; Blok et al., 1992c) and Leydig cells (Namiki et al., 1991). Using specific ligand binding, Grootegoed et al. (1977) and Anthony et al. (1989) showed that spermatocytes and spermatids did not contain ARs.

In view of the aforementioned FSH-induced short-term down-regulation of ARmRNA in total testis, we postulated that this down-regulation also might involve AR containing peritubular myoid cells. Because Sertoli cells are the only testicular cells expressing FSH receptors (Dorrington et al., 1975; Dorrington and Armstrong, 1979; Bortolussi et al., 1990; Heckert and Griswold, 1991), this would imply the action of a paracrine factor, produced by Sertoli cells and acting on peritubular myoid cells. In the present investigation, we have used cultured immature Sertoli cells and peritubular myoid cells to investigate this putative, paracrine interaction that might affect AR mRNA expression in peritubular myoid cells.

MATERIALS AND METHODS

Sertoli cells were isolated from 21-day-old rats, and cultured as described by Themmen et al. (1991). Peritubular myoid cells were obtained from the same testes as the Sertoli cells, as described by Blok et al. (1992c). Both cell types were cultured in Eagle's Minimum Essential Medium (Gibco BRL, Middlesex, U.K.), supplemented with antibiotics and non-essential amino acids (MEM; Oonk et al., 1985) containing 1% (Sertoli cells) or 10% (peritubular myoid cells) foetal calf serum (FCS). The medium was replaced by MEM containing 0.1% bovine serum albumin (BSA; fraction V, Sigma) 24 h before the start of the incubations, and all incubations (5 h) were performed in MEM containing 0.1% BSA, as indicated in Results and Discussion.

Total RNA was isolated from Sertoli and peritubular myoid cells as described by Blok et al. (1992a). The mRNAs were hybridized to 500 bp of the complete human AR cDNA (Trapman et al., 1988).

RESULTS AND DISCUSSION

AR mRNA is present in most tissues and cell types in the male body. Only the spleen (Blok et al., 1992a) and immature liver (Song et al., 1991) have been reported to be devoid of ARmRNA. In Fig. 1, several tissues and cells from immature rats, and tumour cell lines have been analyzed for AR mRNA expression. It was observed that in addition to spleen and liver, also spermatids (and spermatocytes, not shown) did not express detectable amounts of AR mRNA (Fig. 1). This is in agreement with experiments using specific ligand binding to germ cells (Grootegoed et al., 1977; Anthony et al., 1989). In adult testes a low level of AR mRNA was found when compared to the AR mRNA level in testes from 21-day-old rats. This may partly be caused by the presence of large amounts of non-AR containing spermatids in the mature testis (a dilution effect).

Freshly isolated interstitial cells (30% Leydig cells), MA-10 cells (a mouse Leydig tumor cell line; Ascoli, 1981) and H-540 tumour tissue (Cooke et al., 1984) contained a 10 kb AR mRNA band (Fig. 1). These findings are in concordance with other observations showing immunodetection of AR protein in Leydig cells (Takane et al., 1989; Sar et al., 1990; Hardy et al., 1991) and the presence of AR mRNA in purified cultured human Leydig cells (Namiki et al., 1991). Cultured peritubular myoid cells contained a relatively large amount of AR mRNA, compared to the other somatic cell types in the testis. In situ hybridization has thusfar not been performed on the immature testis, but using antibodies peritubular myoid cells were shown to contain a considerable amount of AR protein. Therefore, it is very likely that peritubular myoid cells in situ contain a substantial amount of AR mRNA. Furthermore, Sertoli cells (either freshly isolated or cultured for 3 days), and TM4 cells (a mouse Sertoli cell line; Mather, 1980) also contained AR mRNA (Fig. 1).

The present data indicate that in the immature rat testis, Sertoli cells, Leydig cells and peritubular myoid cells contain androgen receptor mRNA.

In the Introduction, a factor was postulated, produced by Sertoli cells and acting on peritubular myoid cells. In order to investigate the action of this putative paracrine factor, peritubular myoid cells were cultured in the presence of Sertoli cell conditioned medium (SCCM) for 5 h (medium taken from Sertoli cells after 72 h of culture). A marked SCCM-induced reduction in AR mRNA expression in these peritubular myoid cells was observed (Fig. 2b). These findings indicate that the cultured Sertoli cells secrete one or more factors

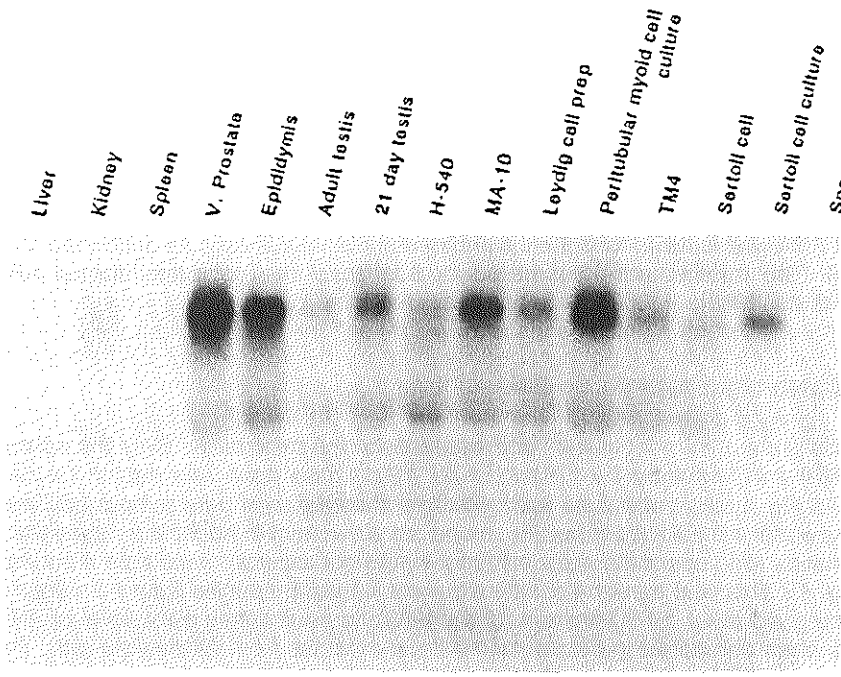

Fig. 1. AR mRNA expression in different cells, tissues and tumour cell lines. Liver, kidney, spleen, ventral prostate, epididymis, Leydig cells, peritubular myoid cells and Sertoli cells were all isolated from 21-day-old rats. Spermatids were isolated from 42-day-old rats. Leydig cells were freshly isolated; Sertoli cells were either freshly isolated or maintained in culture for 3 days; peritubular myoid cells were cultured for 3 days. H-540 cells were cultured according to Cooke et al. (1979), MA-10 cells according to Ascoli (1981), and TM4 cells according to Mather (1980). Equal amounts of total RNA (20 µg) were applied to each lane of the gel and hybridized to a probe, containing 500 bp of the human androgen receptor cDNA (Trapman et al., 1988).

that have an effect on AR mRNA expression in peritubular myoid cells.

Recently, Schlatt et al. (1992) showed a marked FSH-induced increase in actin immunostaining in the testicular peritubular myoid cells of FSH and testosterone treated intact juvenile male rhesus monkeys. From these experiments, Schlatt et al. (1992) also postulated paracrine communication between Sertoli cells and peritubular myoid cells. In order to obtain information on the nature of the paracrine factor(s) that might be involved in the interaction between Sertoli cells and peritubular myoid cells, a number of different growth factors (EGF, TGFß, insulin, and IGF-I) were added to the peritubular myoid cells. Only the addition of EGF (5h) resulted in a marked reduction in AR mRNA expression, comparable to the reduction observed when Sertoli cell conditioned medium was added (Fig. 3a). Furthermore, the addition of foetal calf serum (FCS) to the peritubular myoid cells also inhibited the AR mRNA expression level (Fig. 3b).

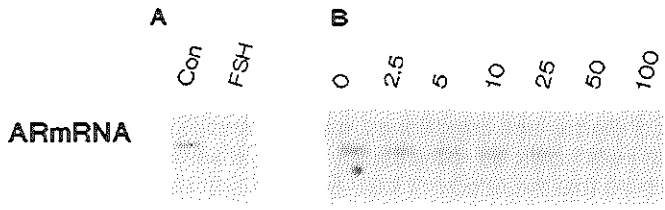

Fig. 2. (A) Effect of FSH on AR mRNA expression in immature testis. Immature rats were injected with vehicle (Con) or FSH (i.p., 1 μg/g body weight) and killed 6 h later; (B) Effect of Sertoli cell conditioned medium on androgen receptor mRNA expression in peritubular myoid cells. Peritubular myoid cells were cultured in the presence of 100, 50, 25, 10, 5, 2.5 or 0% Sertoli cell conditioned medium for 5 h. Equal amounts of total RNA (20 μg) were applied to each lane of the gel and hybridized to a probe containing 500 bp of the human androgen receptor cDNA (Trapman et al., 1988).

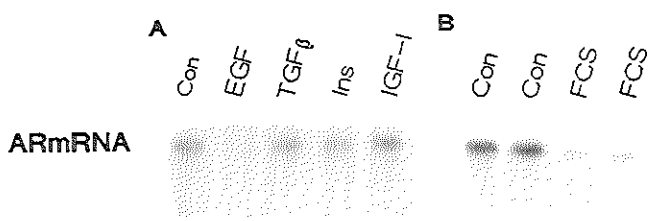

Fig. 3. Effect of growth factors (A) and fetal calf serum (B) on AR mRNA expression in peritubular myoid cells. (A) Peritubular myoid cells were cultured in the presence of EGF, TGFß, insulin, and IGF-I for 5 h, (B) Peritubular myoid cells were cultured in the presence (FCS) of absence (Con) of 10% fetal calf serum. Equal amounts of total RNA (20 μg) were applied to each lane of the gel and hybridized to a probe containing 500 bp of the human androgen receptor cDNA (Trapman et al., 1988).

EGF effects on the testis are of interest because EGF has been reported to play a significant role in spermatogenesis. In mice, it was reported that sialoadenectomy (removal of the submandibular glands, which are important sources of EGF) resulted in impaired spermatogenesis (Tsutsumi et al., 1986). When EGF was substituted in physiological concentrations to the sialoadenectomized mice, spermatogenesis returned to normal. This result, however, could only partly be reproduced by Russell et al. (1990), who found only small effects of sialoadenectomy. Currently, an important role is postulated for locally produced EGF and EGF-like substances. Holmes et al. (1986) reported the production of EGF-like substances by Sertoli cells, and Skinner et al. (1989) found that TGFα was one of the EGF-like Sertoli cell products. TGFα binds to the EGF receptor and is reported to have a number of similar biological activities as EGF (Derynck, 1986). EGF receptors have been

reported to be present on all somatic cells in the testis (Skinner et al., 1989; Suarez-Quian et al., 1989; Suarez-Quian and Niklinski, 1990; Stubbs et al., 1990; Foresta et al., 1991). Therefore, it is possible that EGF, secreted by the Sertoli cells upon FSH injection, is responsible for the reduction of ARmRNA in other testicular cells. However, there is considerable redundancy of the biological activities of various growth factors, indicating that other growth factors may be involved.

The effect of Sertoli cell conditioned medium and EGF on AR mRNA expression in peritubular myoid cells could be mimicked by fetal calf serum (FCS; Fig. 3b). Skinner et al. (1989) also observed that the effects of EGF and TGFα on peritubular myoid cell proliferation were mimicked by fetal calf serum (FCS). Isolated and cultured peritubular myoid cells show active proliferation in the presence of serum. Therefore, decreased AR mRNA expression might be correlated with cell proliferation. Future experiments will be performed to investigate whether the putative paracrine factor secreted by Sertoli cells, may stimulate peritubular myoid cell proliferation.

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CHAPTER 7

Effect of testosterone deprivation on expression of the androgen receptor in rat prostate, epididymis and testis

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Effect of testosterone deprivation on expression of the androgen receptor in rat prostate, epididymis and testis

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Summary

Adult rats were treated with ethane dimethane sulphonate (EDS) to eliminate the Leydig cells. This treatment resulted in very low levels of testosterone in the blood and in the testis. Furthermore, histological evaluation of spermatogenesis showed no marked differences between control and EDS-treated animals.

In the ventral prostate, 5 days after EDS-treatment, a 4.0 ± 0.3-fold up-regulation of androgen receptor (AR) mRNA was observed, together with a 2.2 ± 0.2-fold increase in actin mRNA. In the epididymis, a 2.0 ± 0.5-fold increase in AR mRNA level was observed, without a change in actin mRNA level. In the testes of EDS-treated rats, the AR mRNA level was not changed (1.02 ± 0.17-fold of controls), and there was also no change in actin mRNA level at 5 days after EDS-treatment. These results indicate that AR mRNA expression in the ventral prostate and epididymis is regulated differentially by testosterone when compared to regulation in the testis.

Testicular androgen binding sites were assayed by Scatchard analysis of the binding of ^{3}H-R1881 to a nuclear fraction, that was isolated by a method which involved the use of liquid nitrogen and high sucrose buffer. The number of specific binding sites per testis in EDS-treated rats with testosterone-implants, remained unaltered compared to control rats (9.1 ± 1.4 pmol/testis). In these rats, 20% of the normal testicular testosterone level was sufficient to maintain the androgen receptor in a tight nuclear binding (transformed) form. In testes from EDS-treated rats without testosterone-implants, the AR did not fractionate into the nuclear fraction; however, the total testicular AR content in these animals was close to control levels, as measured by nuclear ^{3}H-R1881 binding after receptor transformation through injection of a high dose of testosterone (10 mg) 2 h before killing the rats (testosterone pulse). In the different experimental groups, FSH was not required to maintain the total testicular AR content (ligand binding).

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Immunoprecipitation and Western blotting of the testicular AR using specific monoclonal and polyclonal antibodies indicated that the total testicular amount of immunodetectable AR protein in long-term testosterone deprived rats was very low when compared to that in control rats or rats with testosterone-implants. This is in disagreement with results obtained in the ligand binding assay, and may point to a structural modification of the AR in the testis that possibly occurs in the prolonged absence of androgens.

Keywords: androgen receptor, epididymis, follicle-stimulating hormone (FSH), prostate, testis, testosterone.

Introduction

During fetal and postnatal life, androgens play a crucial role in male development. In androgen-dependent tissues, testosterone and dihydrotestosterone interact with the androgen receptor and regulate a number of different processes. It is generally thought that, upon androgen binding, the receptor is transformed to a tight nuclear binding form, that regulates the transcription of a number of genes (Mainwaring, 1977; Schrader *et al.*, 1981; Brinkmann *et al.*, 1983). With respect to the expression of the androgen receptor gene itself, it is known that in the ventral prostate, a well studied androgen-dependent tissue, androgen receptor mRNA and protein levels are both regulated by androgens. The number of androgen receptors is decreased in the absence of androgens (measured by ^{3}H-ligand binding), whereas under the same experimental conditions the amount of androgen receptor mRNA is increased (Fiorelli *et al.*, 1989; Quarmby *et al.*, 1990). This has also been observed for LNCaP cells (lymph-node carcinoma derived from the prostate) (Krongrad *et al.*, 1991).

In the testis, testosterone plays a role in the initiation and maintenance of spermatogenesis (Clermont & Harvey, 1967; Steinberger, 1971). In addition, it is clear that FSH is involved in the initiation of germ cell development in the immature testis (Steinberger, 1971; Fritz, 1978; Russell *et al.*, 1987), and has some role to play in the maintenance of spermatogenesis in adult rats, non-human primates and the human male (Matsumoto & Bremner, 1987; Marshall & Nieschlag, 1987; Bartlett *et al.*, 1989).

Regulation of the expression of the androgen receptor in the testis *in vivo* is not documented extensively. Most of the data derives from studies of isolated testicular cell types. Buzek & Sanborn (1988) have described an increase in androgen receptor protein (ligand binding), during postnatal testicular development, in Sertoli cells and Leydig cells but not in peritubular myoid cells, deduced from measurements using isolated and cultured cells. In cultured Sertoli cells from immature rats, Verhoeven & Cailleau (1988) and Blok *et al.* (1989) have shown an additive stimulatory effect of testosterone and FSH on androgen receptor protein (^{3}H-mibolerone or ^{3}H-R1881 binding). Androgen receptor mRNA expression in these cells, however, was stimulated only by FSH but not by testosterone (Blok *et al.*, 1989).

In the present study, EDS was used to deprive adult rats of Leydig cells and thereby of testosterone, as described by Bartlett *et al.* (1986). EDS treatment was

followed by administration of either no hormone, or testosterone and/or FSH, for 5 days, to study regulation of androgen receptor expression in the testis, when compared to that in the ventral prostate and epididymis. Data on androgen receptor mRNA expression were obtained using Northern and dot-blot analysis. Expression and transformation of testicular androgen receptor protein was studied by ^{3}H-R1881 binding, and by immunoprecipitation and Western blotting.

Materials and methods

Animals

All animals were housed, 2–4 per cage, in a controlled environment at 25°C with a light cycle of 12L:12D, and received rat diet *ad libitum*.

Experimental design

Ten groups of animals were included in this study. The intact control group ($n = 5$, Group 1) was treated with vehicle alone, and one group of intact animals ($n = 5$, Group 2) received 10 mg testosterone, as a suspension in saline (i.p.) 2 h prior to killing (testosterone-pulse). The other groups (five rats in each group) (Groups 3–10) were all treated with 75 mg kg^{-1} EDS (i.p.) in DMSO/H_2O (1:3, v/v). These animals were subjected to one of the following treatments:

(a) injection with saline,
(b) testosterone-implant; a total of 15 cm (3 × 5 cm) silastic implants filled with testosterone (i.d. = 3.35 mm, o.d. = 4.65 mm; Dow Corning International Ltd., Düsseldorf, F.R.G.),
(c) injection with FSH; twice daily injection of a purified preparation of human FSH (5 IU per injection, i.e. 10 IU per day; Fertinorm, Serono, Freiburg, F.R.G.). The purity of the FSH preparation has been assessed previously (Bartlett *et al.*, 1989).
(d) testosterone-pulse; single injection of 10 mg testosterone (i.p.) 2 h prior to killing.

The following groups were formed, receiving one or a combination of these treatments:

Group 1: Control;
Group 2: Control + testosterone-pulse;
Group 3: EDS;
Group 4: EDS + testosterone-pulse;
Group 5: EDS + testosterone-implant;
Group 6: EDS + testosterone-implant + testosterone-pulse;
Group 7: EDS + testosterone-implant + FSH;
Group 8: EDS + testosterone-implant + FSH + testosterone-pulse;
Group 9: EDS + FSH;
Group 10: EDS + FSH + testosterone-pulse.

The experimental groups 1–10 were maintained under these treatment protocols for 5 days, after which all animals were killed by decapitation in a rando-

mized design. From all animals, serum and testes were obtained for hormonal analysis, weighing, histological evaluation and determination of androgen receptor mRNA and protein. Ventral prostates and epididymides were weighed and used for androgen receptor mRNA analysis. The tissues were flash-frozen in liquid nitrogen within 2–3 min of killing the rats. Data on androgen receptor mRNA and protein (^{3}H-R1881 binding and Western blotting) were obtained from pooled testes. Three nuclear fractions, used for Scatchard analysis of ^{3}H-R1881 binding were prepared for each experimental group. In addition, ^{3}H-R1881 binding data and immunoprecipitation data of Groups 1 and 3 were obtained for five individual animals.

Hormone assays

Serum and testicular testosterone were measured by luminescence-immunoassay (LIA) following ether extraction as described previously (Bartlett *et al.*, 1989). The mean intra-assay variations were 5.8 and 4.9% and the inter-assay variations were 4.8 and 7.9% in the ranges 10–12 and 20–25 nmol l^{-1} respectively. Serum LH and FSH were measured by radioimmunoassay using reagents kindly supplied by the NIADDK (Bethesda MD, U.S.A.). The standard preparations used were FSH-RP-2 and LH-RP-1; tracers were prepared from LH-I-6 and FSH-I-6, and the antisera were anti-rLH-S-9 and anti-rFSH-S-11. The limit of detection in all assays was 1.6 ng ml^{-1}. For each hormone, all samples were analysed in a single assay, and the intra-assay variation was 6.5% for LH and 9.2% for FSH.

RNA extraction and hybridization

Total RNA was extracted from frozen ventral prostate, epididymis or testicular tissue in a solution containing 3 M LiCl and 6 M ureum, a method which allows for extraction of large mRNAs (Auffray & Rougeon, 1980), followed by extractions with phenol and chloroform. Total RNA was precipitated in ethanol containing 0.16 M sodium acetate, and stored in 70% ethanol at −20°C. RNA was separated by electrophoresis (3 h, 40 mA) in a denaturing agarose gel (0.7% agarose; Sigma, St. Louis, MO, USA) containing ethidium bromide (0.1 mg 150 ml^{-1} gel). The RNA was blotted (overnight) onto a Biotrans nylon membrane (ICN, Irvin, CA, U.S.A.) (Davis *et al.*, 1986). The blots were fixed and hybridized (48 h, 42°C), in a hydridization solution containing 45% v/v formamide (J. T. Baker B.V., Deventer, The Netherlands), 0.5% w/v sodium dodecyl sulfate, 10% Denhardts solution (0.1% w/v Ficoll, 0.1% w/v polyvinylpyrrolidone and 0.1% w/v bovine serum albumin), 10 mM phosphate buffer, 15% Dextran sulphate (Pharmacia LKB Biotechnology, Uppsala, Sweden) and 100 mg l^{-1} Salmon sperm DNA (Sigma). Androgen receptor mRNA was hybridized to a 0.5 kb (EcoR1-EcoR1) human androgen receptor cDNA probe (Trapman *et al.*, 1988). This probe corresponds to a part of the steroid binding domain and the 3′ untranslated region of the human androgen receptor mRNA. Because a heterologous probe was used, the blots were washed at low stringency; 1 × SCC (150 mM NaCl and 15 mM Na-citrate) with 0.25% sodium dodecyl sulphate for 40 min at 42°C before autoradiography (Hyper-film-MP, Amersham International plc, Little Chalfont, Buckinghamshire, U.K.). The films were scanned using a video densitometer (BIO-RAD, model 620). The results represent the mean ± SD of three different blots.

Isolation of nuclei

Testes were pulverized under liquid nitrogen (Barberis *et al.*, 1989). The resulting powder was suspended on ice in 5.5 ml homogenization buffer A (0.32 M sucrose, 10 mM Hepes, 15 mM KCl, 0.15 mM spermine, 0.5 mM spermidine (Sigma), 0.1% (v/v) Triton X-100 and 4 mM EDTA, pH 7.6). Proteolytic breakdown was inhibited by addition of different protease inhibitors; 0.6 mM bacitracin (Janssen Chimica, Beerse, Belgium), 0.6 mM PMSF and 0.25 mM leupeptin (Sigma). Triton X-100 (0.1% v/v) was added to facilitate cell lysis, and milk protein (1% w/v) (Elk, DMV Campina BV., Eindhoven, The Netherlands) was added to decrease proteolytic breakdown of nuclear proteins. The nuclear suspension was kept on ice for 5 min, homogenized (30 strokes) in a dounce tissue grinder (Wheaton Scientific, Millville, N.J., USA) and mixed with 11 ml homogenization buffer B (buffer A, but containing 2 M sucrose and 10% v/v glycerol). The mixture, with a final sucrose concentration of 1.44 M, was applied to a 4 ml cushion of homogenization buffer B and centrifuged using a Beckman SW 40 Ti rotor (Beckman, Geneva, Switzerland) (48 000 *g*, 45 min, 0°C). The nuclear pellet was used for estimation of nuclear androgen binding (Gorski *et al.*, 1986).

Estimation of nuclear ^{3}H-R1881 binding

Nuclear proteins were extracted from the isolated nuclei using a high-salt buffer (0.5 M NaCl in 40 mM Tris buffer, containing 10% glycerol and 1 mM EDTA, pH 8.5) in the presence of protease inhibitors as described above, and kept on ice for 1 h. Subsequently, the nuclear lysate was centrifuged (100 000 *g*, 30 min, 0°C) to pellet DNA and non-extractable material, and the supernatant was treated with Dextran-coated charcoal to remove steroids. The charcoal-treated supernatant was then pre-incubated for 30 min at 6°C with a 500-fold excess of triamcinolone acetonide (Sigma), to block binding of androgens to progesterone receptors (Zava *et al.*, 1979). Subsequently, 50 µl portions of the supernatant were incubated with different concentrations of ^{3}H-R1881 (0.25, 0.5, 1.0, 2.0, 4.0, 8.0, 16.0 and 32.0 nM; specific activity: 81.8 Ci mmol^{-1}) (NEN research products, Stevenage, Hertfordshire, U.K.), with or without a 100-fold excess of non-radioactive R1881 (NEN research products) to estimate specific ligand binding. Samples were incubated for 72 h at 6°C to allow exchange between bound and free ligand; this incubation was terminated by precipitation of proteins with protamine sulphate (Organon B.V., Oss, The Netherlands) (4.5% v/v in 40 mM Tris buffer containing 10% v/v glycerol, 1 mM EDTA and 10 mM dithiothreitol, pH 8.1). The precipitate was washed four times with 40 mM Tris buffer and dissolved in 0.5 ml Soluene (Packard Instrument Company Inc., Downers Grove, IL, U.S.A.). The samples were counted after addition of 10 ml scintillation fluid (Ultima Gold, Packard, Groningen, The Netherlands). B_{max} and K_d of androgen binding were estimated from Scatchard plots.

Androgen receptor detection using antibodies

Testes from Groups 1 and 3 were pulverized under liquid nitrogen (Barberis *et al.*, 1989). The resulting powder was suspended in 1 ml high salt buffer (0.5 M NaCl, 40 mM Tris, 10% glycerol, 1 mM EDTA, pH 8.5) in the presence of different inhibitors of proteolytic breakdown (0.6 mM bacitracin and 0.6 mM PMSF). Immunopre-

cipitation and Western blotting was performed using the same methods and antibodies as described by Van Laar *et al.* (1991).

Testicular histology
Testes were fixed in Bouins solution, dehydrated, embedded in Paraplast (Lancer Ltd., Oxford, U.K.) and stained with periodic acid-Schiff's reagent and haematoxylin.

Statistics
Data were analysed using one-way and multi-factor analysis of variance and Tukey's test to determine significant differences.

Results

Ventral prostate, epididymis and testis weights
EDS ± hormone treatment did not result, within 5 days, in any change in bodyweight (not shown). Ventral prostate weight was increased or decreased, according to changes in circulating testosterone (Tables 1 and 2). Paired epididymal weight showed no significant change between the groups (Table 1). Paired testes weight was decreased slightly in EDS-treated rats not receiving testosterone (Table 1).

Table 1. Weight of the ventral prostate, epididymis and testis in animals treated with EDS and substituted with testosterone (T) and/or FSH

		Organ weight (mg)		
Group No.	Treatment	Paired testes	Ventral prostate	Paired epididymides
(1, 2)	Control	3103 ± 155	501 ± 121	1280 ± 86
(3, 4)	EDS	2854 ± 104*	295 ± 68*	1186 ± 273
(5, 6)	EDS + T	3073 ± 104	664 ± 65*	1191 ± 153
(7, 6)	EDS + T + FSH	3212 ± 324	732 ± 158*	1288 ± 199
(9, 10)	EDS + FSH	2856 ± 177*	290 ± 60*	1168 ± 318

EDS = killed 5 days after EDS treatment; T = substitution with testosterone for 5 days (15 cm silastic implants); FSH = substitution with FSH for 5 days (2 × 5 IU injected per day). Results represent mean ± SD of 10 rats (the data of the groups treated or not treated with a T-pulse were combined).

*$P<0.01$, compared with Control group.

Histological evaluation of spermatogenesis showed no marked differences between control animals (Groups 1 and 2) and EDS-treated animals (Groups 3–10) (not shown).

Hormone levels in the serum and testis
In Group 3 animals, 5 days after EDS-treatment, the serum level of testosterone was undetectable (<0.3 nmol l^{-1}). When EDS-treated animals were substituted with testosterone using silastic implants, the serum testosterone concentration was approximately 6-fold higher than the control level (Group 5). The testosterone-pulse treatment (10 mg, i.p. injection), given 2 h prior to killing the animals,

resulted in a serum testosterone concentration which was up to 50-fold higher than the control level (Table 2).

Table 2. ^{3}H-R1881 binding data and post-mortem hormonal parameters from animals treated with EDS and substituted with testosterone and/or FSH

Group number and treatment	FSH (ng ml^{-1})	Serum T (nmol l^{-1})	Testicular testosterone (nmol l^{-1})	Nuclear AR (%)
1. Control	8.1 ± 1.8	7.4 ± 6.0	165.0 ± 114.4	100 ± 16
2. Control + T-pulse	6.6 ± 7.2	470.0 ± 110.0	400.3 ± 95.7	104 ± 21
3. EDS	13.3 ± 3.7*	<0.3*	7.6 ± 8.7*	<5***
4. EDS + T-pulse	10.1 ± 1.7*	555.0 ± 87.0	258.0 ± 95.0**	97 ± 17
5. EDS + T	2.3 ± 0.6*	45.3 ± 8.1*	36.1 ± 3.5*	81 ± 11
6. EDS + T + T-pulse	2.6 ± 0.9*	409.0 ± 163.0	263.5 ± 43.7**	113 ± 24
7. EDS + T + FSH	8.7 ± 0.9	38.4 ± 8.1*	38.5 ± 4.5*	132 ± 35
8. EDS + T + FSH + T-pulse	7.5 ± 1.0	510.0 ± 160.0	344.3 ± 64.8**	139 ± 13
9. EDS + FSH	19.3 ± 2.7*	0.4 ± 0.4*	2.8 ± 4.5*	<5
10. EDS + FSH + T-pulse	17.2 ± 2.4*	449.0 ± 77.2	308.6 ± 60.0**	78 ± 9

EDS = killed 5 days after EDS treatment; T = substitution with testosterone for 5 days (15 cm silastic implants); FSH = substitution with FSH for 5 days (2 × 5 IU injected per day). T-pulse = testosterone injection (10 mg) 2 h prior to killing. The FSH and T values represent mean ± SD of five rats, with * = significantly different from Control group ($P<0.01$), and ** = significantly different from Control + T-pulse group ($P<0.01$).

The nuclear ^{3}H-R1881 binding (Nuclear AR) was estimated in triplicate using three different preparations of testicular nuclei obtained from pooled left testes of five rats (mean ± SD); however, the androgen receptor data of Groups 1 and 3 represent mean ± SD of five different animals, with *** = significantly different from Control group ($P<0.02$).

The testicular testosterone concentration, estimated 5 days after EDS-treatment (Groups 3 and 9), was very low. Testosterone substitution by implant raised the testicular level of testosterone to approximately 20% of that in controls (Groups 5 and 7). Testosterone-pulse treatment (2 h), caused a net increase in the testicular testosterone concentration of approximately 200–300 nmol l^{-1}, both in the absence or presence of a testosterone-implant (Table 2).

Serum levels of FSH were increased in Group 3 (Table 2), presumably as a result of the absence of negative feedback of testosterone on the pituitary gland. In EDS-treated animals substituted with testosterone for 5 days (Groups 5 and 6), the serum FSH concentration was decreased to 30% of the control value. When testosterone substituted animals also received FSH injections, the circulating FSH concentration, measured 12 h after the last injection of FSH, was comparable to the control value (Table 2). There were no effects of FSH treatment on testicular or blood levels of testosterone.

Androgen receptor mRNA

Expression of androgen receptor mRNA was estimated in ventral prostate, epididymis and testis. These tissues contained a 10 kb androgen receptor mRNA, and in addition a minor 4–5 kb band was observed on Northern blots (Fig. 1a). The 10 kb band represents the intact androgen receptor mRNA, with a length that is in

agreement with the length of the cloned cDNAs and the genomic organization of the androgen receptor (Chang *et al.*, 1988; Baarends *et al.*, 1990). A 4–5 kb band has also been observed by other authors, and was thought to represent a specific hybridization (Tan *et al.*, 1988; Lubahn *et al.*, 1989; Quarmby *et al.*, 1990; Tilley *et al.*, 1990; Gaspar *et al.*, 1990). The following observations indicate that the 4–5 kb RNA band, in the present experiments, however, is a breakdown product of the 10 kb androgen receptor mRNA. First, spleen RNA, which is thought to contain a very low amount of androgen receptor mRNA, showed virtually no binding of the probe to either band. Second, when RNA from ventral prostate was incubated for an increasing period of time at room temperature, the androgen receptor mRNA was nicked, presumably at a specific site, as shown by a shift in hybridization from the 10 kb androgen receptor mRNA band to the 4–5 kb RNA band (Fig. 1a). Dot blot analysis of the prostate mRNA preparation, containing both 10 and 4–5 kb RNA species, revealed no differences in hybridization intensity between the different samples (Fig. 1b). The above validates the use of dot-blot analysis of total RNA to estimate androgen receptor mRNA expression.

A pronounced change in androgen receptor mRNA level was observed in ventral prostate from animals treated with EDS and not substituted with testosterone for 5 days; the mRNA level was elevated 4.0 ± 0.3-fold (Groups 3, 4, 9 and 10) (Fig. 2a). However, this 4-fold increase was accompanied by a 2.2 ± 0.2-fold

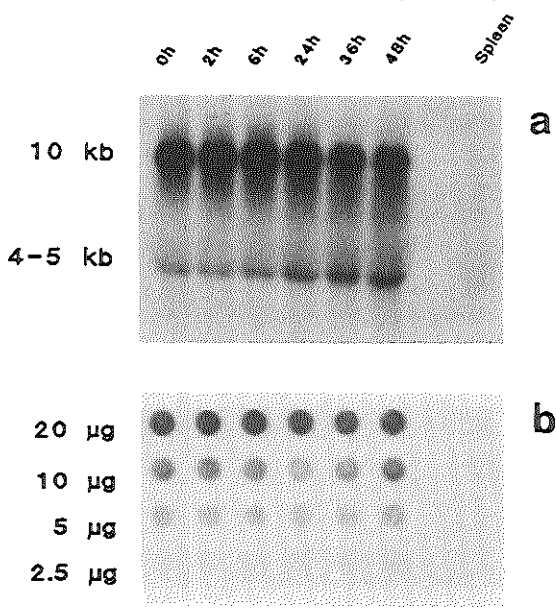

Fig. 1. Northern (a) and dot-blot (b) analysis of total RNA extracted from spleen and ventral prostate. The ventral prostate RNA (35 µg) was kept for 0, 2, 6, 24, 36 and 48 h at room temperature. For Northern analysis, 35 µg of total ventral prostate and spleen RNA was applied per lane and analysed using a human androgen receptor cDNA probe (a). For the dot-blot analysis (b), 20 µg RNA was applied to the filter in a two-fold dilution series and analysed using a human androgen receptor cDNA probe.

elevation of actin mRNA (Fig. 3a). Ethidium bromide staining of 28S and 18S rRNA showed that equal amounts of total RNA were applied to each lane of the gel (not shown). Changes in the cellular composition of the prostate may play a role in this up-regulation of actin mRNA expression (see Discussion). Therefore, actin mRNA levels were not used to correct androgen receptor mRNA levels in the ventral prostate. Special care was taken to apply equal amounts of total RNA to the gel lanes and dot blots.

In animals substituted with testosterone-implants following EDS-treatment, the androgen receptor mRNA level in ventral prostate was suppressed to slightly below control levels. Testosterone-pulse treatment (2 h prior to killing of the animals) did

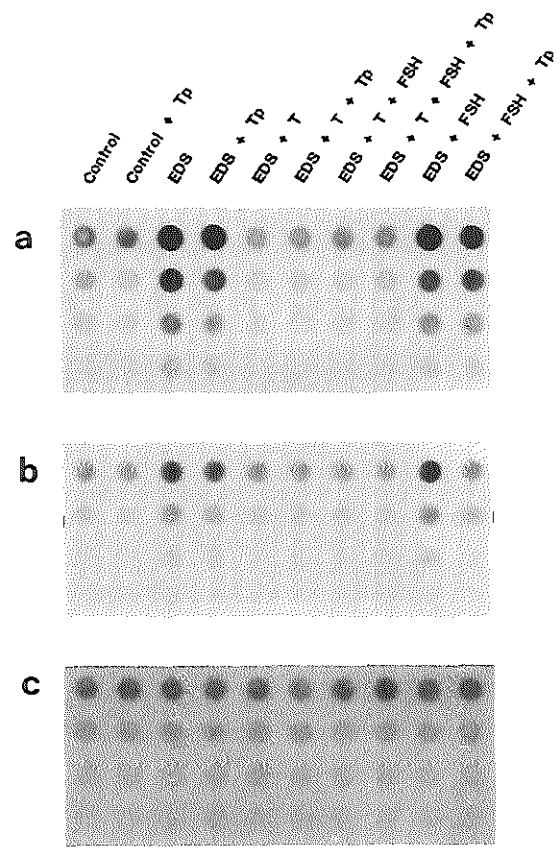

Fig. 2. Hormonal regulation of androgen receptor mRNA expression in ventral prostate (a), epididymis (b) and testis (c). Rats were treated as follows: *Tp* = testosterone injection (10 mg) 2 h prior to killing; *EDS* = killed 5 days after EDS-treatment; *T* = substitution with testosterone for 5 days (15 cm silastic implants); *FSH* = substitution with FSH for 5 days (2 × 5 IU injected per day). For dot blot analysis, 20 µg total RNA was applied to the filter in a two-fold dilution series and analysed using a human androgen receptor cDNA probe.

not result in a change in androgen receptor mRNA expression when compared to groups not receiving a testosterone-pulse (Fig. 2a).

In the epididymis, a 2.0 ± 0.5-fold increase of the androgen receptor mRNA level was observed in EDS-treated animals not substituted with testosterone (Fig. 2b). No change in actin mRNA level was observed for the epididymis (Fig. 3b).

In the testis, no marked alteration of androgen receptor mRNA expression was observed (Fig. 2c). Scanning of the dot blot showed a similar amount of androgen receptor mRNA in control animals (1.00 ± 0.18) and EDS-treated animals not substituted with testosterone (1.02 ± 0.17). No change in actin mRNA was observed for the testis (Fig. 3c).

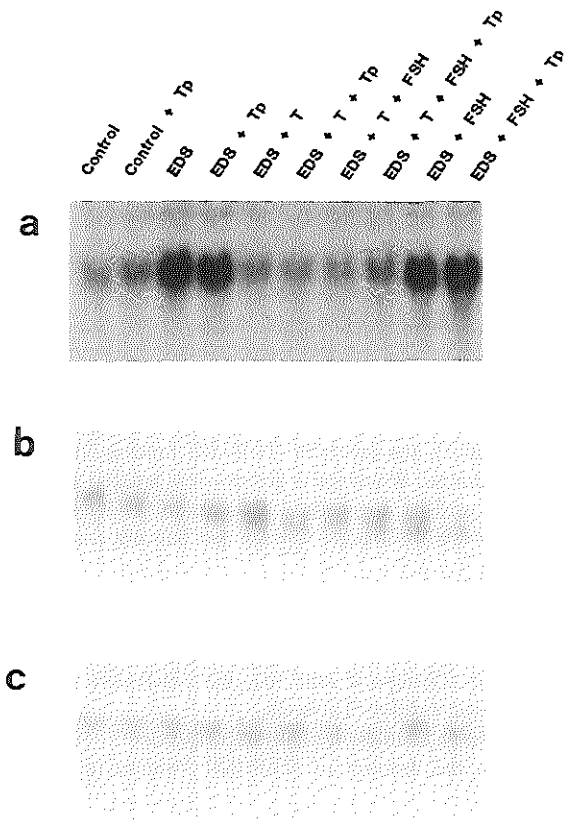

Fig. 3. Up-regulation of actin mRNA level in ventral prostate. The rats were treated as described in the legend to Figure 2. Total RNA was extracted from ventral prostate (a), epididymis (b) and testis (c). A total of 35 μg RNA was applied per lane (Northern blot) and analysed using a hamster actin cDNA probe.

Testicular androgen receptor: ligand binding

To study the biological variation between individual animals, nuclear fractions were prepared from the left testis of five control animals (Group 1) and five EDS-treated rats (Group 3) (10 nuclear preparations). The binding measured by Scatchard analysis in these nuclear preparations was 9.1 ± 1.4 pmol/testis (Control; $n = 5$) and <0.5 pmol/testis (EDS; $n = 5$) (Fig. 4). The statistical difference between control and EDS-treated groups was significant ($P<0.02$). To allow repeated determinations of mRNA (dot blot) and androgen receptor protein (^{3}H-R1881 binding and Western blot) in the same tissue, all further experiments were carried out using pooled left testes from five animals in each group.

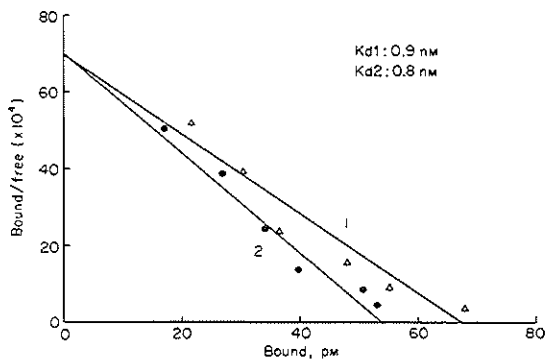

Fig. 4. Specimen Scatchard plots of specific ^{3}H-R1881 binding to total testicular nuclear extracts. The Kd value was estimated using two different nuclear extracts from two control animals. Specific binding was expressed as the ratio bound/free and plotted as a function of ^{3}H-R1881 bound.

In control animals and in EDS-treated animals that received a testosterone-implant (Groups 1 and 5), the levels of ^{3}H-R1881 binding in the nuclear fraction were within the same range. When these groups were treated with the 2 h testosterone-pulse (Groups 2 and 6) no marked changes in ^{3}H-R1881 binding could be observed in comparison with Groups 1 and 5 that had not received a testosterone-pulse (Table 2).

In EDS-treated animals that did not receive a testosterone-implant (Groups 3 and 9), there was little or no ^{3}H-R1881 binding detectable in the nuclear extracts (significantly different from control; $P<0.02$). However, after treatment with the 2 h testosterone-pulse (Groups 4 and 10), the ^{3}H-R1881 binding activity was recovered in the nuclear extract (Table 2).

FSH administration to EDS-treated animals (Groups 7–10) did not give rise to significant changes in the amount of ^{3}H-R1881 binding in the nuclear extracts (Table 2).

Testicular androgen receptor: immunoprecipitation

Immunoprecipitation of testicular androgen receptors followed by Western blotting, revealed a 116 kD androgen receptor protein. Using an aspecific monoclonal antibody (raised against the murine FOS-protein) for the immunoprecipitation

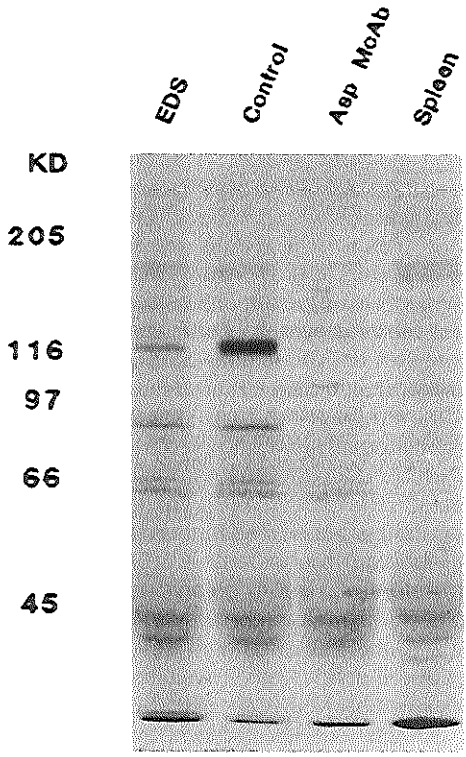

Fig. 5. Western blot analysis of the androgen receptor. The androgen receptor was immunoprecipitated from 1g tissue. *EDS* = testis from animals killed 5 days after EDS-treatment. *Control* = control testis. *Asp McAb* = control testis immunoprecipitated using an aspecific antibody (antibody against the murine FOS protein). *Spleen* = spleen tissue.

step, no such protein band was observed. Furthermore, no androgen receptor protein was detected in spleen (Fig. 5).

In testes from EDS-treated rats, only a very small amount of androgen receptor protein was detected when compared to testes from control rats using immunoprecipitation and Western blotting (Fig. 5). Injection of a high dose of testosterone 2 h before killing of the rats (testosterone-pulse), did not result in a higher amount of immunodetectable androgen receptor protein in testes from EDS-treated rats (Group 4; EDS + Tp). Animals that received a testosterone-implant after EDS-treatment (Groups 5–8), showed a level of immunodetectable androgen receptor that was comparable to the level found in control animals (not shown). These results seem to indicate a loss of immunodetectable testicular androgen receptor in the long-term absence of testosterone.

The outcome of the immunodetection experiments is in disagreement with the results obtained in the ^{3}H-R1881 binding experiments, showing no marked loss of

total androgen receptor (nuclear ligand binding after testosterone-pulse treatment), 5 days after EDS-treatment, in rats not carrying a testosterone-implant.

Discussion

Androgen receptor mRNA in ventral prostate, epididymis and testis

Treatment of adult rats with EDS and substitution for five days with testosterone, caused changes in ventral prostate weight that corresponded with the serum concentration of testosterone. This is in agreement with findings using castrated rats (Deklerck & Coffey, 1978; Huttunen *et al.*, 1981), and it is concluded that elimination of Leydig cells using EDS has an effect on the ventral prostate comparable to that of castration. Changes in tissue weight or cellular composition can give rise to difficulties in the interpretation of data. In the present experimental design, alterations in ventral prostate weight and composition were expected because the ventral prostate is an androgen-sensitive organ that responds rapidly to androgen deprivation. The duration of the period of androgen deprivation was limited to 5 days, to prevent marked alterations of the weight of the testis or the composition of the seminiferous epithelium.

Often, actin mRNA is used as a control to verify whether equal amounts of total mRNA are applied per lane on the gel. In the ventral prostate, however, the amount of actin mRNA (per total RNA) turned out to be influenced by the concentration of circulating testosterone. This may be caused by changes in cellular composition of the ventral prostate tissue upon testosterone deprivation (Deklerck & Coffey, 1978; Huttunen *et al.*, 1981). These changing levels of actin mRNA have not been reported by other authors (Tan *et al.*, 1988; Lubahn *et al.*, 1989; Quarmby *et al.*, 1990; Shan *et al.*, 1990). In the presentation of our results we felt that it might not be justifiable to use actin mRNA levels to correct for the efficiency of androgen receptor mRNA extraction from the ventral prostate.

In the epididymis, a two-fold up-regulation of the amount of androgen receptor mRNA was observed, without a change in actin mRNA level.

Together with data from the literature (Tan *et al.*, 1988; Lubahn *et al.*, 1989; Quarmby *et al.*, 1990; Shan *et al.*, 1990), the present results indicate that testosterone down-regulates its own mRNA in a number of tissues (ventral prostate, epididymis, brain, kidney) and in at least one cell line (LNCaP, Quarmby *et al.*, 1990; Krongrad *et al.*, 1991). Down-regulation of androgen receptor mRNA expression by testosterone, however, is not a general phenomenon. Blok *et al.* (1989), using cultured immature rat Sertoli cells, and Husmann *et al.* (1991), studying prostate tissue from immature neonatally castrated rats, have shown that, under certain experimental conditions, androgen receptor mRNA levels are not regulated by testosterone. In the present experiments, in testes from adult rats, no testosterone-dependent change in androgen receptor mRNA expression was observed.

In future experiments, we will use androgen receptor gene promoter constructs (Baarends *et al.*, 1990) to study regulation of androgen receptor gene expression in different cell types. Such studies may provide further evidence that regulation of androgen receptor gene transcription in testicular cells, is regulated differentially when compared to, for example, prostate cells.

Androgen receptor protein in testis: specific ^{3}H-R1881 binding

For estimation of specific ^{3}H-R1881 binding sites (Scatchard analysis), a nuclear fraction was isolated from total testis. The procedure for isolation of the nuclear fraction included tissue fractionation under liquid nitrogen (Barberis *et al.*, 1989) and the use of buffers containing a high concentration of sucrose (Gorski *et al.*, 1986), in order to reduce loss of nuclear androgen receptors. Using this method, and applying various ways to inhibit proteolysis, 9.1 ± 1.4 pmol of androgen binding was observed per testis (mean ± SD of five control adult rats). This is higher than the total amount of androgen binding observed by Buzek & Sanborn (1988), in a nuclear fraction from adult rat testis.

EDS-treated rats with a testosterone-implant for 5 days, had a testicular testosterone level of approximately 20% of the control value. The level of ^{3}H-R1881 binding to the testicular nuclear fraction of these testosterone-substituted animals, however, remained unaltered compared to control animals. This suggests that a testicular testosterone level as low as 20% of the control value is sufficient to maintain a normal level of transformed androgen receptor (as measured by specific ^{3}H-R1881 binding). Sharpe *et al.* (1988) have evaluated spermatogenesis in EDS-treated rats substituted with a testosterone ester for 3 weeks. These authors found that a testicular testosterone level of 24% of control was sufficient to maintain spermatogenesis, which is in agreement with the present findings. Using the EDS-model and the method to estimate nuclear androgen receptors described herein, it will be possible to study what testicular testosterone concentration is needed to transform the androgen receptor to an active tight nuclear binding form. Such a study may yield information concerning the relationship between testicular testosterone concentration and maintenance of spermatogenesis, and the transformation and activity of the androgen receptor.

In EDS-treated animals that had not received a testosterone-implant, very low binding of ^{3}H-R1881 in the nuclear fraction was observed. These results might suggest that the unoccupied androgen receptor is not located in the nucleus. Simental *et al.* (1991) found that in COS cells, transfected with androgen receptor cDNA, the unoccupied androgen receptor was located in the perinuclear region. However, Husmann *et al.* (1990) reported nuclear localization of the androgen receptor in the presence as well as in the absence of ligand. Discussion about the subcellular localization of the unoccupied androgen receptor may not have ended. In the present experiments, we have taken the position that untransformed (unoccupied) androgen receptor molecules, that are not in the tight nuclear binding form, are lost from the nuclei upon cell fractionation. Therefore, rats in the different experimental groups were treated with a very high dose of testosterone (10 mg) 2 h prior to killing (testosterone-pulse), to transform unoccupied androgen receptors to the occupied, tight nuclear binding form. Using this approach, the number of occupied androgen receptors (without testosterone-pulse) and the total amount of androgen receptors (with testosterone-pulse) could be measured in the nuclear fraction. The results show that, even in EDS-treated rats that did not have a testosterone-implant, testicular androgen receptors were still present. These receptors became transformed to a tight nuclear binding form upon testosterone-pulse treatment, and then fractionated into the nuclear fraction.

The serum FSH level was low in EDS-treated rats with a testosterone-implant. Therefore, FSH was also administered to the experimental groups. FSH treatment had no effect on testicular androgen receptor mRNA, and only minor effects on the androgen receptor protein level were observed. This does not correspond with data on cultured Sertoli cells, in which up-regulation of androgen receptor mRNA as well as protein by FSH has been reported (Verhoeven & Cailleau, 1988; Blok *et al.*, 1989). However, this can be explained, because the present study has measured total testicular androgen receptor in adult animals, rather than androgen receptor in cultured Sertoli cells from immature rats.

The present results on ^{3}H-R1881 binding seem to indicate that the untransformed androgen receptor in the testis does not show decreased stability in the prolonged absence of testosterone. This result is rather surprising, in view of reports that testosterone exerts an effect on the stability of the androgen receptor in various cell culture systems (Grino *et al.*, 1990; Syms *et al.*, 1985). Therefore, the presence of androgen receptor protein was also studied using Western blotting.

Androgen receptor protein in testis: immunodetection

Using monoclonal and polyclonal androgen receptor antibodies in immunoprecipitation and Western blotting, a 116 kD band was observed in the adult rat testis. Using the same method and the same antibodies, human LNCaP cells were found to contain a slightly smaller (110 kD) androgen receptor protein (van Laar *et al.*, 1991). This was confirmed in the present experiments. The controls presented herein suggest strongly that the 116 kD band represents the rat androgen receptor.

An intriguing discrepancy was observed between the results from the ligand binding and the immunoprecipitation experiments. Using immunoprecipitation, the animals subjected to prolonged testosterone deprivation (Groups 3, 4, 9, 10), showed very low levels of immunodetectable androgen receptor protein, even after testosterone-pulse treatment. However, ^{3}H-R1881 binding data, using nuclear preparations from these groups, showed the presence of high levels of untransformed receptors. Apparently, the antibodies bind poorly to the androgen receptor from long-term testosterone depleted testes. This result indicates that the untransformed form of the androgen receptor in the testosterone-deprived testis may undergo a structural modification, which does not occur in the presence of testosterone.

Acknowledgments

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Supplement[1] to Chapter 7.

In Chapter 7, immunoprecipitation and Western blotting of the androgen receptor was performed to confirm the maintenance of the androgen receptor protein in testosterone deprived testis. Using monoclonal and polyclonal antibodies directed against the N-terminal domain of the human androgen receptor (van Laar et al., 1991), a 116 KD band was observed (Fig. 5). In this supplement, the Western blot of the immunoprecipitated testicular androgen receptor from the experimental groups 1-10 is shown (Fig. 6).

In animals treated with EDS and receiving testosterone implants (groups 5-8), a comparable level of immunoprecipitable androgen receptor was observed as in control animals (groups 1-2) (Fig. 6). However, animals treated with EDS but not receiving testosterone implants (groups 3, 4, 9, 10), had very low levels of immunoprecipitable androgen receptor. It should be noted that the Western blot represents immunoprecipitation from total testis homogenates, so that loss of androgen receptor from the nuclear fraction does not have an influence on the outcome of the experiments.

The discrepancy between the results from the ligand binding (Chapter 7; Table 2) and immunoprecipitation experiments (Figures 5 and 6) can not readily be explained. Using a monoclonal antibody directed against the C-terminal domain of the human androgen receptor, the same results were obtained. Moreover, immunoprecipitation of the androgen receptor from nuclear lysates, which were used in the ligand-binding assay, also yielded the same results. Apparently, the antibodies poorly bind to the androgen receptor from testosterone depleted testis (also not after retransformation, 2 h after injection of a high dose of testosterone). This may point to a permanent structural modification of the untransformed androgen receptor that occurs in the prolonged absence of testosterone. One other point of consideration is that the antibodies were directed against the human androgen receptor. It is possible that even small structural changes in the rat androgen receptor protein, occurring upon prolonged testosterone deprivation, may result in loss of receptor-antibody recognition.

[1]*The information in this supplement has been published as part of an article in the Proceedings of the Eighth International Congress on Hormonal Steroids, The Hague, The Netherlands, September 1990*
(Blok LJ, Bartlett JMS, Bolt-de Vries J, Themmen APN, Brinkmann AO, Weinbauer GF, Nieschlag E and Grootegoed JA (1991) Regulation of androgen receptor mRNA and protein in the rat testis by testosterone. J Steroid Biochem Molec Biol 40:343-348)

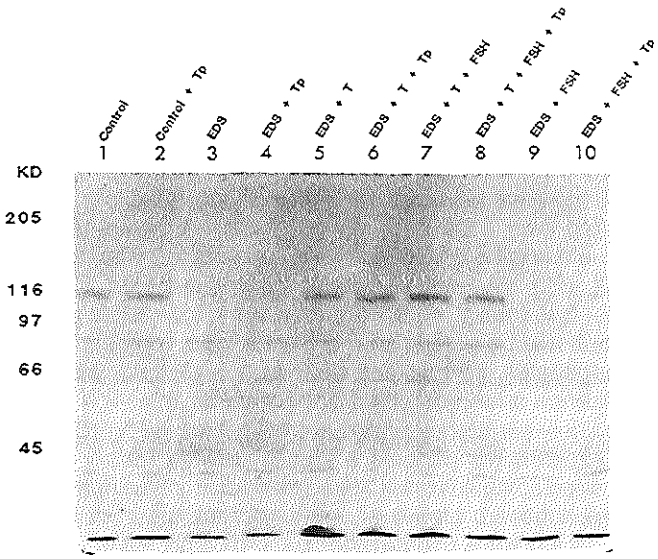

Fig. 6. Western blot analysis of the androgen receptor immunoprecipitated from total testis lysates. Tp = testosterone injection (10 mg) 2 h prior to killing; EDS = killed 5 days after EDS treatment; T = substitution with testosterone for 5 days (15 cm silastic implants); FSH = substitution with FSH for 5 days (2 x 5 IU injected per day).

CHAPTER 8

GENERAL DISCUSSION

8.1 Testosterone

Testosterone and FSH are important hormonal regulators of mammalian spermatogenesis (Chapter 1). In the next paragraphs, the emphasis will be on the role of testicular testosterone in rat and monkey.

8.1.1 The testicular testosterone concentration and spermatogenesis in adult rats

Leydig cells produce large amounts of testosterone. This results in a high testicular testosterone concentration as measured when the steroids are extracted from total testis tissue, or when the testosterone concentration is determined in interstitial fluid, seminiferous fluid, or in testicular venous blood. In adult rats, the measured testicular testosterone concentration is approximately 20- to 50-fold higher than the testosterone concentration in serum and in peripheral organs (testicular testosterone concentration: 150 - 500 nM; circulating testosterone concentration: 5 -15 nM, taken from references discussed below). Whether or not the high testicular testosterone level is a prerequisite for normal spermatogenesis, is a question which many investigators have tried to answer in the past decades, using different approaches. In the next paragraphs we have summarized some of these reports.

1. Cunningham and Huckins (1979) have injected adult rats daily with testosterone. As a result of this treatment, the circulating LH level was reduced to undetectable values, and the testicular testosterone level declined to **below 10%** of normal. After 39 days of treatment, they observed qualitatively and almost quantitatively normal spermatogenesis (qualitatively = all germ cell types are present; quantitatively = development of normal numbers of all germ cell types, so that the sperm counts will be normal). The FSH levels were reduced only by 30% as a result of the treatment. Their general conclusion was that, in the presence of very low testicular testosterone levels, complete spermatogenesis could persist.
2. Zirkin et al. (1989) reported that in similar experiments **at least 20%** of the normal intact testicular testosterone concentration was necessary to maintain a normal daily sperm production during 8 weeks of treatment. In these animals the FSH levels were reported to be reduced by 50%.
3. Huang et al. (1987) and Sun et al. (1989) extended the experiments of Cunningham and Huckins (1979) by substituting hypophysectomized adult rats with increasing amounts of testosterone, for respectively 13 and 7 weeks. Sun et al. (1989) found that testicular testosterone levels of **around 10%** of the normal control values could qualitatively maintain spermatogenesis. When the testicular testosterone levels were increased, spermatogenesis was improved, but normal sperm counts could never be achieved (Sun et al., 1989). Huang et al. (1987) found that **25%** of the normal testicular testosterone concentration could maintain qualitatively normal spermatogenesis at a maximal level. FSH could not be detected in these animals.
4. Active immunisation of adult rats against GnRH resulted in undetectable concentrations of circulating testosterone, LH and FSH. As a result, spermatogenesis became impaired. Readministration of testosterone to the GnRH-immunized adult rats (silastic implants, resulting in a testicular testosterone level of **20%** of normal), resulted in a restart of spermatogenesis, and after 8 weeks normal sperm counts were observed (Awoniyi et al., 1989). Maintenance of spermatogenesis, measured under similar conditions was also complete (Awoniyi et al., 1992). The FSH levels in these animals were very low or undetectable.

5. When EDS-treated animals were substituted with testosterone for 3 or 10 weeks (Sharpe et al., 1988a and b), spermatogenesis could be maintained if the testicular testosterone level was maintained **above 25%** of normal intact testicular testosterone concentrations. FSH levels dropped to respectively 50% of normal or just above the detection level in these experiments. Sprando et al. (1990) found that 8 weeks after EDS treatment, spermatogenesis could only qualitatively be maintained by testosterone substitution, but not quantitatively; the sperm counts were decreased by 30%.

6. Bartlett et al. (1989) substituted hypophysectomized rats with FSH (twice-daily injections with 5IU human FSH) and testosterone. In these animals the testicular testosterone level was decreased to **below 5%** of the normal intact value, while spermatogenesis was almost quantitatively maintained. When only FSH or testosterone was substituted, spermatogenesis was markedly reduced.

The above summarized reports agree that normal testicular testosterone concentrations are at least 4- to 5-fold and perhaps 10- to 20-fold higher than needed for spermatogenesis.

Biochemical data supporting the view that a very high testicular testosterone level is not a prerequisite for normal spermatogenesis came from experiments using cultured testicular cells. Buzek and Sanborn (1990) found that binding of ligand to androgen receptors in cultured Sertoli and peritubular myoid cells, reached a maximum at testosterone concentrations around 3 nM (Kd = 0.7 nM). This indicated that 3 nM testosterone saturates the androgen receptor and generates a maximal androgen signal in the cultured cells. In the testis, the testosterone concentrations are 50- to 150-fold higher. Of course, the conditions in the testis in vivo are very much different from those in cultured testicular cells in vitro, but the fact remains that the testicular androgen receptor does not seem to need high local testosterone levels to transduce the androgen signal. In the following paragraphs (and in Chapter 7) further biochemical data are provided, from in vivo experiments, that support the hypothesis that the normal testicular testosterone concentration is far in excess over that required for spermatogenesis.

In Chapter 7, we have varied circulating and testicular testosterone levels, and measured the occupancy of the androgen receptor (the EDS model used in these experiments has been described in detail in Paragraph 1.4.3 and in Chapter 7). In Table 1 (ventral prostate data) and in Table 2 (testis data) some of the results from these studies are summarized.

It was observed that in ventral prostate, under control conditions in intact animals, only 40% of the androgen receptors was occupied with ligand. Furthermore, we observed that the occupancy of the androgen receptor with ligand (Table 1; occupied AR %/g) became higher, concomitant with the increase of circulating levels of testosterone, and was lowered following a decrease in circulating levels of testosterone. This fits the observation that in ventral prostate in vivo, epithelial cell numbers and to a lesser extent stromal cell numbers are regulated by an increase or decrease in circulating testosterone (DeKlerk and Coffey, 1978; Huttunen et al., 1980).

Occupied androgen receptors can bind to consensus DNA sequences and regulate gene transcription (Beato et al., 1990; Wahli and Martinez, 1991). This means that with an increase in occupancy from 40% to 100%, more androgen receptors become active as transcription factors. In the ventral prostate, an example of transcriptional regulation of a gene by changes in circulating testosterone is the androgen receptor gene (Fig. 1). When the androgen level is increased by substituting testosterone after EDS treatment, the androgen

receptor mRNA expression is decreased, and vice versa (in Chapter 5, strong indications are provided for transcriptional regulation of the androgen receptor gene by androgens).

Table 1. Androgen receptor content of ventral prostate

Treatment	Serum T nM	Occupied AR %/g	Total AR %/g	Prost. weight g
Control	7.4 ± 6.0	42.3	100	501 ± 121
EDS	< 0.3	< 5	56	295 ± 68
EDS + T-implant	45.3 ± 8.1	110	112	664 ± 65

Serum T = the concentration of serum testosterone; Occupied AR = percentage of androgen receptors that are transformed to the tight nuclear binding form; Total AR = total amount of androgen receptor as a percentage of the control, expressed per gram tissue; Prost. weight g = ventral prostate weight in grammes; EDS = sacrificed 5 days after EDS treatment; T-implant (or T) = substitution with 15 cm silastic testosterone implants for 5 days. The androgen receptor (AR) data represents the mean of two experiments.

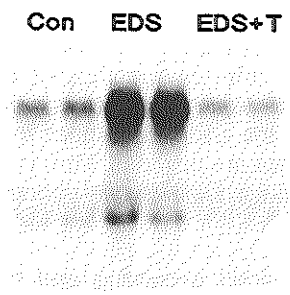

Fig. 1. Regulation of androgen receptor mRNA expression in adult rat ventral prostate by different levels of testosterone. Total tissue RNA was extracted in duplicate. For hormonal and biochemical parameters, and for the legend, see Table 1.

In the adult rat testis (Table 2) the situation is different. Under control conditions, the androgen receptor is fully occupied with ligand and probably has attained its maximal capacity to regulate gene transcription. This situation does not change when the testicular testosterone concentration is decreased to 20% of normal.

These data indicate that all androgen actions in the testis mediated via the nuclear androgen receptor can be fully maintained at a testicular testosterone concentration of 20% of the normal intact control concentration. It is expected (and currently investigated) that this level can be further decreased to 5 - 10% of control.

Table 2. Androgen receptor content of testes.

Treatment	Testicular T nM	Occupied AR %/g	Total AR %/g	Testes weight g
Control	165 ±114.4	100±16	104±21	3103±155
EDS	7.6± 8.7	<5	97±17	2854±104
EDS + T-implant	36.1± 3.5	81±11	113±24	3073±104

Testicular T = the concentration of testicular testosterone; Occupied AR = percentage of androgen receptors that are transformed to the tight nuclear binding form; Total AR = total amount of androgen receptor as a percentage of the control, expressed per gram tissue; Testes weight g = paired testes weight in grammes; EDS = sacrificed 5 days after EDS treatment; T-implant = substitution with 15 cm silastic testosterone implants for 5 days. For serum testosterone concentrations see Table 1. More details are provided in Chapter 7.

8.1.2 Androgen effects not mediated via nuclear receptors

Some of the experiments summarized in Paragraph 8.1.1 indicate that the testis needs at least 5 to 10% of its normal testosterone concentration for quantitative maintenance of spermatogenesis. Yet, this testosterone level is considerably higher than the circulating testosterone level and possibly also higher than the testosterone level needed to occupy all testicular androgen receptors. The question is how this difference in testosterone requirement between the testis and for example the ventral prostate can be explained?

In the ventral prostate the active androgen is dihydrotestosterone (DHT), which binds with a much higher affinity to the androgen receptor than testosterone (Wilbert et al., 1983). In the adult testis, there is no high rate of conversion of testosterone to DHT, and it is hypothesized that testosterone acts as the active androgen (Baker et al., 1977). This difference may account partly for the fact that the testis needs more testosterone than the ventral prostate, but other factors may be involved.

In this paragraph, data are presented that suggest the existence of an androgen receptor located on the cell plasma membrane, that binds androgen with a moderate affinity (10^{-8}M), and subsequently rapidly affects calcium and/or cAMP levels in the cell. For other steroids similar mechanisms have been proposed.

Koenig et al. (1989) examined the early response of heart myocytes to administration of testosterone and found an acute effect on calcium influx and distribution in the cells, induced by testosterone concentrations of 1 - 10 nM. One of the first effects of the calcium influx was the activation of ornithine decarboxylase (1 min), resulting in a rapid increase in polyamine concentration (2 min) and a somewhat delayed increase in the uptake of horseradish peroxidase, 2-deoxyglucose and α-aminoisobutyrate (markers respectively for endocytosis, amino acid transport and hexose transport). The polyamines are thought to trigger the stimulation of endocytosis, hexose transport, and amino acid transport. The testosterone-induced calcium influx and the resulting effects in the myocytes could be blocked by EGTA (calcium chelator), La^{+3} (calcium antagonist), or verapamil (calcium transport blocker). The calcium ionophore A23187 mimicked the testosterone effects on the myocytes.

Effects of testosterone administration on intracellular calcium have also been documented for LNCaP cells. Steinsapir et al. (1991) observed a rapid calcium influx (2 min) upon stimulation of LNCaP cells with a synthetic androgen (10^{-6} - 10^{-12} M mibolerone).

There is some evidence that the described effects of testosterone on intracellular calcium in myocytes and LNCaP cells are mediated via a protein that may resemble the androgen receptor. With respect to the calcium influx in myocytes, Koenig et al. (1982) observed steroid specificity, hormone concentration dependency, and the absence of the effect in testicular feminized (Tfm) mutant mice in which the androgen gene is defect. Steinsapir et al (1991) showed inhibition of androgen-induced calcium influx by the antiandrogen hydroxyflutamide (10^{-6}M). However, this putative plasma membrane androgen receptor is most likely not identical to the cloned nuclear androgen receptor.

Non-nuclear receptor mediated effects of androgens are not extensively studied sofar, but there are reports of non-nuclear actions of other steroids. Estrogen (estradiol-17ß) administration to uterine endometrial cells, for example, resulted in enhanced calcium uptake over a 10-30 min period (Pietras and Szego, 1975). Furthermore, progesterone addition to sperm was found to initiate calcium uptake, and consequently to induce the acrosome reaction (Osman et al., 1989). Recently, Blackmore and Lattanzio (1991) have found indications for a progesterone receptor on the plasma membrane of the sperm head. In oocytes, progesterone also causes a rapid increase in calcium uptake (Moreau et al., 1980; Wasserman et al., 1980).

The non-nuclear actions of 1,25-dihydroxy-vitamin D3 (vit-D) have been studied more thoroughly (Nemere and Norman, 1991). An altered calcium and phospholipid metabolism in reaction to vit-D administration has been reported in a great number of cells in different species: intestinal and colonic epithelium, kidney tubules, muscle cells, lymphoid cells, mammary tissue, hepatocytes and osteoblasts (for a review, see Nemere and Norman, 1991). On the basis of the studies on vit-D, Nemere and Norman (1991) postulated a model for non-nuclear receptor mediated steroid actions on calcium homeostasis in cells, involving steroid binding sites on the plasma membrane (Fig. 2).

Other support for the existence of steroid hormone actions on the plasma membrane comes from studies on sex hormone binding proteins. Sex hormone binding protein (SBP or SHBG) is a protein that binds testosterone and DHT with moderate affinity (Kd of 1 - 10 nM) and 17ß-estradiol (E2) with a lower affinity. SBP is present in the plasma of many species, but is reported to be absent in mature rat and mouse plasma (other plasma proteins may bind androgens and estrogens in these species). For SBP, two specific binding places on the plasma membrane have been reported [Kd = 2×10^{-8} M and $1.5. \times 10^{-9}$ M (Hryb et al., 1989)]; ^{125}I-SBP binding has been demonstrated in isolated membranes from decidual endometrium, benign prostatic hyperplasia and LNCaP cells. Most data indicate that the steroid-bound SBP binds with very high affinity (Kd = 10^{-12} M) to the membranes (Natalja et al., 1990; Avvakumov et al., 1986). Rosner et al. (1991) reported, however, that the unliganded SBP also binds to LNCaP cell membrane with a high affinity. Upon addition of DHT (10 nM, 15 min) to LNCaP cells preincubated with SBP, the cAMP content of the cells was increased by approximately 50% (calcium influx has not been studied so far). As a control, DHT was added to LNCaP cells not preincubated with SBP, and no effects on cAMP content were observed. Hence, SBP acts as a mediator of this steroid hormone effect on the plasma membrane.

Taking all these data together, there are grounds to consider androgen actions which do not directly involve the nuclear androgen receptor. However, for the testis, these theories are highly speculative and thusfar lack experimental proof. It is therefore too early to postulate the existence of functional plasma membrane androgen receptors in the testis, which might explain the need for relatively high levels of testicular testosterone for ongoing spermatogenesis.

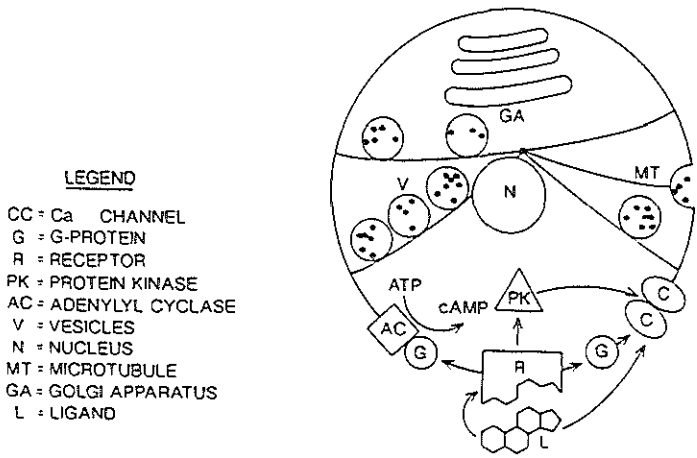

Fig. 2. Model for steroid action not mediated via nuclear receptors. The hypothesis which is suggested includes a possible direct interaction of the steroid with Ca^{+2} channels, or receptor mediated activation of Ca^{+2} channels through G proteins, formation of cAMP or activation of protein kinases. Please note that the receptor is in the plasma membrane. (Part of a figure from Nemere and Norman, 1991).

8.1.3 Regulation of male fertility

The development of a hormonal method for male contraception has been the subject of much research that started more than a decade ago (Swerdloff et al., 1978). In the course of clinical experiments, it became clear that it may be difficult to develop a male contraceptive that will prove useful for large groups of people. Good male contraceptives should combine several characteristics:

- the blockade of male fertility must be complete but also reversible,
- male secondary sexual characteristics must be maintained,
- there should be no side effects,
- and the contraceptive should be long acting and easy to apply (user friendly).

At the moment, several studies are being undertaken to test or develop hormonal methods to suppress sperm production (Waites, 1990). These studies focus on three main aspects. First, suppression of the secretion of the gonadotropins; second, the maintenance of normal androgen levels in the circulation without re-stimulating spermatogenesis; and third, the evaluation of the fertilizing capacity of residual sperm (in case the hormonal treatment does not achieve azoospermia).

A central point in these investigations is the testosterone concentrations in the circulation and in the testis. Circulating testosterone should be sufficiently high to maintain male secondary sexual characteristics, whereas testicular testosterone levels should be markedly decreased to reduce spermatogenesis. As is indicated in Paragraph 8.1.1, the testicular

testosterone level may not always be a true indication of androgen action. Therefore, measuring androgen receptor occupancy in peripheral organs and in the testis of treated animals can provide fundamental information about the biological activity of the androgen levels in these organs.

One group of investigational drugs studied in the context of male contraception are GnRH antagonists. A brief summary of the studies is given below.

GnRH antagonists bind to the pituitary GnRH receptor (Heber et al., 1982), but do not stimulate gonadotropin release. Furthermore, GnRH antagonist binding prevents GnRH binding to the receptor, and consequently LH and FSH release is inhibited. Because of low LH levels, the testicular testosterone production is also reduced. When GnRH antagonist was injected daily during 10 weeks in the non-human primate Macaca fascicularis, complete loss of sperm production was achieved (Bind Akhtar et al., 1985; Weinbauer et al., 1984; 1987; 1988). When antagonist treatment was stopped, sperm production returned to the normal intact level within 12 weeks. During treatment with GnRH antagonist, circulating LH and testosterone concentrations were decreased to very low levels. The low testosterone concentration, however, was not sufficient to maintain male secondary sexual characteristics.

In order to support male secondary sexual characteristics, GnRH antagonist treatment requires testosterone substitution. Unfortunately, in monkeys receiving both antagonist and androgen at the same time, some sperm production was detected (Weinbauer et al., 1988). It remains to be established what the fertilizing capacity is of this residual sperm. Sperm counts, however, could be reduced to zero, introducing a delay of 6 weeks between the start of GnRH antagonist treatment and the start of testosterone substitution (Weinbauer et al., 1989). The idea behind this treatment is that spermatogenesis can not be re-initiated by a relatively low concentration of testosterone alone; FSH is required in addition to testosterone to initiate spermatogenesis.

An interesting observation in the experiments of Weinbauer et al. (1988) was that the testicular testosterone levels of the GnRH antagonist-treated monkeys (measured 15 weeks after the start of the GnRH antagonist treatment) did not fall below 30% of the normal value, although circulating LH and serum testosterone levels were very low. Furthermore, substitution of circulating testosterone (to maintain sexual characteristics) had no significant effect on this testicular testosterone level.

As described in Chapter 7, a residual testosterone level was also observed in the testis of EDS-treated rats (7.6 $\pm$ 8.7 nM testosterone; Table 2). When we measured androgen receptor occupancy, this residual testosterone did not transform a measurable amount of androgen receptors to the tight nuclear binding form. In other words, the remaining testosterone seems to represent an inactive pool with respect to receptor binding. In the testis of GnRH antagonist-treated monkeys, the same analysis might be carried out to determine whether the remaining testicular testosterone (30%) indeed represents an inactive testosterone pool, with respect to transformation of testicular androgen receptors.

8.2 FSH

FSH and testosterone are both involved in the regulation of spermatogenesis. For androgens, we have discussed (8.1) that testosterone levels in the testis are so high that, within a broad range of concentrations (20% to 100%) the activity of the androgen receptor as a transcription

factor may remain at its maximum. About the actual FSH level in the testis, not much information is available. The circulating FSH levels have been described to be relatively low from day 5 to day 15 after birth. Thereafter, the concentration starts to increase approximately 3-fold (around day 40) and subsequently returns to a lower value (Ketelslegers et al., 1978). Most studies concerning the role of FSH in spermatogenesis use methods to reduce the level of FSH to almost zero and then substitute with high amounts of FSH.

In the experiments described in Chapter 4, we have injected intact 21-day-old rats with FSH (i.p. injection with 1 μg/g bodyweight ovine FSH) and observed marked effects of this single injection on androgen and FSH receptor mRNA expression in the whole testis (Fig. 3).

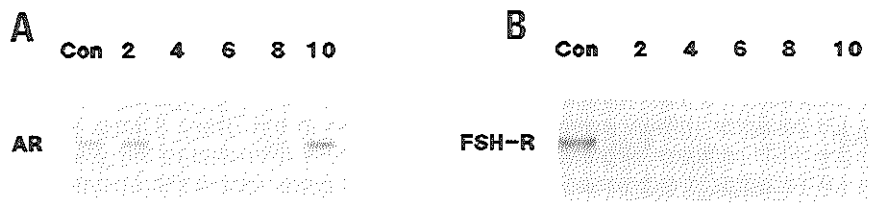

Fig. 3. (A) Effect of FSH on testicular androgen receptor mRNA and (B) FSH receptor mRNA expression. The 21-day-old rats were injected (i.p.) with 1 µg ovine FSH-S16/g body weight and sacrificed 2, 4, 6, 8 or 10 h later. Con = control; saline injected and sacrificed 4 h later. For Northern analysis (A:androgen receptor mRNA), 20 µg of total RNA, isolated from total testis, was applied per lane and analyzed using a human androgen receptor cDNA probe. For RNase-protection (B: FSH receptor mRNA) 10 µg of total RNA was used to hybridize with a 400 bp FSH receptor probe. The FSH receptor mRNA data have not been published so far.

These effects offer no explanation for the function of FSH in spermatogenesis, but the observations do illustrate that FSH stimulation of immature Sertoli cell activity in vivo is not at its maximum.

8.2.1 Regulation of gonadotropin receptors

The pituitary gonadotropins are key hormones in the regulation of gametogenesis and steroidogenesis in the gonads. In this paragraph, the regulation of FSH and LH receptors in ovary and testis will be discussed. In Chapter 1, we have described similarities between FSH and LH, and between their receptors. It may therefore be expected that there exist certain conformities in the regulation of both receptors.

In the literature, several reports describe that ovarian gonadotropin receptors, in particular the LH receptor, are highly regulated when follicles are recruited, mature, ovulate and luteinize (Richards, 1980). During maturation of the follicle, there is a large increase in LH receptor expression, which is followed by a marked decrease after the preovulatory LH surge. During the ensuing luteinisation, LH receptor expression is increased again. FSH receptor expression is less pronounced, and differences in expression between stages of follicle

development are not so evident (Richards, 1980).

Regulation of the expression of LH and FSH receptor mRNAs during the estrous cycle was studied using in situ hybridisation (Camp et al., 1991). In ovaries from immature rats, it was observed that FSH receptor mRNA expression is confined to the granulosa cells, while LH receptor mRNA is only found in thecal cells. Upon maturation of the follicle (either induced by hormone stimulation, but also during the estrous cycle), a high level of LH receptor mRNA was found in granulosa cells. Using cultured granulosa cells, it appeared that FSH was responsible for this marked stimulation of LH receptor mRNA expression (Piquette et al., 1991). After the ovulation-inducing preovulatory LH surge, but also when ovulation was stimulated with human chorionic gonadotropin (LaPolt et al., 1991 and 1992), LH receptor mRNA expression in granulosa cells was rapidly decreased to below the level of detection. The situation in the corpora lutea is variable; in some corpora lutea LH receptor mRNA is expressed, but other corpora lutea did not show this mRNA. In the thecal and interstitial cells, the expression of LH receptor mRNA is only moderately increased during the days before, and on the day of the preovulatory LH surge (metestrus, diestrus and proestrus).

The expression of FSH receptor mRNA is much lower, and the regulation less articulate than the LH receptor mRNA expression and regulation in the ovary (Camp et al., 1991). Furthermore, FSH receptor expression in the adult ovary is restricted to the granulosa cells, while the LH receptor is expressed in most ovarian cell types (not in the oocyte).

For the testis, LH receptor regulation has been studied using the MA-10 mouse Leydig cell tumor cell line as a model (Wang et al., 1991). Addition of LH to the culture medium of these cells resulted in a rapid decline in ligand binding, due to internalization of the receptor, which was followed by a decrease in LH receptor mRNA and protein expression. These results are not very different from the results concerning FSH regulation of FSH receptor expression in cultured Sertoli cells (discussed in 8.2.2), that showed a rapid desensitization of FSH signal transduction and a decrease in FSH receptor mRNA and protein expression.

From these results, it is suggested that some aspects of the regulation of the LH and FSH receptor expression in the testis and ovary involve similar mechanisms.

8.2.2 FSH regulation of androgen and FSH receptor expression in the testis

Using cultured Sertoli cells from immature rats, the in vivo down-regulatory effect of FSH injection on androgen and FSH receptor mRNA expression in immature rat testis could be mimicked. It is known that cAMP plays a significant role in the signal transduction of FSH. In concordance with this, a rapid decrease in androgen and FSH receptor mRNA expression in Sertoli cells was also observed after addition of dibutyryl cAMP (dbcAMP) to the culture medium. In the here discussed experiments, we have used dbcAMP to stimulate the Sertoli cells in order to avoid refractoriness to FSH stimulation due to receptor internalization (for details on these effects see Chapters 1 and 5).

Following the rapid down-regulation of FSH receptor and androgen receptor mRNA within the first 5 h of culture in the presence of dbcAMP, clear differences between the regulation of androgen and FSH receptor mRNA expression became apparent. Androgen receptor mRNA expression was restored to control levels after 7 - 8 h, and gradually increased when the culture time was extended to 72 h. In contrast, the FSH receptor mRNA expression level remained reduced at approximately 40% of the control level during culture in the presence of dbcAMP (Fig. 4a).

Using cycloheximide, an inhibitor of protein synthesis, it was found that de novo protein

synthesis was not a prerequisite for the FSH/dbcAMP-induced decrease in androgen and FSH receptor mRNA expression. Nuclear transcription run-on experiments indicated that the decline in androgen receptor mRNA expression was not the result of decreased androgen receptor gene transcription. Similarly, the decline in FSH receptor mRNA expression was not caused by a decreased rate of FSH receptor gene transcription.

It can be postulated, therefore, that FSH, using cAMP as the predominant second messenger, activates (one or more) short-lived and preexisting proteins, that through an unknown mechanism decrease the stability of the androgen and FSH receptor mRNAs.

After this initial phase, the androgen receptor mRNA expression is restored to control level and then slowly increased (increased gene transcription), whereas the FSH-receptor mRNA expression reaches a new steady state at approximately 40% of the control value (no increase of the rate of gene transcription).

The rapid FSH/dbcAMP-induced changes in FSH and androgen receptor mRNA expression were not immediately translated into corresponding differences at the protein level (Fig. 4b). For the androgen receptor there are indications that the protein stability is much higher than the mRNA stability (Chapter 3). Therefore, rapid and marked differences in mRNA expression are translated slowly into small differences in protein expression. The FSH receptor has not been thoroughly studied in this respect.

Since the short-term down-regulation of androgen receptor mRNA expression was of no major consequence for receptor regulation, one may ask the question what might be the physiological relevance of this FSH-induced down-regulation. As far as we know, in Sertoli cells such a short-term effect of FSH has only been observed for androgen and FSH receptor mRNAs, and not for other mRNAs. For example actin and GAPDH mRNAs are not regulated by FSH, whereas cAMP-dependent protein kinase subunit mRNA (Oyen et al., 1988) and c-fos mRNA expression (Hall et al., 1988) are transiently increased after FSH addition to the cells. For inhibin α-subunit mRNA, a prolonged increase in mRNA expression was described (Klaij et al., 1990).

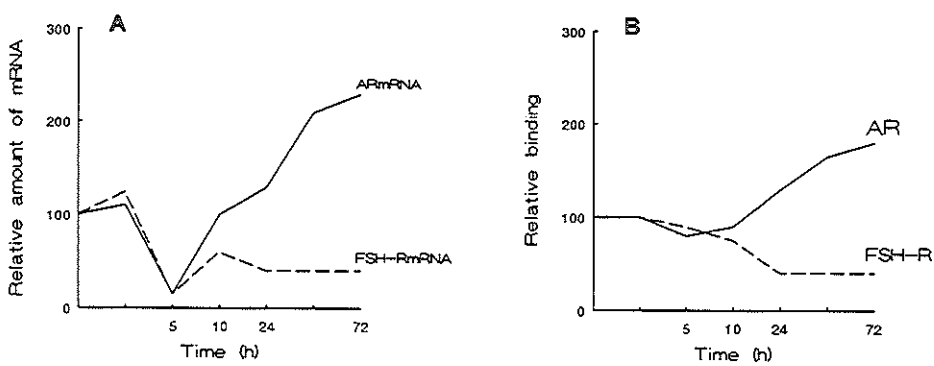

Fig. 4. Regulation of androgen and FSH receptor mRNA (A) and protein (B) expression in cultured Sertoli cells in the presence of dbcAMP; extrapolation of the results described in Chapters 3 and 4.

Within certain limits, subtle short-term down-regulation of androgen and FSH receptor

mRNA expression may play a role in physiology. However, when we inject extra FSH in immature rats, or when we add FSH to Sertoli cells in culture, the cells are exposed to a high dose of hormone. This high FSH dose may accelerate processes that, in vivo under physiological conditions, take place at a much lower rate.

Sertoli cells stimulated for longer periods of time with FSH or dbcAMP show a good correlation between androgen receptor mRNA and protein levels, and between FSH receptor mRNA and protein levels. It is speculated that under normal physiological conditions, FSH plays a long-term regulatory role in the testicular expression of androgen and FSH receptors in immature rats.

8.3 Interaction between peritubular myoid cells and Sertoli cells

The importance of the interaction between stromal (mesenchymal) and epithelial cells was recognized many years ago (Grobstein, 1953). A classical experiment in this respect is the analysis of androgen-induced mammary epithelial regression in mice (Kratochwil and Schwartz, 1976). In male mouse embryos, mammary epithelial development is totally inhibited by androgens that are secreted by the fetal testes (Ohno, 1979). This inhibition takes place irrespective of the presence of functional or dysfunctional androgen receptors in the regressing epithelial cells. However, the presence of normal functional androgen receptors in the underlaying mesenchymal tissue is essential. From these experiments, it was concluded that developmental effects of androgens on mammary epithelial gland tissue were mediated via the underlaying mesenchyme (Cunha et al., 1983; Kooistra, 1991).

The concept of epithelial cell development, modulated by androgen-induced factors from mesenchymal cells, has also been recognized in the prostate. The development of the prostate from urogenital sinus tissue, and the maintenance of its adult structure and function, fully depends on the presence of functional androgen receptors and sufficient amounts of androgens. During fetal prostate development, the role of the mesenchyme in modulating androgen effects on epithelial cell growth and development is evident, because the mesenchymal cells contain androgen receptors whereas the epithelial cells do not (Shannon and Cunha, 1983; Takeda et al., 1985; Husmann et al., 1991).

Irrespective of the presence of androgens, the prostate epithelial cells begin to express androgen receptors starting at day 4 - 6 after birth (Cunha et al., 1983). In order to study the role of the mesenchyme in development and maintenance of the epithelial cells in the prostate after birth, tissue recombinants were made between mesenchymal and epithelial cells of urogenital sinus origin, derived from normal intact animals or animals expressing defective androgen receptors (testicular feminized animals; Tfm). The tissue recombinants were implanted into intact male hosts and allowed to grow to mature structures. Wild-type mesenchyme/Tfm epithelial recombinants and wild-type mesenchyme/wild-type epithelial recombinants were analized.

It was observed that in all cases, differentiated epithelial cells developed next to fibroblasts and smooth muscle cells of mesenchymal origin. Furthermore, the secretory products of the wild-type mesenchyme/Tfm epithelium structures were highly similar to secretory products from normal intact prostates (Cunha and Chung, 1981; Norman et al., 1986; Sugimura et al., 1986). Androgen deprivation, resulted in comparable changes in histology in recombinant and normal prostate tissue cultures. It is clear that during prostate development, but also in the adult prostate, a number of epithelial characteristics is regulated by androgens via the stromal (mesenchymal) cells.

In the testis, the importance of cell-cell interactions has also been recognized. Here we will focus on the interaction between the peritubular myoid cells and the Sertoli cells as an example of a mesenchymal-epithelial interaction.

In the fetal testis, Sertoli cell clusters are surrounded by peritubular cell sheaths. With the onset of spermatogenesis, the peritubular cells develop towards a more mature appearance, while the Sertoli cells stop dividing and start differentiating to their adult form. The development of the peritubular myoid cells is dependent on androgens, but also on FSH (Bressler and Ross, 1972). It is clear that the action of FSH is mediated via the Sertoli cells.

Sertoli cells co-operate on a structural level with peritubular myoid cells, in forming a complex intracellular matrix in between the two cell types. This extracellular matrix consists of components produced by Sertoli cells and produced by peritubular myoid cells (Skinner et al., 1985) and provides structural integrity for the tubules and assists in the maintenance of an efficient blood-testis barrier (Dym and Fawcett, 1970). Furthermore, extracellular matrix components have also been reported to play a significant role in regulating the biological activities of growth factors (Ruoslahti and Yamaguchi, 1991).

On a regulatory level, it seems that androgen stimulation of Sertoli cells is partly achieved via the peritubular myoid cells. Peritubular myoid cells have been reported to secrete an important protein which modulates Sertoli cell function: PmodS (Hutson and Stocco, 1981; Skinner and Fritz, 1985a and b) (see also Paragraph 1.1.2). The production and secretion of PmodS by cultured peritubular myoid cells is increased upon stimulation with androgens. When Sertoli cells are cultured in the presence of semi-purified PmodS, the production of transferrin and androgen binding protein is markedly increased. Here we can observe a certain analogy with the described effects of androgens on the prostate: the action of androgens on androgen receptor containing epithelial cells (Sertoli cells) is modulated by the effect of androgens on mesenchymal cells (peritubular myoid cells).

In Chapter 6, a putative Sertoli cell factor which might play a role in the interactions between Sertoli cells and peritubular myoid cells, is described. At this early stage of the investigations to characterize the putative paracrine factor, it is difficult to speculate on its role in physiology.

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SUMMARY

Sertoli cells play an important role in the regulation of spermatogenesis and are referred to as the nursing cells of the germinal epithelium. FSH and testosterone, the main regulatory hormones of spermatogenesis, act on Sertoli cells. FSH acts directly, because the Sertoli cell is the only cell type in the male body expressing FSH receptors. Germ cells do not express androgen receptors and androgen action on spermatogenesis, therefore, involves androgen action on Sertoli cells. Androgen receptors are also present in testicular peritubular myoid cells, and it is likely that these cells also play a role in the regulation of spermatogenesis by androgens.

In this thesis, investigations are described on the regulation of FSH receptor and androgen receptor gene expression in the testis in relation to spermatogenesis. Expression of the receptor genes was studied at the level of gene transcription, and by estimation of mRNA and protein concentrations.

In Chapters 2, 3 and 5, it is shown that FSH can regulate androgen receptor gene expression in testicular cells. When FSH was added to the medium of cultured Sertoli cells from immature rats, two effects on androgen receptor mRNA expression were observed:

- first, there was a short-term (5h) down-regulation of the amount of androgen receptor mRNA, caused by a decreased androgen receptor mRNA stability. Androgen receptor protein expression was not affected, because this down-regulation of mRNA was transient and the androgen receptor protein has a longer half-life than the androgen receptor mRNA.
- second, there was a long-term (24-72h) up-regulation of androgen receptor mRNA and protein expression, caused by an increased rate of androgen receptor gene transcription.

A short-term (4 - 6h) down-regulatory effect on androgen receptor mRNA expression was also observed in testes of immature rats, after the rats had been injected with FSH. This indicates that in vivo (in intact testes), FSH may exert similar effects on androgen receptor gene expression as observed in vitro (using cultured Sertoli cells).

Peritubular myoid cells, together with Sertoli cells, are a substantial source of androgen receptor mRNA and protein in the testis. Since we observed a marked short-term reduction in androgen receptor mRNA expression in total testis of FSH-injected immature rats, it was speculated that Sertoli cell factors might reduce androgen receptor mRNA levels in peritubular myoid cells. In Chapter 6, it is shown that in peritubular myoid cells, cultured in the presence of Sertoli cell-conditioned medium, the androgen receptor mRNA expression indeed became reduced. Peritubular myoid cells cultured in the presence of EGF or 10% FCS showed a similar decrease in androgen receptor mRNA expression.

Experiments dealing with the regulation of FSH receptor gene expression in Sertoli cells are described in Chapters 4 and 8. Initial down-regulation of FSH receptor mRNA expression, 4 - 6h after addition of FSH to cultured Sertoli cells (but also in the testes of FSH injected immature animals), showed similarities to the regulation of androgen receptor mRNA expression. The rapid FSH-induced decrease in FSH-receptor mRNA expression did not involve inhibition of FSH receptor gene transcription, but rather mRNA stability, and did not affect the protein level.

When FSH was added to the cultures for longer time periods (24 h), FSH receptor mRNA and protein levels were reduced. In the literature, desensitization of cultured Sertoli cells generated by the continuous presence of high doses of FSH has been described as resulting from receptor internalization upon ligand binding, and decreased second messenger

activity through activation of phosphodiesterase activity. The current results indicate that diminished FSH receptor gene expression also plays a role in Sertoli cell desensitization by FSH.

When immature Sertoli cells were cultured in the presence of testosterone, androgen receptor protein stability was markedly increased (Chapters 2 and 3). Androgen receptor mRNA expression in cultured Sertoli cells (and peritubular myoid cells), however, was not affected by the addition of testosterone to the culture medium (Chapters 2, 3 and 5). In Chapters 7 and 8, it is described that androgen receptor mRNA expression in the testis was also not affected in the prolonged absence of testosterone in vivo (5 days). These experiments indicate that in vivo (in the rat testis), and in vitro (in cultured rat Sertoli and peritubular myoid cells), androgen receptor mRNA expression is not affected by androgens. This contrasts with results from experiments using other cell types, described in the following paragraph.

When LNCaP cells were cultured in the absence of androgens, an increased androgen receptor mRNA expression was observed, which was found to be caused by an increased rate of androgen receptor gene transcription (Chapter 5). In Chapters 7 and 8, in in vivo experiments, androgen receptor mRNA expression in the ventral prostate and epididymis was markedly increased upon androgen deprivation and lowered upon androgen resubstitution (in the literature, there are similar observations described for other organs). These experiments indicate that androgen receptor gene transcription in ventral prostate (and also in LNCaP cells) and in epididymis, in contrast to transcription of this gene in the testis, is regulated by androgens.

Androgen receptor protein expression in the testis (Chapter 7) was measured by specific ligand binding in isolated nuclei. In addition to occupied androgen receptors, also the total amount of androgen receptors was measured. When the testicular testosterone concentration was experimentally reduced to a very low level, androgen receptors became unoccupied, but the total amount of androgen receptor protein (measured by ligand binding assay) remained at the control level. The interaction of unoccupied androgen receptors with antibodies, however, was altered, indicating a structural modification of the receptor molecule in the prolonged absence of androgens. From these results it was concluded that in vivo (as was also observed in vitro, in cultured Sertoli cells), androgen receptors are unstable in the prolonged absence of ligand.

Another important observation described in Chapter 7, is that a testicular testosterone level of 20% of the control level is sufficient to occupy all androgen receptors, which is in agreement with experimental data showing that the normal testicular testosterone level is at least 4- to 5-fold higher than what is needed to maintain spermatogenesis.

FSH and testosterone are involved in regulation of spermatogenesis. FSH is required for the initiation and re-initiation of spermatogenesis, while testosterone alone can maintain spermatogenesis. In the experiments described in this thesis, it is observed that regulation of FSH and androgen receptor gene expression may play an important role in the regulation of spermatogenesis by FSH and testosterone.

SAMENVATTING

Sertoli cellen zijn van essentieel belang voor regulatie van de spermatogenese. Het functioneren van Sertoli cellen wordt in belangrijke mate bepaald door de hormonen FSH en testosteron. Van FSH is bekend dat dit hormoon een directe invloed uitoefent op de Sertoli cel, omdat dit de enige cel in het mannelijk lichaam is die FSH receptoren tot expressie brengt. Aangezien Sertoli cellen androgeen receptoren bevatten kan ook testosteron direct inwerken op Sertoli cellen. Germinale cellen bevatten geen androgeen receptoren. Naast Sertoli cellen zijn de androgeen receptor bevattende peritubulaire myoid cellen, indirect, betrokken bij de regulatie van spermatogenese.

In dit proefschrift wordt ingegaan op de regulatie van de expressie van het FSH receptor gen en het androgeen receptor gen in relatie tot de regulatie van de spermatogenese. De expressie van beide genen werd onderzocht op het niveau van gen transcriptie, alsmede door het meten van mRNA en eiwit concentraties.

In Hoofdstukken 2, 3 en 5 is beschreven wat het effect is van FSH toediening aan gekweekte Sertoli cellen op de expressie van het androgeen receptor gen. Twee effecten op androgeen receptor gen expressie werden gevonden:

- ten eerste nam de hoeveelheid androgeen receptor mRNA, door destabilisatie van het mRNA, binnen 5 uur af tot bijna nul. De hoeveelheid androgeen receptor eiwit veranderde niet, omdat de halfwaarde tijd van het androgeen receptor eiwit veel groter is dan van het androgeen receptor mRNA.
- ten tweede nam in de periode 24 - 72 uur na FSH toediening de hoeveelheid androgeen receptor mRNA en eiwit toe, door stimulatie van androgeen receptor gen transcriptie.

Een korte termijn effect (4 - 6 uur) van FSH toediening op androgeen receptor mRNA expressie in gekweekte Sertoli cellen bleek ook op te treden in de gehele testis wanneer immature ratten werden ingespoten met FSH. Dit resultaat kan betekenen dat FSH in vivo (in de intacte testis) de androgeen receptor gen expressie op eenzelfde manier kan regelen als in vitro (in gekweekte cellen).

Naast Sertoli cellen bevatten ook peritubulaire cellen androgeen receptoren. Gezien de observatie dat in ratten geïnjecteerd met FSH de androgeen receptor mRNA expressie in de gehele testis werd geremd, ligt het voor de hand te veronderstellen dat Sertoli cellen een factor produceren die androgeen receptor mRNA expressie in de peritubulaire myoid cellen kan reduceren. In Hoofdstuk 6 zijn experimenten beschreven die laten zien dat androgeen receptor mRNA expressie in peritubulaire myoid cellen inderdaad werd gereduceerd, wanneer deze cellen werden gekweekt in aanwezigheid van Sertoli cel geconditioneerd medium. Wanneer de peritubulaire myoid cellen werden gekweekt in aanwezigheid van EGF of 10% FCS, werd eenzelfde effect op de androgeen receptor mRNA expressie waargenomen.

Zoals beschreven in de Hoofdstukken 4 en 8, wordt ook de FSH receptor gen expressie in Sertoli cellen gereguleerd door FSH. De korte termijn regulatie (4 - 6 uur) van FSH receptor mRNA expressie in gekweekte Sertoli cellen (maar eveneens in de testes van FSH behandelde ratten) vertoont gelijkenis met de regulatie van androgeen receptor mRNA expressie. Wanneer FSH werd toegediend aan Sertoli cellen in kweek nam de FSH receptor mRNA expressie snel af. Deze afname werd niet teruggevonden in het gemeten FSH receptor eiwit niveau, en was niet het gevolg van een afname in transcriptie snelheid van het FSH receptor gen.

Langdurig stimuleren van Sertoli cel kweken door FSH (24 uur), resulteerde in een afname in de FSH receptor mRNA en eiwit niveaus. In de literatuur wordt beschreven dat deze desensitizatie van Sertoli cellen door langdurige kweek in aanwezigheid van FSH, het gevolg is van receptor internalisatie door ligand binding en verminderde second messenger activiteit door toegenomen fosfodiesterase activiteit. De hier beschreven resultaten wijzen erop dat FSH receptor gen expressie ook een rol speelt bij de desensitizatie van Sertoli cellen door FSH.

Wanneer testosteron werd toegevoegd aan immature Sertoli cellen in kweek, nam de stabiliteit van het androgeen receptor eiwit toe (Hoofdstukken 2 en 3), maar de hoeveelheid androgeen receptor mRNA in gekweekte Sertoli cellen (en peritubulaire myoid cellen) veranderde niet. In Hoofdstukken 7 en 8, wordt beschreven dat in afwezigheid van testosteron in vivo (5 dagen), androgeen receptor mRNA expressie in de testis ook niet veranderde. Uit deze experimenten blijkt dat, in vivo (in de ratten testis), maar ook in vitro (in gekweekte ratten Sertoli en peritubulaire myoid cellen), androgeen receptor mRNA expressie niet wordt beinvloed door androgenen. Dit is in tegenstelling tot bevindingen met andere cellen en weefsels, beschreven in de onderstaande paragraaf.

Wanneer LNCaP cellen werden gekweekt in afwezigheid van androgenen nam de androgeen receptor mRNA expressie toe. Dit is het gevolg van een verhoogde transcriptie van het androgeen receptor gen. In in vivo experimenten, beschreven in Hoofdstukken 7 en 8 hebben we een soortgelijke observatie gedaan; in de ventrale prostaat en in de epidydimis van testosteron gedepriveerde ratten nam de androgeen receptor mRNA expressie significant toe (soortgelijke observaties zijn in de literatuur ook beschreven voor andere organen). Deze resultaten geven aan dat in de ventrale prostaat (en ook in LNCaP cellen) en in de epididymis, in tegenstelling tot de testis, androgeen receptor gen transcriptie wordt gereguleerd door androgenen.

Door middel van een specifieke ligand-bindingsassay konden de androgeen receptoren in geisoleerde kernen uit de testis worden gemeten. Naast de bezette receptoren is ook het totaal aantal receptoren gemeten. Uit de experimenten blijkt dat wanneer de testiculaire testosteron concentratie werd verlaagd tot een zeer laag niveau, alle androgeen receptoren onbezet raakten. Het totale aantal aanwezige androgeen receptoren echter, bleef gelijk. Detectie van receptoren die langere tijd onbezet waren, met behulp van antilichamen, was niet mogelijk, hetgeen wijst op een structurele verandering in het receptor molecuul.

In dezelfde reeks experimenten werd ook gevonden dat een testiculair testosteron niveau van 20% van het normale niveau voldoende was om alle aanwezige androgeen receptoren in de testis te bezetten. Deze resultaten wijzen er op dat het normale testiculaire testosteron niveau zeker 4 tot 5 maal hoger is dan nodig voor het handhaven van spermatogenese.

FSH en testosteron zijn nodig voor regulatie van de spermatogenese. FSH is van belang bij het opstarten van spermatogenese, terwijl testosteron in afwezigheid van FSH de voortgang van de spermatogenese kan waarborgen. Uit de in dit proefschrift beschreven experimenten kan worden afgeleid dat regulatie van FSH en androgeen receptoren een belangrijke rol kan spelen in de regulatie van spermatogenese door FSH en testosteron.

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CURRICULUM VITAE

Leen Blok was born on the 8th of January 1962 in the town of Fijnaart en Heijningen in The Netherlands. His secondary education (HAVO-VWO) was taken at the Nassau Scholen Gemeenschap in Breda. In 1981 he started a Biology study at the Agricultural University in Wageningen, from which he graduated with distinction in 1988. In March 1988 a start was made with the PhD research at the Erasmus University Rotterdam. The research has been performed under supervision of and in close collaboration with Dr. J. Anton Grootegoed, head of the Department of Endocrinology & Reproduction. The writer of this thesis has been awarded a NATO-fellowship by The Netherlands Organisation for Scientific Research and a research fellowship by the Mayo Rochester Research Committee, in order to perform research over a two year period with Dr. Donald J. Tindall, at the Department of Urology Research, Mayo Clinic, Rochester, Minnesota, USA, starting December 1992.